(19)日本国特許庁(JP)　(12)公表特許公報(A)　(11)特許出願公表番号
特表2004-524831
(P2004-524831A)
(43)公表日　平成16年8月19日(2004.8.19)

(51)Int.Cl.[7]	F I		テーマコード（参考）
C12N 15/09	C12N 15/00	ZNAA	2G045
A61K 31/5415	A61K 31/5415		4B024
A61K 45/00	A61K 45/00		4B063
A61K 47/22	A61K 47/22		4B065
A61P 25/14	A61P 25/14		4C076

審査請求 未請求　予備審査請求 有　（全 341 頁）　最終頁に続く

(21)出願番号　特願2002-556766(P2002-556766)
(86)(22)出願日　平成14年1月15日(2002.1.15)
(85)翻訳文提出日　平成15年7月15日(2003.7.15)
(86)国際出願番号　PCT/GB2002/000153
(87)国際公開番号　WO2002/055720
(87)国際公開日　平成14年7月18日(2002.7.18)
(31)優先権主張番号　0101049.5
(32)優先日　平成13年1月15日(2001.1.15)
(33)優先権主張国　英国(GB)

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(54)【発明の名称】神経変性疾患におけるタンパク質凝集に関する材料および方法

(57)【要約】
安定した細胞系において、前駆体タンパク質が病理学的に凝集する（例えばタウオパチー）病態に伴う前駆体タンパク質（例えばタウ）を産生物フラグメント（例えば１２ｋＤフラグメント）にタンパク質分解的に変換する方法を開示し、そして方法は（ａ）（ｉ）鋳型フラグメントが細胞に対して有毒でないレベルで細胞において構成的に発現されるような前駆体タンパク質の鋳型フラグメント；および（ｉｉ）刺激に応答してタンパク質が細胞において誘導的に発現される前駆体タンパク質；をコードする核酸でトランスフェクトされた安定した細胞系を提供することを含み、それにより鋳型フラグメントと前駆体タンパク質との相互作用が、前駆体タンパク質における立体配座変化を引き起こし、前駆体タンパク質の産生物フラグメントへの凝集およびタンパク質分解性プロセシングを引き起こす。好ましくは、方法を用いて産生物のバンドまたは複数のバンドの産生（または産生の調整）のモニターによる凝集過程のモジュレーターをスクリーニングするために用いられる。またアッセイにおいて用いられる材料、加えて医薬品、並びに本発明のアッセイにおいて高度な活性を示す化合物、例えば還元型ジアミノフェノチアジンに基づく関連する使用および方法もまた提供される。

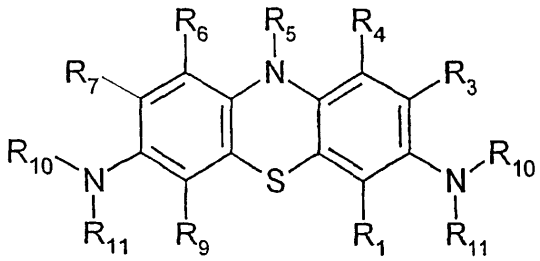
【特許請求の範囲】
【請求項１】
安定した細胞系における前駆体タンパク質を産生物フラグメントにタンパク質分解的に変換する方法であって、前駆体タンパク質は、前駆体タンパク質が病理学的に凝集する病態に伴い、
下記工程：
（ａ）下記：
（ｉ）鋳型フラグメントが、細胞に対して有毒でないレベルで、細胞において構成的に発現されるような前駆体タンパク質の鋳型フラグメント；および
（ｉｉ）タンパク質が刺激に応答して細胞において誘導的に発現される、前駆体タンパク質；
をコードする核酸でトランスフェクションされた安定した細胞系を提供すること
を含み、それにより鋳型フラグメントと前駆体タンパク質との相互作用が、前駆体タンパク質の産生物フラグメントへの凝集およびタンパク質分解性プロセシングのような前駆体タンパク質における立体配座変化を引き起こす方法。
【請求項２】
病理学的凝集が、神経変性および／または臨床痴呆に伴う病態における前駆体タンパク質のタンパク質分解性プロセシングを導く、請求項１記載の方法。
【請求項３】
病態における前駆体タンパク質の病理学的凝集が、コアドメインフラグメントおよび少なくとも鋳型タンパク質のコアフラグメントを含む鋳型フラグメントへのタンパク質分解性プロセシングを導く、請求項１または請求項２記載の方法。
【請求項４】
鋳型フラグメントが、実質的にコアフラグメントからなる、請求項３記載の方法。
【請求項５】
細胞において産生される産生物フラグメントが有毒である、請求項１～４のいずれか一項記載の方法。
【請求項６】
産生物フラグメントが、鋳型フラグメントと同一である、請求項１～５のいずれか一項記載の方法。
【請求項７】
複数の異なる産生物フラグメントが産生される、請求項１～５のいずれか一項記載の方法。
【請求項８】
細胞において前駆体タンパク質を誘導的に発現させるような刺激に細胞を供する工程を含む、請求項１～７のいずれか一項記載の方法。
【請求項９】
少なくとも一つの産生物フラグメントの産生をモニターする、請求項１～８のいずれか一項記載の方法。
【請求項１０】
病態に伴う前駆体タンパク質の凝集および／またはタンパク質分解性プロセシングのモジュレーターを同定する方法であって、下記：
（ａ）凝集を調整することができることが推測される作用物質を提供すること、
（ｂ）作用物質の存在下で請求項９記載の方法を実施すること、
（ｃ）モニターされた産生物または各々の産生物フラグメントの産生を作用物質の調整活性と相関させること
を含む方法。
【請求項１１】
工程（ｂ）が、
（ａ）細胞を一つ以上のプレート上で培養すること、

（ｂ）作用物質が、細胞内に入るのに十分な時間、細胞を作用物質と共にインキュベーションすること
により実施される、請求項１０記載の方法。
【請求項１２】
作用物質を細胞に導入して最終濃度を１～５０μＭにする、請求項１１記載の方法。
【請求項１３】
複数の異なる産生物フラグメントの産生をモニターする、請求項１０～１２のいずれか一項記載の方法。
【請求項１４】
モニターされたその産生物または各々の産生物フラグメントの産生を基準値と比較する、請求項１０～１３のいずれか一項記載の方法。
【請求項１５】
作用物質の不在下でこの方法を実施することにより基準値が得られる、請求項１４記載の方法。
【請求項１６】
提供される作用物質が、血液脳関門を通過することができるように選択される、請求項１０～１５のいずれか一項記載の方法。
【請求項１７】
作用物質の拡散係数を測定し、そして拡散係数を作用物質阻止可能性と相関させることにより、提供される作用物質を選択する工程を含む、請求項１０～１６のいずれか一項記載の方法。
【請求項１８】
更に作用物質に関してＢ５０を計算する工程を含む、請求項１０～１７のいずれか一項記載の方法。
【請求項１９】
更に作用物質の細胞生存性に及ぼす影響を評価する工程を含む、請求項１０～１８のいずれか一項記載の方法。
【請求項２０】
更に作用物質に関してＬＤ５０を計算する工程を含む、請求項１９記載の方法。
【請求項２１】
作用物質に関して治療指数を計算する工程を含む、請求項１８および２０記載の方法。
【請求項２２】
前駆体タンパク質が、タウタンパク質である、請求項１～２１のいずれか一項記載の方法。
【請求項２３】
鋳型フラグメントが、タウのコアフラグメントを含む、請求項２２記載の方法。
【請求項２４】
鋳型フラグメントが、図７に示す全長タウタンパク質のアミノ酸１８６～２９７から３９０～４４１に広がるタウのフラグメントを含む、請求項２３記載の方法。
【請求項２５】
鋳型フラグメントが、図７に示す全長タウタンパク質のアミノ酸２９５、２９６または２９７からアミノ酸残基３９０または３９１に広がるタウのフラグメントからなる、請求項２４記載の方法。
【請求項２６】
タウのおよそ１２、１４、２５、２７、３０、３２、３６、３８、４２または４４kDaの産生物フラグメントの産生をモニターする、請求項２２～２５のいずれか一項記載の方法。
【請求項２７】
タウのおよそ１２kDaの産生物フラグメントの産生をモニターする、請求項２６記載の方法。

【請求項２８】
その産生物または各々の有毒産生物フラグメントの産生をＳＤＳ　ＰＡＧＥでモニターする、請求項２２～２７のいずれか一項記載の方法。
【請求項２９】
その産生物または各々の有毒産生物フラグメントの産生を免疫学的にモニターする、請求項２２～２８のいずれか一項記載の方法。
【請求項３０】
（ｉ）タウのＧｌｙ－１６およびＧｌｎ－２６の間の領域に位置するヒト特異的エピトープに特異的であるか、（ｉｉ）Ｇｌｕ－３９１で断端されたコアタウフラグメントに特異的であるか、（ｉｉｉ）反復ドメインの一般的タウエピトープに特異的であるか、または（ｉｖ）Ｓｅｒ－２０８～Ｓｅｒ－２３８の間に位置する種非特異的一般的タウエピトープに特異的である、モノクローナル抗体から選択される抗体をモニターで用いる、請求項２９記載の方法。
【請求項３１】
可溶性全長タウを捕捉するために固相基質に吸収されているコア反復ドメインに対応するタウのフラグメントの能力を調整する作用物質の能力を決定することにより提供される作用物質を選択する工程を含む、請求項２２～３０のいずれか一項記載の方法。
【請求項３２】
作用物質が、フェノチアジンである、請求項２２～３１のいずれか一項記載の方法。
【請求項３３】
作用物質が、還元型フェノチアジンである、請求項３２記載の方法。
【請求項３４】
（ａ）請求項２２～３２のいずれか一項記載の方法を実施すること、
（ｂ）２を越える治療指数を有するモジュレーターを選択すること
を含むタウオパチーの処置のための治療用または予後用物質として使用するための医薬品をスクリーニングする方法。
【請求項３５】
「疾患」が、アルツハイマー病、運動ニューロン病、レビ小体疾患、ピック病または進行性核上性麻痺から選択される、請求項３４記載の方法。
【請求項３６】
（ａ）請求項３４または請求項３５記載の方法を実施して医薬品を同定すること、
（ｂ）単離された形態の医薬用物質を提供すること
を含むタウオパチーの処置のための治療用または予後用モジュレーターとして使用するための医薬品を製造する方法。
【請求項３７】
更にタウオパチーの処置に使用するための医薬用組成物として作用物質を処方することを含む、請求項３６記載の方法。
【請求項３８】
更にタウオパチーの処置の方法における医薬用組成物の使用を含む、請求項３７記載の方法。
【請求項３９】
調製が少なくとも８０、９０、９５、または９９％還元（ロイコ）形態で存在するようにフェノチアジンを前還元する工程を含む、タウオパチーの処置または予防に使用するための医薬用組成物の調製におけるフェノチアジンの使用。
【請求項４０】
フェノチアジンが、外来性還元剤を添加することにより前還元される、請求項３９記載の使用。
【請求項４１】
還元形態が、安定剤の添加により還元状態で安定化される、請求項４０記載の使用。
【請求項４２】

還元形態が、安定剤と共に凍結乾燥されている、請求項４１記載の使用。
【請求項４３】
タウオパチーの処置または予防において使用するための医薬用組成物の調製における前還元されたフェノチアジンの使用であって、ここで医薬品が、少なくとも８０、９０、９５、または９９％のフェノチアジンの還元（ロイコ）形態を含む、前還元されたフェノチアジンの使用。
【請求項４４】
医薬用組成物が、更に下記：
医薬上許容される賦形剤、担体またはバッファーの１つ以上を含む、請求項３９～４３のいずれか一項記載の使用。
【請求項４５】
医薬用組成物が、徐放性処方として調製される、請求項４４記載の使用。
【請求項４６】
フェノチアジンが、ジアミノフェノチアジンである、請求項３９～４５のいずれか一項記載の使用。
【請求項４７】
前還元された（ロイコ）フェノチアジンが、式：
【化１】

(I)



（式中、Ｒ１、Ｒ３、Ｒ４、Ｒ６、Ｒ７およびＲ９は、独立して水素、ハロゲン、ヒドロキシ、カルボキシ、置換されたまたは置換されていないアルキル、ハロアルキルまたはアルコキシから選択され；Ｒ５は、水素、ヒドロキシ、カルボキシ、置換されたまたは置換されていないアルキル、ハロアルキルまたはアルコキシから選択され；並びにＲ１０およびＲ１１は、独立して水素、ヒドロキシ、カルボキシ、置換されたまたは置換されていないアルキル、ハロアルキルまたはアルコキシから選択される）
を有するか、またはその医薬上許容される塩である、請求項３９～４６のいずれか一項記載の使用。
【請求項４８】
Ｒ１、Ｒ３、Ｒ４、Ｒ６、Ｒ７およびＲ９は、独立して水素、－CH_3、－C_2H_5または－C_3H_7から選択され；Ｒ１０およびＲ１１は、独立して水素、－CH_3、－C_2H_5または－C_3H_7から選択され；そして
Ｒ５は、水素、－CH_3、－C_2H_5または－C_3H_7である、請求項４７記載の使用。
【請求項４９】
フェノチアジンが、ジアミノフェノチアジン核の回りに０、２、３、または４個のメチル基を有しているジアミノフェノチアジンである、請求項４６～４８のいずれか一項記載の使用。
【請求項５０】
フェノチアジンが、非対称的にメチル化されているジアミノフェノチアジンである、請求項４６から４９のいずれか一項記載の使用。
【請求項５１】

フェノチアジンが、塩化トロニウム、アズールＡ、アズールＢおよびチオニンである、請求項５０記載の使用。
【請求項５２】
フェノチアジンが、メチレンブルー、トルイジンブルーＯまたは１，９－ジメチルメチレンブルーから選択される、請求項４６～４９のいずれか一項記載の使用。
【請求項５３】
フェノチアジンが、少なくとも８０、９０、９５、または９９％の還元（ロイコ）形態である、請求項４７～５２のいずれか一項記載の前還元されたフェノチアジンを安定剤と組み合わせて含む、医薬用組成物。
【請求項５４】
安定剤と共に凍結乾燥されている、請求項５３記載の医薬用組成物。
【請求項５５】
安定剤が、アスコルビン酸塩である、請求項５３または請求項５４記載の医薬用組成物。
【請求項５６】
タウオパチーの処置または予防に使用するための、請求項５３～５５のいずれか一項記載の医薬用組成物。
【請求項５７】
請求項５３～５５のいずれか一項記載の医薬用組成物の使用を含むタウオパチーの処置方法。
【請求項５８】
治療または予防が、予防的有効量または治療的有効量の医薬用組成物を同一物を必要とする患者に与えることを含む、請求項３４～５２、請求項５６または請求項５７のいずれか一項記載の方法、使用または組成物。
【請求項５９】
治療または予防が、同一物を必要とする患者に全溶解固形物２０mg、全溶解固形物５０mg、または全溶解固形物１００mgを２倍のmg比率のアスコルビン酸と組み合わせて、摂取前にフェノチアジンが９０％を超える還元を達成するような様式で与えることを含む、請求項３９～５２、請求項５６または請求項５７のいずれか一項記載の方法、使用または組成物。
【請求項６０】
治療または予防が、チオニンであるフェノチアジンを患者に与えることを含み、そしてこれを患者に一日当たりの用量で１～１０００mgを、場合によっては１～８単位に分割して与える、請求項３９～５２、請求項５６または請求項５７のいずれか一項記載の方法、使用または医薬用組成物。
【請求項６１】
治療または予防が患者にメチレンブルーであるフェノチアジンを患者に与えることを含み、そして一日当たりの用量がおよそ３．２～３．５mg/kgである、請求項３９～５２、請求項５６または請求項５７のいずれか一項記載の方法、使用または医薬用組成物。
【請求項６２】
方法が、（ｉ）鋳型フラグメントが、細胞に対して有毒でないレベルで構成的に細胞において発現されるような前駆体タンパク質の鋳型フラグメント；および
（ｉｉ）タンパク質が、刺激に応答して細胞において誘導的に発現される前駆体タンパク質；
をコードする核酸を細胞に導入する工程を含む、請求項１～３８のいずれか一項記載の方法において使用するための安定した細胞を産生する方法。
【請求項６３】
前駆体タンパク質をコードする核酸が、ｌａｃ誘導性プロモーターに作動可能に連結されている、請求項６２記載の方法。
【請求項６４】
前駆体タンパク質の発現が、ＩＰＴＧを１～５０mMで添加することにより誘導される、請

求項６３記載の方法。
【請求項６５】
鋳型フラグメントをコードする核酸が、サイトメガロウイルスプロモーター配列に作動可能に連結されている、請求項６２～６４のいずれか一項記載の方法。
【請求項６６】
鋳型フラグメントをコードする核酸が、鋳型ベクターとして導入され、そして前駆体タンパク質をコードする核酸が、別個の前駆体タンパク質ベクターとして導入される、請求項６２～６５のいずれか一項記載の方法。
【請求項６７】
前駆体タンパク質ベクターが、前駆体タンパク質をコードする核酸がクローン化されているｐＯＰＲＳＶＩＣＡＴベクターに由来する、請求項６６記載の方法。
【請求項６８】
鋳型フラグメントベクターが、前駆体タンパク質をコードする核酸がクローン化されているプラスミドｐＺｅｏ２９５～３９１ベクターに由来する、請求項６６または請求項６７記載の方法。
【請求項６９】
前駆体タンパク質が、タウである、請求項６２～６８のいずれか一項記載の方法。
【請求項７０】
鋳型フラグメントをコードする核酸が、タウのコアフラグメントをコードする、請求項６９記載の方法。
【請求項７１】
鋳型フラグメントをコードする核酸が、全長タンパク質のアミノ酸１８６～２９６から３９０～４４１の間に広がるタウのフラグメントをコードする、請求項７０記載の方法。
【請求項７２】
鋳型フラグメントをコードする核酸が、図７で示す全長タウタンパク質のアミノ酸２９５、２９６または２９７からアミノ酸残基３９０または３９１に広がるタウのフラグメントをコードする、請求項７１記載の方法。
【請求項７３】
鋳型フラグメントをコードする核酸が、図７で示すアミノ酸残基２９５～３９１に広がるタウのフラグメントをコードする、請求項７２記載の方法。
【請求項７４】
（ｉ）鋳型フラグメントが、細胞に対して有毒でないレベルで細胞において構成的に発現されるような前駆体タンパク質の鋳型フラグメント；および
（ｉｉ）疾患タンパク質が、刺激に応答して細胞において誘導的に発現される前駆体タンパク質；
をコードする核酸を含む物質の組成物であって、その核酸は、請求項６２～７２のいずれか一項に記載されている、組成物。
【請求項７５】
（ｉ）鋳型フラグメントが、細胞に対して有毒でないレベルで細胞において構成的に発現されるような前駆体タンパク質の鋳型フラグメント；および
（ｉｉ）疾患タンパク質が、刺激に応答して細胞において誘導的に発現される前駆体タンパク質；
を発現するように、請求項７４の核酸で形質転換された哺乳動物宿主細胞。
【請求項７６】
ニューロン細胞系または繊維芽細胞系に由来する、請求項７５記載の細胞。
【請求項７７】
以下の細胞系：３Ｔ３；ＮＩＥ－１１５；３Ｔ６；Ｎ２Ａ；ＳＹ５Ｙ；ＣＯＳ－７から選択される、請求項７６記載の細胞。
【請求項７８】
前駆体タンパク質の刺激性産生のための作用物質または前駆体タンパク質の鋳型フラグメ

ントとの相互作用を検出するための作用物質から選択される少なくとも一つの別の成分に加えて、請求項７５～７７のいずれか一項記載の宿主細胞を含むキット。
【請求項７９】
検出用作用物質が、抗体である、請求項７８記載のキット。
【請求項８０】
下記：
５’－３’　Ｔ４０－ＮｏｔＩ
５’－ｇｔｃ　ｇａｃ　ｔｃｔ　ａｇａ　ｇｇｃ　ｇｇｃ　ｃｇｃ　ＡＴＧ　ＧＣＴ　ＧＡＧ　ＣＣＣ　ＣＧＧ　ＣＡＧ　ＧＡＧ－３’
３’－５’　Ｔ４０－ＮｏｔＩ
５’－ａｃｔ　ｃｔｔ　ａａｇ　ｇｇｔ　ｃｇｃ　ｇｇｃ　ｃｇｃ　ＴＣＡ　ＣＡＡ　ＣＡＡ　ＡＣＣ　ＣＴＧ　ＣＴＴ　ＧＧＣ　ＣＡＧ－３’
２９５センスプライマー
５’－ＣＧＧ　ＡＡＴ　ＴＣＣ　ＡＣＣ　ＡＴＧ　ＧＡＴ　ＡＡＴ　ＡＴＣ　ＡＡＡ　ＣＡＣ　ＧＴＣ　ＣＣＧ－３’
３９１アンチセンスプライマー
５’－Ｃ　ＧＣＧ　ＧＧＡ　ＴＣＣ　ＴＣＡ　ＣＴＣ　ＣＧＣ　ＣＣＣ　ＧＴＧ　ＧＴＣ　ＴＧＴ　ＣＴＴ　ＧＧＣ－３’
から選択される核酸プライマー。
【発明の詳細な説明】
【技術分野】
【０００１】
本発明は、細胞ベースのモデルおよび神経変性疾患に伴うタンパク質の凝集をモデル化するその他の試験系に関する。更に本発明は、かかる凝集を調整できる化合物に関する。
【０００２】
痴呆、例えばアルツハイマー病（ＡＤ）の状態は、患う患者の脳におけるタンパク質様構造、例えばβ－アミロイドプラークおよび神経原繊維濃縮体の細胞内および／または細胞外沈着の蓄積の進行をしばしば特徴とする。これらの損傷の外観は、主に病理学的神経原繊維変性および脳萎縮症並びに認識障害に相関する（Ｍｕｋａｅｔｏｖａ－Ｌａｄｉｎｓｋａ，Ｅ．Ｂ．ら、Ａｍ．Ｊ．Ｐａｔｈｏｌ．１５７（２）：６２３～６３６（２０００）。
【０００３】
神経炎プラークおよび神経原繊維濃縮体の双方は、ペアードヘリカルフィラメント（ＰＨＦ）を含有し、その主要な構成要素は、微小管関連タンパク質タウである（Ｗｉｓｃｈｉｋら、ＰＮＡＳ　ＵＳＡ８５；４５０６（１９８８））。プラークは、またアミロイド前駆体タンパク質のプロセシング異常から誘導される細胞外β－アミロイド筋原繊維をも含有する（ＡＰＰ；Ｋａｎｇら、Ｎａｔｕｒｅ　３２５：７３３（１９８７））。Ｗｉｓｃｈｉｋらの論文（「Ｎｅｕｒｏｂｉｏｌｏｇｙ　ｏｆ　Ａｌｚｈｅｉｍｅｒ’ｓ　Ｄｉｓｅａｓｅ」第２版（２０００年）、Ｄａｗｂａｒｎ，Ｄ．およびＡｌｌｅｎ，Ｓ．Ｊ．編、分子および細胞神経生物学シリーズ、バイオス・サイエンティフィック・パブリッシャーズ、オックスフォード）では、神経変性痴呆の病因におけるタウタンパク質の推定される役割が詳細に論じられている。
【０００４】
アルツハイマー病の研究によりタウの正常の形態の喪失（Ｍｕｋａｅｔｏｖａ－Ｌａｄｉｎｓｋａら、Ａｍ．Ｊ．Ｐａｔｈｏｌ．１４３：５６５（１９９３）；Ｗｉｓｃｈｉｋら、Ｎｅｕｒｏｂｉｏｌ．Ａｇｅｉｎｇ　１６：４０９（１９９５ａ））；Ｌａｉら、Ｎｅｕｒｏｂｉｏｌ．Ａｇｅｉｎｇ　１６：４３３（１９９５ｂ））、病理学的なＰＨＦの蓄積（Ｍｕｋａｅｔｏｖａ－Ｌａｄｉｎｓｋａら、前出（１９９３）；Ｈａｒｒｉｎｇｔｏｎら、Ｄｅｍｅｎｔｉａ　５：２１５（１９９４ａ）；Ｈａｒｒｉｎｇｔｏｎら、Ａｍ．Ｊ．Ｐａｔｈｏｌ．１４５：１４７２（１９９４ｂ）；Ｗｉｓｃｈｉｋら、前出（１９９

５ａ））および中前頭皮質のシナプス喪失（Ｔｅｒｒｙら、Ａｎｎ．Ｎｅｕｒｏｌ．３０：５７２（１９９１））が、随伴認識障害と相関することが示されている。更に、シナプスの喪失（Ｔｅｒｒｙら、前出）および錐体細胞の喪失（Ｂｏｎｄａｒｅｆｆら、Ａｒｃｈ．Ｇｅｎ．Ｐｓｙｃｈｉａｔｒｙ ５０：３５０（１９９３））の双方は、タウ反応性神経原繊維病理の形態測定に相関し、これは、分子レベルではアルツハイマー病におけるタウタンパク質プールの可溶性形態から重合形態（ＰＨＦ）にほとんどすべてが再配分されることと並行している（Ｍｕｋａｅｔｏｖａ－Ｌａｄｉｎｓｋａら、前出（１９９３）；Ｌａｉら、前出（１９９５））。

【０００５】

タウは、選択的スプライシングアイソフォームに存在し、これは、微小管結合ドメインに対応する反復配列の三つまたは四つのコピーを含有する（Ｇｏｒｄｅｒｔ，Ｍ．ら、ＥＭＢＯ Ｊ．８：３９３～３９９（１９８９）；Ｇｏｅｄｅｒｔ，Ｍら、Ｎｅｕｒｏｎ ３：５１９～５２６（１９８９））。ＰＨＦのタウは、タンパク質分解的にプロセシングされて反復ドメインの位相変化したものから構成されるコアドメインになり（Ｗｉｓｃｈｉｋ，Ｃ．Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８５：４８８４～４８８８（１９８８）；Ｗｉｓｃｈｉｋ，ら、ＰＮＡＳ ＵＳＡ ８５：４５０６～４５１０（１９８８）；Ｎｏｖａｃ，Ｍ．ら、ＥＭＢＯ Ｊ．１２：３６５～３７０（１９９３））；三つの反復のみが安定したタウ－タウ相互作用に関係する（Ｊａｋｅｓ，Ｒ．ら、ＥＭＢＯ Ｊ．１０：２７２５～２７２９（１９９１））。一度形成されると、ＰＨＦ様タウ凝集物は、さらなる捕捉の種として作用し、全長タウタンパク質のタンパク質分解性プロセシングの鋳型を提供する。（Ｗｉｓｃｈｉｋら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９３：１１２１３～１１２１８（１９９６））。

【０００６】

ペアードヘリカルフィラメント（ＰＨＦ）は、その形成および蓄積中に最初に細胞質内で集合し、おそらく、ＰＨＦ集合の前または集合中に断端されるようになる初期のタウオリゴマーから無晶形凝集物を形成する（Ｍｅｎａ，Ｒ．ら、Ａｃｔａ Ｎｅｕｒｏｐａｔｈｏｌ．８９：５０～５６（１９９６）；（Ｍｅｎａ，Ｒ．ら、Ａｃｔａ Ｎｅｕｒｏｐａｔｈｏｌ．９１：６３３～６４１（１９９６））。次いでこれらのフィラメントは、典型的な細胞内神経原繊維濃縮体を形成し続ける。この状態では、ＰＨＦは、断端されたタウのコアおよび全長タウを含有する微毛性外被から成る。（Ｗｉｓｃｈｉｋ，Ｃ．Ｍ．ら、前出（１９９６））。集合過程は、指数関数的であり、正常な機能のタウの細胞性プールを消費し、そして欠損を補うために新規タウの合成を誘起する。（Ｌａｉ，Ｒ．Ｙ．Ｋ．ら、Ｎｅｕｒｏｂｉｏｌｏｇｙ ｏｆ Ａｇｅｉｎｇ １６（３）：４３３～４４５（１９９５））。実際に、ニューロンの作動可能障害は、細胞外濃縮体を残して、細胞死に至るまで発達する。細胞死は、細胞外濃縮体の数と高度に相関する（Ｗｉｓｃｈｉｋら、前出（２０００））。濃縮体は、細胞外空間まで押し出されるので、対応するＮ－末端タウ免疫反応性の損失を伴うニューロンの微毛性外被の進行性の損失があるが、タウ免疫反応性の保存は、ＰＨＦコアに伴う（図１；Ｂｏｎｄａｒｅｆｆ，Ｗ．ら、Ｊ．Ｎｅｕｒｏｐａｔｈ．Ｅｘｐｅｒ．Ｎｅｕｒｏｌ．５３（２）：１５８～１６４（１９９４））。

【０００７】

ＰＨＦに組み込まれたタウの反復ドメインにおいて認められた位相変化は、反復ドメインが、フィラメントへの組み込みの間に誘起される立体配座変化を被ることを示唆している。アルツハイマー病の発症の間に、この立体配座変化が、病理学的基質、例えば損傷を受けたか、または突然変異した膜タンパク質へのタウの結合により開始され得ることが認識されている。（図２－またＷｉｓｃｈｉｋ，Ｃ．Ｍ．ら、Ｍｉｃｒｏｔｕｂｕｌｅ－ａｓｓｏｃｉａｔｅｄ ｐｒｏｔｅｉｎｓ：ｍｏｄｉｆｉｃａｔｉｏｎｓ ｉｎ ｄｉｓｅａｓｅ、Ａｖｉｌａ，Ｊ．、Ｂｒａｎｄｔ，Ｒ．およびＫｏｓｉｋ，Ｋ．Ｓ．編（ハーウッド・アカデミック・パブリッシャーズ、アムステルダム）１８５～２４１頁）をも参照）。

【０００８】

アルツハイマー病の場合、現在の医薬的治療は、神経変性の結果であるコリン作動性伝達の喪失の症状の処置に焦点が置かれている（Ｍａｙｅｕｘ，Ｒ．ら、Ｎｅｗ　Ｅｎｇ．Ｊ．Ｍｅｄ．３４１：１６７０～１６７９（１９９９））。しかしながら、利用可能な処置は、疾患の進行を最大６～８カ月遅らせるが、それを防御することはない。神経変性に至るタウの凝集を防御する薬物の発見により、凝集を開始する多様な上流の事象の即座な知識を必要としない、予防または疾患の進行の阻害のための更に効果的な計画が提供されるであろう（図３参照）。

【０００９】

モデルおよびアッセイ

ＷＯ９６／３０７６６は、固相基質に吸収されているコア反復ドメインに対応するタウフラグメントが可溶性全長タウを捕捉し、高親和性でタウと結合できる、タウ凝集に関するインビトロアッセイについて記載している（図４参照）。この結合は、凝集したタウ分子の反復ドメインにおけるタンパク質分解性消化に対する安定性を付与する。過程は、自己伝播性であり、典型的な医薬品により選択的に遮断され得る（Ｗｉｓｃｈｉｋら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　９３：１１２１３～１１２１８（１９９６））。

【００１０】

ＷＯ９６／３０７６６に記載されたインビトロアッセイによりタウ－タウ結合のインヒビターまたはモジュレーターの同定が可能になるが、本発明は、アルツハイマー病様タンパク質凝集の細胞ベースのモデルが有用であるとも認識している。かかる細胞性モデルを、タウ－タウ凝集のモジュレーター候補物質の１次スクリーニング、およびＷＯ９６／３０７６６のインビトロアッセイにおいて既に同定された化合物の２次スクリーニングの双方において用いることができる。更に、細胞におけるタウ凝集の実証により、病理学的なタウ凝集の開始に関与する正常な細胞基質の同定も助けられ、その基質は、それ自体医薬的介入の標的になり得る。

【００１１】

しかしながら、多くの文献により、組織培養モデルにおける種々のタウ構築物の発現は、凝集の実証に失敗したと報告されている（例えばＢａｕｍ，Ｌ．ら、Ｍｏｌ．Ｂｒａｉｎ　Ｒｅｓ．３４：１～１７（１９９５））。例えば、３Ｔ３マウス繊維芽細胞は、タウタンパク質を有さず、したがって組換えタウが、内因性マウスタウとは独立して発現され得る細胞環境を提示する。種々細胞系のトランスフェクションが、以前に報告されている（Ｋａｎａｉら（１９８９）；ＧｏｅｄｅｒｔおよびＪａｋｅｓ（１９９０）；Ｋｎｏｐｓら（１９９１）；ＬｅｅおよびＲｏｏｋ（１９９２）；Ｇａｌｌｏら（１９９２）；Ｌｏら（１９９３）；Ｍｏｎｔｅｊｏ　ｄｅ　Ｇａｒｃｉｎｉら（１９９４）；Ｆａｓｕｌｏら（１９９６））。しかしながら、かかる細胞系の断端されたタウの安定した長期間の発現は、達成されなかった。例えば、１６４または１７３～３３８または３５２までの残基のためのタウ構築物は、タンパク質を発現しなかった（ＬｅｅおよびＲｏｏｋ（１９９２））。

【００１２】

Ｆａｓｕｌｏら（Ａｌｚｈｅｉｍｅｒ’ｓ　Ｒｅｓｅａｒｃｈ　２：１９５～２００（１９９６））は、ＣＯＳ細胞における断端されたタウの一過性の発現について報告したが、このタウの安定した長期間の発現に関するデータは、示されていなかった。これらの研究者は、一過性のトランスフェクション系の使用から、断端されたタウ単独の発現が薬物を試験するのに適した様式でタウ凝集を誘起するのに十分ではなかった、と結論づけた。

【００１３】

これまでのところ、インビトロでの可溶性タウの凝集は、非生理学的条件下で、および高濃度で達成されただけである。（Ｗｉｓｃｈｉｋ、前出（２０００）を再考されたい）ＷＯ９６／３０７６６は、また細胞環境におけるタウ凝集を研究するための二つの研究法をも記載している。第１の研究法では、全長タウまたはタウのフラグメントが、細胞において安定して発現された。第２の研究法では、リポフェクチンの使用により凝集したタウ

が一過的に細胞にトランスフェクトされた。

【００１４】

これらの研究法は、共にタウ－タウ凝集の研究に関して有用であるが、これらには、ある限界がある。リポフェクションを用いる、凝集したタウの細胞へのトランスフェクションは、凝集したタウそのもののインビトロ産生と同様に効率が変化しやすい。更に、タウ凝集のための最も有効な種であるコアタウフラグメントは、細胞において安定して発現される場合、有毒であることが見出され、低レベル発現に導かれる。このように、真核細胞におけるＰＨＦコアの断端されたタウフラグメントの構成的発現は、達成するのが困難である。一過性発現系によりタウ発現の最適化が可能になるが、フラグメントの固有の有毒によりこれらの系が信頼性のないものになっている。タウのより長いフラグメントは有毒が低いが、これらは、細胞において発現される場合に凝集が不確実である。

【００１５】

したがって、例えばタウ分子等の間の相互作用が、安定した、そして制御可能な細胞系において生理学的条件下で調査され得る、そしてアルツハイマー病のごとき症状の可能性のある診断、予後または治療用物質に関するスクリーニングに用いることができる代替のモデル系が開発されるのが望ましい。

【００１６】

発明の開示

本発明者らは、その凝集が、神経変性疾患に伴うタンパク質の鋳型発動タンパク質分解性プロセシングをモデル化するために用いることができる安定した細胞試験系を考案した。タウタンパク質を実例に挙げた一つの試験系では、タウタンパク質のフラグメントの非常に低レベルの構成的発現を全長タウの誘導発現と組み合わせた。全長タウの誘導により、タンパク質分解性変換が導かれ、プロセシングされたフラグメントに至り、これによりタウの「鋳型によるタンパク質分解性プロセシング」が生じたことが確認される。誘導された全長タウからプロセシングされた１２ｋＤのフラグメントの産生の阻害によるタウ凝集インヒビターの影響の実証が、系により容易に可能になる。

【００１７】

１２ｋＤフラグメントの固有の毒性特性にかかわらず、かかる安定した系が達成され得るということは特に驚くべきことである。例えば、以下の実施例で実証されるように、全長タウのＮ－末端またはＣ－末端のいずれかでの部分的なトランケーションが結果的に安定した発現が維持される細胞系をもたらし、これらのより長い構築物は、微小管ネットワークへの結合よりもむしろ凝集に対する弱い性向のみを示す。タウフラグメントの組み合わせの安定した発現は、細胞の細胞質内で凝集を生じるが、この系は、再現性を維持することはできない。１２ｋＤフラグメントの誘導発現に基づく系は、フラグメントの予測できない細胞内凝集の結果として有毒をもたらす。

【００１８】

したがって、生存可能又は生存不能のいずれかである細胞系を産生する、凝集を誘導することそして一方では毒性と、タウが低い凝集に対する性向を有する生存可能細胞を維持することとの間には、二律背反があると思われる。これにもかかわらず、本発明の誘導タウ発現系は、安定で、そしてしかもスクリーニング等で用いるための制御されたタンパク質の凝集を提供できる。

【００１９】

加えて、アッセイの使用によりタンパク質凝集の特定のインヒビター（例えばフェノチアジン）の作用メカニズムは、先行技術に基づいて推測されていたように、実質的な酸化還元よりもむしろ主に自然状態での立体構造であるという証拠が提供されている。この発見は、本明細書で論じられる疾患という面で、かかる化合物の使用の選択、評価、処方および使用に関する予期しなかった含蓄を有している。拡散係数を測定することにより生得的固有に評価されたパラメーターは、インヒビターの可能性に高度に相関し得るので、とりわけ、拡散係数の評価により推定されるインヒビターを同定するか、または公知のインヒビターの構造または状態を最適化するための有用なスクリーニングが提供され得る。

【００２０】
更に還元形態のフェノチアジンの使用は、その阻害特性を増強するのに有利であることがアッセイにより示される。これらの観察は、本発明のさらなる側面の基礎を成すものである。
【００２１】
概して本発明は、安定した細胞系でタンパク質分解性プロセシングを介して前駆体タンパク質を前駆体タンパク質のフラグメント産生物に変換するための方法を提供し、該方法は、（ａ）（ｉ）鋳型フラグメントが、細胞に対して有毒でないレベルで細胞において構成的に発現されるような前駆体タンパク質の鋳型フラグメント；および（ｉｉ）刺激に応答して細胞においてタンパク質の発現が誘導される前駆体タンパク質をコードする核酸でトランスフェクトされた安定した細胞系を提供することの工程を含み、それにより鋳型フラグメントと前駆体タンパク質との相互作用が、前駆体タンパク質において、例えば凝集および前駆体タンパク質のフラグメント産生物へのタンパク質分解性プロセシングを引き起こすような立体配座変化を引き起こす。
【００２２】
この方法は、前駆体タンパク質が細胞内で発現されるように、細胞を刺激物質に供することを含んでよい。しかしながら刺激の不在下でさえ、低レベルであるが検出可能なレベルの発現が引き起こされる誘導プロモーターを用いる態様では、次の刺激工程を排除できる。
【００２３】
概して、前駆体タンパク質は、インビボで立体配座の重合化相互作用（自己伝播様式で）を被ることができるものであり、最終的には、産生物フラグメントを含み、そして病態に伴う凝集物の形成に至る。本明細書で提供される方法で得られる産生物フラグメントは、病理学的凝集および、インビボで一つまたはそれ以上の有毒産生物の産生および病態に至るタンパク質分解産生物プロセシングの測定である。本発明の方法の産生物フラグメント（または一つ以上のフラグメント）は、有毒でよいか、または単純に病理学的な凝集過程の指標として用いることができる。
【００２４】
この方法が根拠とするタンパク質および相互作用について以下でより詳細に論じる。
本発明者らは、細胞系に対して有毒でない、すなわち細胞系が、生存可能である（第１の）濃度で鋳型フラグメントを構成的に発現することは、思いがけずに可能であることを実証した。例えば、ＷＯ９６／３０７６６の図２９で示される種類の細胞異常をも示さない。
【００２５】
それにもかかわらず（例えば規定の時間での刺激の添加により)、細胞に対して有毒であり、病態に対応する（第２の、より高度な）濃度まで、このように蓄積できる産生物フラグメント（これは鋳型フラグメントと同一、広義には等価、または全く異なる）まで前駆体タンパク質をプロセシングする種をまくことができる。今度は、これにより産生物フラグメントの影響に伴う病態をモデル化し、そして産生物フラグメントの作製に及ぼすモジュレーターの影響を評価するための便利な方法が提供される。
【００２６】
種々のその他の別個の態様では、本発明により前駆体タンパク質のタンパク質分解性プロセシングおよび場合によっては前駆体タンパク質の産生物フラグメントへの凝集の開始、種まき、または制御のいずれかのための対応する方法が提供される。
各々の場合、方法は、前駆体タンパク質のタンパク質分解性プロセシングのレベルを（直接的または間接的に）モニターすることを含んでよい。
【００２７】
本発明の一つの態様では、繊維芽細胞（３Ｔ６）は、誘導プロモーターの制御下で全長タウ（「Ｔ４０」）および構成レベルの低いＰＨＦコアタウフラグメント（１２ｋＤフラグメント）を発現する。Ｔ４０発現が、この系で誘導される場合、これは、細胞内でＮ－末

端ではアミノ酸２９５～およびＣ－末端ではアミノ酸３９０～で凝集依存的トランケーションを受け、それにより、高レベルの１２ｋＤ　ＰＨＦコアドメインフラグメントが産生される。１２ｋＤフラグメントの産生は、タウ凝集インヒビターにより容量依存的な様式で遮断され得る。実際に、細胞内における１２ｋＤフラグメントのタンパク質分解的産生に関する化合物の阻害活性の定量化を、インビトロでタウ－タウ結合性の阻害を記載する同一のパラメーターという形で完全に記載することができる。すなわち、細胞内での１２ｋＤフラグメントのタンパク質分解的産生の程度は、反復ドメインによるタウ－タウ結合の程度により完全に決定される。細胞内の関連するプロテアーゼの有用性は無制限である。

【００２８】

前駆体タンパク質および疾患（タウオパチーを含む）

前記したように、本発明は、タンパク質が誘導された立体配座重合相互作用を受ける、すなわちタンパク質またはそのフラグメントにおける立体配座変化が、自己伝播の様式で別の（前駆体）タンパク質分子の鋳型結合および凝集を生じる疾患に伴ういずれかのタンパク質の使用周辺を根拠としてもよい。

一度核形成が開始されると、立体配座変化が凝集物に、別のタンパク質分解に対するさらなる抵抗性を与え得る、別のタンパク質分子の立体配座重合の誘起に関与する凝集カスケードが続いて起こり、さらなるタンパク質分解に対して実質的に抵抗する凝集物における有毒産生物フラグメントの形成に至る。このように形成されたタンパク質凝集物は、神経変性、臨床痴呆およびこの群の疾患のその他の病理学的症状の最も近い原因であると考えられる。

【００２９】

本発明の好ましい態様では、本発明は、タウタンパク質に基づく。本明細書で用いる「タウタンパク質」なる用語は、一般にタウタンパク質ファミリーのいずれかのタンパク質を意味する。タウタンパク質は、集合および分解の反復サイクル中に微小管と同時精製される多くのタンパク質ファミリーのなかの一つとして特徴付けられ（Ｓｈｅｌａｎｓｋｉら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　７０：７６５～７６８（１９７３））、微小管関連タンパク質（ＭＡＰ）として公知である。タウファミリーのメンバーは、特徴的なＮ－末端セグメント、脳において発達中に制御されるＮ－末端セグメントに挿入されたおよそ５０個のアミノ酸の配列、３１～３２個のアミノ酸の３または４タンデム反復から成る特徴的なタンデム反復領域、およびＣ－末端テールを共有する。

【００３０】

ＭＡＰ２は、細胞体樹状突起区画における主要な微小管関連タンパク質である（Ｍａｔｕｓ，Ａ．、「Ｍｉｃｒｏｔｕｂｕｌｅｓ」（ＨｙａｍｓおよびＬｌｏｙｄ編）１５５～１６６頁、ジョン・ウィレイ・アンド・サンズ、ニューヨーク）。ＭＡＰ２アイソフォームは、タンデム反復領域ではタウタンパク質とほとんど同一であるが、Ｎ－末端ドメインの配列および範囲で、双方は、実質的に異なる（ＫｉｎｄｌｅｒおよびＧａｒｎｅｒ、Ｍｏｌ．Ｂｒａｉｎ　Ｒｅｓ．２６：２１８～２２４（１９９４））。それにもかかわらず、タンデム反復領域における凝集は、タウ反応ドメインに選択的ではない。したがって本明細書におけるタウタンパク質またはタウ－タウ凝集に関連するいずれかの論考は、タウ－ＭＡＰ２凝集およびＭＡＰ２－ＭＡＰ２凝集等にも関連すると考えるべきであることは理解されよう。

【００３１】

図５は、本発明で用いることができる種々のその他の疾患随伴凝集タンパク質を列挙する表を示す。各々の場合で、（複数の）タンパク質の凝集および／または突然変異の開始が、役割を果たし得る疾患または複数の疾患もまた列挙される。疾患の活性の原因となるドメインまたは突然変異が列挙され、そして好ましくは、少なくともタンパク質のこの最小部分のすべてまたは一部が、本発明において用いられる鋳型フラグメントに含まれる。

表から見られるように、病理学的タンパク質凝集により特徴づけられる疾患の実例には運動ニューロン疾患およびレビ小体疾患が含まれる。

【００３２】
タウタンパク質（およびその機能またはプロセシング異常）が役割を果たし得るのはアルツハイマー病のみではないことは注目される。ニューロン変性性障害、例えばピック病および進行性核上性麻痺（ＰＳＰ）の病因は、各々新皮質の歯状回および星状錐体細胞における病理学的に断端されたタウ凝集物の蓄積に相関するようである。その他の痴呆症には前頭側頭性痴呆（ＦＴＤ）；染色体１７に関連したパーキンソン症候群（ＦＴＤＰ－１７）；脱抑制パーキンソン痴呆筋萎縮症候群（ＤＤＰＡＣ）；淡蒼球・橋・黒質変性（ＰＰＮＤ）；グアム－ＡＬＳ症候群；淡蒼球・黒質・ルイジアン変性（ＰＮＬＤ）；皮質基底変性（ＣＢＤ）およびその他のものなどがある（Ｗｉｓｃｈｉｋら、前出（２０００）、詳細な論議に関してはとりわけ表５．１参照）。これらの疾患は、すべてタウ凝集異常により主にまたは部分的に特徴づけられ、本明細書では「タウオパチー」と称する。

【００３３】
したがって、タウタンパク質またはタウ様タンパク質（例えばＭＡＰ２）に関して本発明の態様が記載される前記の論考を鑑みて（そしてそれ以外を要求する状況は除く)、記載は、前記で論じた別のタンパク質（例えばβアミロイド、シヌクレイン、プリオン等）または凝集の伝播に必須であるドメインにおける立体配座変化により類似の病理学的凝集が開始または行われ得る、またはこの形成された凝集物にタンパク質分解安定性を与えるその他のタンパク質に同等に適用されると考えるべきであると理解されよう（Ｗｉｓｃｈｉｋら、による文献（「Ｎｅｕｒｏｂｉｏｌｏｇｙ ｏｆ Ａｒｚｈｅｉｍｅｒ’ｓ Ｄｉｓｅａｓｅ」第２版（２０００）、Ｄａｗｂａｒｎ，Ｄ．およびＡｌｌｅｎ，Ｓ．Ｊ．編、分子および細胞神経生物学シリーズ、バイオス・サイエンティフィック・パブリッシャーズ、オックスフォード））。すべてのかかるタンパク質を本明細書では「凝集疾患タンパク質」と称し得る。

【００３４】
本明細書で「タウ－タウ凝集」等の記載を行ったのと同様に、これはまた別の「凝集タンパク質凝集」、例えばβアミロイド凝集、プリオン凝集およびシヌクレイン等にも適用できると考えられる。「タウタンパク質分解性分解」等も同様である。

【００３５】
鋳型フラグメント
本発明の好ましい態様では、鋳型フラグメントが前駆体タンパク質の「コアフラグメント」を含む、実質的に「コアフラグメント」からなるか、または「コアフラグメント」からなり、この用語は、前駆体タンパク質に結合して前記したタンパク質分解および凝集を開始または伝播できるタンパク質のその部分を意味する。
凝集する疾患タンパク質の場合、かかるコアフラグメントは、また凝集のタンパク質分解安定性に寄与するものである可能性がある。

【００３６】
したがって、例えば「タウコアフラグメント」は、タンデム反復領域から誘導される断端されたタウタンパク質配列を含むタウフラグメントであり、これは適当な条件下で、別のタウタンパク質またはＭＡＰ２タンパク質のタンデム反復領域に高い親和性で結合できる。したがってタウの場合、好ましいフラグメントは、実例を挙げると、非限定例としてはアルツハイマー病の脳のＰＨＦ（および、究極的には神経原繊維濃縮体）に存在するタウフラグメントがある。

【００３７】
したがって好ましいタウフラグメントは、約（およそ）２９５～２９７から約３９０～３９１に伸びてよいが（図６の「ｄＧＡＥ」参照)、以下で論じるように、かかるフラグメントの変種をもまた用いることができる。
ＡＰＰ（アミロイド前駆体タンパク質）の場合、例えば融合タンパク質として１～４０または１～４２アミノ酸のＡβドメインを含むＡＰＰのフラグメントの発現が好ましい。

【００３８】
その他のコアフラグメントは、例えば図５に関して論じられたドメインに基づいてよい。

鋳型フラグメントは、これらのタンパク質の二つまたは二つ以上（例えば融合体として）からのドメインを含んでよい。
鋳型フラグメントの全長は、アッセイおよび用いられている凝集疾患タンパク質コアフラグメントに適しているいずれの長さでもよいが、一般に、約２０、３０、４０、５０、６０、７０、８０、９０個などのアミノ酸の長さ以上である。しかしながら、望ましい場合、１００、２００または更には５００個以上でもよい態様もある。
【００３９】
誘導体
指定したタンパク質（例えば前駆体タンパク質、鋳型またはコアフラグメント）または列挙した核酸配列について論じる本明細書の全例において、対応する参照タンパク質（または核酸）の誘導体またはその他の変種が、参照配列の適当な特性を保持する場合、必要に応じてそれを用いてよい。かかる誘導体は、また参照配列と配列同一性を共有する。
例えば用いたタンパク質には、Ｎ－またはＣ－末端伸長が含まれ、その伸長は、タンパク質配列に対して異種性でよい。同様に、誘導体は、参照配列のアミノ酸挿入、欠失または付加の方法によるものである。例えば、タウタンパク質またはタウコアフラグメント、誘導体は、少なくともタウタンパク質のタンデム反復領域に類似する部分アミノ酸配列を含むが、天然のタウもしくはそのフラグメントの一つ以上のアミノ酸が置換されているか、もしくは削除されているか、または別のアミノ酸が挿入されている。
【００４０】
結合活性を増強するか、または除去するために、かかる変化を行うことができる（後者の場合は対照実験に有用である）。対照は、配列またはドメインの欠失を含有し、これらが有する凝集に及ぼす影響を調べることができる。
好ましい誘導体は、病態に伴うことが解っているか、または推測されるものに対応する突然変異を組み込むものでよい。これらは、タウ配列内のＰ３０１Ｓに対応する変化を含有できる（図７参照）。別の突然変異には、Ｇ２７２Ｖ、Ｇ３８９Ｒ、Ｐ３０１Ｌ、Ｎ２７９Ｋ、Ｓ３０５Ｎ、Ｖ３３７Ｍ、Ｇ２７２Ｖ、Ｋ２８０Δ、Ｒ４０６Ｗ（Ｗｉｓｃｈｉｋら、前出（２０００）をも参照）などがある。
【００４１】
別の好ましい誘導体には、前記で論じたコアフラグメントのタンデム反復、またはこれらのフラグメント内の結合ドメインなどがある。
更に別の誘導体は、配列が混合されているか、または組み合わされている複数の関連する疾患タンパク質に基づくキメラ産生物に基づくことができる。例えばタウの制限酵素フラグメントは、ＭＡＰ２のフラグメントまたは関連性のない遺伝子のフラグメントとでさえライゲートして組換え誘導体を作製することができる。コアフラグメントを修飾するための代替の試験計画は、Ｈｏら、Ｇｅｎｅ　７７：５１～５９（１９８９）に記載されるようなＰＣＲ、またはＤＮＡ混ぜ合わせ（Ｃｒａｍｅｒｉら、Ｎａｔｕｒｅ　３９１（１９９８））を用いる。
【００４２】
核酸構築物の使用
本発明の核酸、または本発明で使用するための核酸は、実質的に純粋もしくは均質な形態で、または元来の種の別の核酸を含まずに、もしくは実質的に含まずに、その天然の環境から単離および／または精製された形態で提供され得る。本明細書で用いる場合、「単離された」なる用語は、これらの可能性をすべて含む。例えば鋳型フラグメントをコードする核酸は、それが天然で一緒に見出されない（隣接して作動しない）が、人工的にライゲートされているかそうでなければ組み合わされている核酸配列を含むという点で、少なくとも部分的に合成性である。
【００４３】
本発明による核酸は、ｃＤＮＡ、ＲＮＡ、ゲノムＤＮＡおよび修飾された核酸または核酸アナログの形態であるか、またはそこから誘導され得る。ＤＮＡ配列が、例えば図を参照して指定されている場合、側面がそれ以外を要求するのでなければ、そこでＴがＵに置換

されているＲＮＡ等価物は含まれる。

【００４４】

前記したように、核酸は、問題の参照配列と相同性を共有する誘導体またはその他の変種をコードできる。好ましくは、問題の核酸および／またはアミノ酸配列は、変種が基づく配列の約５０％、または６０％、または７０％、または８０％、最も好ましくは少なくとも約９０％、９５％、９６％、９７％、９８％、または９９％の同一性を共有する。類似性または相同性は、技術分野で標準使用であるＡｌｔｓｃｈｕｌら、Ｊ．Ｍｏｌ．Ｂｉｏｌ．２１５：４０３～４１０（１９９０）のＴＢＬＡＳＴＮプログラム、または、そしてこれが好ましいのであるが、デフォルト・パラメーターを用いる標準的なプログラムＢｅｓｔＦｉｔ（ウィスコンシン・パッケージ、バージョン８、１９９４年１１月（ジェネティクス・コンピューター・グループ、５７５　サイエンス・ドライブ、マジソン、ウィスコンシン、米国、ウィスコンシン５３７１１）の一部である）により定義および決定できる。特定の配列相同性の核酸分子間のハイブリダイゼーションを達成するのに必要なストリンジェンシー条件を計算するための一般式は：T_m＝８１．５℃＋１６．６Ｌｏｇ［Na^+］＋０．４１（％　Ｇ＋Ｃ）－０．６３（％ホルムアミド）－６００／二重鎖の＃ｂｐ。

【００４５】

本明細書に含まれる情報および参照並びに技術分野で公知の技術（例えばＳａｍｂｒｏｏｋ、ＦｒｉｔｓｃｈおよびＭａｎｉａｔｉｓ、「Ｍｏｌｅｃｕｌａｒ　Ｃｌｏｎｉｎｇ、Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ」（１９８９）、コールド・スプリング・ハーバー・ラボラトリー・プレス、並びにＡｕｓｕｂｅｌら、Ｓｈｏｒｔ　Ｐｒｏｔｏｃｏｌｓ　ｉｎ　Ｍｏｌｅｃｕｌａｒ　Ｂｉｏｌｏｇｙ（１９９２）、ジョン・ウェレイ・アンド・サンズ、参照）を用いて技術者は、適当なタンパク質またはポリペプチドをコードする核酸配列を容易に調製できる。これらの技術には、（ｉ）関連する核酸、例えばゲノム供給源からのサンプルを増幅するためのポリメラーゼ連鎖反応（ＰＣＲ）の使用、（ｉｉ）化学的合成、または（ｉｉｉ）ｃＤＮＡ配列の調製などがある。

【００４６】

例えばタウコアフラグメントをコードするＤＮＡを作製でき、これを、コード化ＤＮＡを取ること、発現されるべき部分のいずれかの側の適当な制限酵素認識部位を同定すること、およびこのＤＮＡから上記部分を切り取ることによることを含む、当業者に公知のいずれかの適当な方法に用いることができる。タンパク質（例えばタウ）コード化配列への修飾を、例えば特定部位突然変異誘発を用いて作製することができる。

【００４７】

構築物

したがって、別の側面では、本発明はまた適当な前駆体および鋳型フラグメントタンパク質をコードする核酸分子にも関する。以下で論じるように、これらは、同一のまたは異なる構築物に存在してよく、そして後者の場合、組成物は、二つまたは二つ以上の型の構築物を含む組成物もまた提供される。

【００４８】

ベクターが一つまたはそれ以上の選択された宿主細胞において複製されることを可能にする核酸配列は、種々の細菌、酵母およびウイルスに関して公知である。例えば、種々のウイルス起源（ＳＶ４０、ポリオーマ、アデノウイルス、ＶＳＶまたはＢＰＶ）は、哺乳動物細胞におけるクローニングベクターとして有用である。本明細書で記載する核酸を含む発現ベクター、例えば細胞により取り込まれ得るプラスミド、コスミド、ウイルス粒子、ファージ、またはいずれかその他の適当なベクターもしくは構築物の形態でよく、そしてこれを適当に発現させる。

【００４９】

発現ベクターは、ｍＲＮＡ合成を指向するように、通常目的のタンパク質コード化核酸配列に作動可能に連結されるプロモーターを含有する。種々の可能性のある宿主細胞により認識されるプロモーターは、公知である。「作動可能に連結された」とは、同一の核酸分

子の一部として結合され、適当に配置され、そして転写が、プロモーターから開始されるように指向されていることを意味する。プロモーターに作動可能に連結されたＤＮＡは、プロモーターの「転写制御下」にある。哺乳動物宿主細胞におけるベクターからの転写は、ウイルス、例えばポリオーマウイルス、鶏痘ウイルス、アデノウイルス（例えばアデノウイルス２)、ウシ乳頭腫ウイルス、トリ肉腫ウイルス、サイトメガロウイルス、レトロウイルス、Ｂ型肝炎ウイルスおよびシミアンウイルス４０（ＳＶ４０）のゲノムから、異種性哺乳動物プロモーター、例えばアクチンプロモーターまたは免疫グロブリンプロモーターから、および熱ショックプロモーターから得られるプロモーターにより、かかるプロモーターが宿主細胞に適合する場合に制御される。真核宿主細胞（酵母、菌類、昆虫、植物、動物、ヒト、またはその他の多細胞生物からの有核細胞）において用いられる発現ベクターは、転写の終止およびｍＲＮＡの安定化に必要な配列をも含有する。

【００５０】

鋳型フラグメントに用いるプロモーターは、「構成性」である。このプロモーターは、十分弱く、その凝集およびタンパク質分解性プロセシングに至る前駆体タンパク質に及ぼすその影響による（間接的に）以外は、細胞で発現される鋳型フラグメントのレベルは、慣用される技術を用いてそれ自体（直接的に）検出できない（すなわち、上記凝集が阻害される場合、有効に検出できない）。当業者は、本開示を鑑みて上記のような過度な負担なしにかかるプロモーターを選択できる。

前駆体タンパク質の場合、プロモーターは、当業者によりよく理解されている、いわゆる「誘導可能」でよく、発現は、適用された刺激に応答して「スイッチ・オン」または増加する。刺激の特性は、プロモーター間で変化する。ある誘導プロモーターは、適当な刺激の不在下では、わずか、または検出不能なレベルの発現（または発現しない）しか引き起こさない。また別の誘導プロモーターは、刺激の不在下で検出可能な構成的発現を引き起こす。刺激の不在下での発現のレベルにかかわらず、いずれかの誘導プロモーターからの発現は、正しい刺激の存在下で増加する。以下の実験では、Ｌａｃ誘導プロモーターを使用している。

【００５１】

本発明の発現ベクターは、一つ以上の選択遺伝子をも含有できる。典型的な選択遺伝子は、（ａ）抗生物質またはその他の毒素、例えばアンピシリン、ネオマイシン、メソトレキセート、またはテトラサイクリンに対する抵抗性を付与する、（ｂ）栄養要求欠損を補足する、または（ｃ）複合培地から利用できない必須栄養を供給するタンパク質をコードし、例えばバチルスのＤ－アラニンラセマーゼをコードする遺伝子である。哺乳動物細胞に適した選択マーカーの実例は、所望のタンパク質コード化核酸を取り込む受容能力のある細胞の同定を可能にするもの、例えばＤＨＦＲまたはチミジンキナーゼである。野生型ＤＨＦＲを用いる場合、適当な宿主細胞は、ＤＨＦＲ作用が欠如したＣＨＯ細胞系であり、Ｕｒｌａｕｂら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　７７：４２１６（１９８０）に記載されるように調製および増殖される。酵母での使用に適した選択遺伝子は、酵母プラスミドＲｐ７に存在するｔｒｐ１遺伝子である［Ｓｔｉｎｃｈｃｏｍｂら、Ｎａｔｕｒｅ　２８２：３９（１９７９）；Ｋｉｎｇｓｍａｎら、Ｇｅｎｅ　７：１４１（１９７９）；Ｔｓｃｈｅｍｐｅｒら、Ｇｅｎｅ　１０：１５７（１９８０）］。ｔｒｐ１遺伝子は、トリプトファン中で成長する能力を欠いた酵母の突然変異株のための選択マーカー、例えばＡＴＣＣ：４４０７６またはＰＥＰ４－１を提供する［Ｊｏｎｅｓ，Ｇｅｎｅｔｉｃｓ　８５：１２（１９７７）］。

【００５２】

したがって、本発明で用いるための典型的なベクターには、複製起点、必要に応じて構成または誘導プロモーターに作動可能に連結された一つまたはそれ以上のタンパク質配列、転写終止配列、エンハンサーエレメント、マーカー遺伝子などが含まれ得る。種々のこれらの成分を含有する適当なベクターの構築には、技術者に公知の標準的なライゲーション技術を用いる。

【００５３】

形質転換

本発明は、また前記した方法において用いるための安定した細胞を産生する方法をも提供し、この方法は：（ａ）（ｉ）鋳型フラグメントが、細胞に対して有毒でないレベルで細胞において構成的に発現されるような前駆体タンパク質の鋳型フラグメント；および（ｉｉ）刺激に応答して疾患の細胞においてタンパク質の発現が誘導されるような前駆体タンパク質をコードする核酸を細胞に導入すること、の工程を含む。

【００５４】

一般に「形質転換」として限定されないで言及され得る導入は、いずれか利用可能な技術を用いることができる。真核細胞では、適当な技術には、リン酸カルシウムトランスフェクション、ＤＥＡＥデキストラン、電気泳動、リポソーム媒介トランスフェクションおよびレトロウイルスもしくはその他のウイルス、例えばワクシニアまたは昆虫細胞に関してはバキュロウイルスを用いる形質導入などがある。Ｓａｍｂｒｏｏｋら、前出に記載された塩化カルシウムを用いるカルシウム処置、またはエレクトロポレーションは、一般に原核細胞または実質的な細胞壁を含有するその他の細胞に関して用いられる。アグロバクテリウム・ツメファシエンス（Ａｇｒｏｂａｃｔｅｒｉｕｍ　ｔｕｍｅｆａｃｉｅｎｓ）での感染は、Ｓｈａｗら、Ｇｅｎｅ　２３：３１５（１９８３）および１９８９年6月２９日発行のＷＯ８９／０５８５９に記載されるように、特定の植物細胞の形質転換に関して用いられる。

【００５５】

かかる細胞壁を有さない哺乳動物細胞に関しては、Ｇｒａｈａｍおよびｖａｎ　ｄｅｒ　Ｅｂ、Ｖｉｒｏｌｏｇｙ　５２：４５６～４５７（１９７８）のリン酸カルシウム沈殿法を用いることができる。哺乳動物細胞宿主系形質転換の一般的な側面は、米国特許第４，３９９，２１６号に記載されている。酵母への形質転換は典型的には、Ｖａｎ　Ｓｏｌｉｎｇｅｎら、Ｊ．Ｂａｃｔ．１３０：９４６（１９７７）およびＨｓｉａｏら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．（ＵＳＡ）　７６：３８２９（１９７９）の方法に従って実施する。しかしながら、細胞へのＤＮＡの導入のためのその他の方法、例えば核マイクロインジェクション、エレクトロポレーション、無傷細胞との細菌性プロトプラスト融合、またはポリカチオン、例えばポリブレン、ポリオルニチンによる方法をも用いることができる。哺乳動物を形質転換するための種々技術に関しては、Ｋｅｏｗｎら、Ｍｅｔｈｏｄｓ　ｉｎ　Ｅｎｚｙｍｏｌｏｇｙ　１８５：５２７（１９９０）およびＭａｎｓｏｕｒら、Ｎａｔｕｒｅ　３３６：３４８～３５２（１９８８）を参照されたい。

【００５６】

宿主細胞

本発明に用いるのに適当な宿主細胞には、細菌、真核細胞、例えば哺乳動物および酵母細胞、並びにバキュロウイルス系などがある。

異種性ポリペプチドの発現のために技術分野で利用可能な哺乳動物細胞系には、繊維芽細胞３Ｔ６細胞、ＨｅＬａ細胞、ベビー・ハムスター腎臓細胞、ＣＯＳ細胞、ＳＶ４０により形質転換されたサル腎臓ＣＶ１系（ＣＯＳ－７、ＡＴＣＣ　ＣＲＬ１６５１）、チャイニーズハムスター卵巣細胞／－ＤＨＦＲ（ＣＨＯ、ＵｒｌａｕｂおよびＣｈａｓｉｎ、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　７７：４２１６（１９８０））；マウスセルトリ細胞（ＴＭ４、Ｍａｔｈｅｒ、Ｂｉｏｌ．Ｒｅｐｒｏｄ．２３：２４３～２５１（１９８０））；ヒト肺細胞（Ｗ１３８、ＡＴＣＣ　ＣＣＬ　７５）；ヒト肝臓細胞（Ｈｅｐ　Ｇ２、ＨＢ８０６５）；マウス乳腺癌細胞（ＭＭＴ　０６０５６２、ＡＴＣＣ　ＣＣＬ５１）；および多くのその他のものなどがある。

【００５７】

適当な原核細胞宿主には、非限定例としては、真性細菌、例えばグラム陰性またはグラム陽性生物、例えばエンテロバクテリアーシー（Ｅｎｔｅｒｏｂａｃｔｅｒｉａｃｅａｅ）、例えば大腸菌（Ｅ．ｃｏｌｉ）などがある。種々の大腸菌株は、公に入手可能であり、例えば大腸菌Ｋ１２　ＭＭ２９４株（ＡＴＣＣ　３１，４４６）；大腸菌Ｘ１７７６（ＡＴＣＣ　３１，５３７）；大腸菌Ｗ３１１０（ＡＴＣＣ　２７３２５）およびＫ５　７７

２（ＡＴＣＣ　５３６３５）である。真核性微生物、例えば糸状菌または酵母もまたベクターに適したクローニングまたは発現宿主である。サッカロミセス・セルビジエ（Ｓａｃｃｈａｒｏｍｙｃｅｓ　ｃｅｒｅｖｉｓｉａｅ）は、一般に用いられる下等真核性宿主微生物である。適当な宿主細胞の選択は、技術分野の技術範囲内であると考えられる。

【００５８】

別の側面では、本発明は、前記で記載された本発明の異種性核酸を含有する宿主細胞を提供する。本発明の核酸を宿主細胞のゲノム（例えば染色体）に組み込むことができる。標準的な技術に従ってゲノムとの組換えを促進する配列を封入することにより組み込みを促進できる。また別に、核酸を細胞内の染色体外ベクター上にあるか、またはそうでなければ細胞に対して認識可能な異種性もしくは外来性であってよい。

【００５９】

細胞は、前記した方法（核酸構築物の導入）により産生されるか、またはかかる細胞の祖先でよい。対応する細胞系もまた提供される。好ましい細胞系は、繊維芽細胞系、例えば３Ｔ６をベースにすることができる。

【００６０】

本明細書で記載した発現またはクローニングベクターでトランスフェクトまたは形質転換された宿主細胞を、プロモーターを誘導し、形質転換体を選択し、または望ましい配列をコードする遺伝子を増幅するために適当に修飾された、慣用される栄養培地中で培養することができる。培養条件、例えば培地、温度、ｐＨ等は、過度な実験を行わないで技術者により選択され得る。概して、細胞培養の生産性を最大にするための原理、プロトコル、および実施技術を「Ｍａｍｍａｌｉａｎ　Ｃｅｌｌ　Ｂｉｏｔｅｃｈｎｏｌｏｇｙ：ａ　Ｐｒａｃｔｉｃａｌ　Ａｐｐｒｏａｃｈ」Ｍ．Ｂｕｔｌｅｒ編、ＪＲＬプレス（１９９１）およびＳａｍｂｒｏｏｋら（前出）に見出すことができる。

【００６１】

遺伝子発現を、例えばサザン・ブロッティング、ｍＲＮＡの転写を定量化するための慣用されるノーザン・ブロッティング［Ｔｈｏｍａｓ、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　７７：５２０１～５２０５（１９８０）］、ドットブロッティング（ＤＮＡ分析）、またはインサイチュー・ハイブリダイゼーションにより、凝集疾患タンパク質の配列に基づいて、適当に標識されたプローブを用いてサンプル中で直接確認することができる。また別に、ＤＮＡ二重鎖、ＲＮＡ二重鎖、およびＤＮＡ－ＲＮＡハイブリッド二重鎖またはＤＮＡ－タンパク質二重鎖などの特異的二重鎖を認識できる抗体を用いることができる。

【００６２】

あるいは、遺伝子発現を免疫学的方法、例えば細胞または組織切片の免疫組織学的染色により、および細胞培養のアッセイにより測定して、遺伝子産生物の発現を直接定量化することができる。サンプル液の免疫組織学的染色および／またはアッセイに有用な抗体はモノクローナルまたはポリクローナルのいずれかでよく、いずれかの哺乳動物において調製することができる。便利なことには、凝集疾患ポリペプチドの元来の配列に対する抗体を調製することができる。

【００６３】

このように本発明の一つの側面は、例えば遺伝子発現のための条件下で（刺激の存在下）産生物フラグメントが産生されるように宿主細胞を培養することにより、本明細書で論じられた核酸からの発現を引き起こすか、または可能にする必要がある。本発明は、また産生物フラグメントを産生する方法をも包含し、方法は、前記した核酸からの発現を含む。

【００６４】

本発明の別の側面は、本明細書で前記した形質転換された細胞または細胞系に少なくとも一つの別の成分、例えば前駆体タンパク質の産生を刺激するための作用物質、または前駆体タンパク質と以下のセクションで記載する鋳型フラグメントとの相互作用を検出するための作用物質を加えたものを含むキットである。

【００６５】

凝集および／またはタンパク質分解性プロセシングおよび／または有毒フラグメントの検出

種々の態様では、以下の種のいずれか一つまたはそれ以上の濃度またはレベルをモニターすることにより、タンパク質分解性プロセシングまたは凝集の進行（またはその調整；以下を参照）を直接または間接的に検出することができる：前駆体タンパク質；産生物フラグメント；過程中に形成された副産物フラグメント；これらのいずれかの凝集（例えば沈降係数に基づいて）。

したがって、特定のタウタンパク質およびフラグメント（２９７～３５１フラグメントおよびＴ４０に基づく）で実例を示すように、主に前駆体タンパク質から誘導される１２ｋＤのプロセシングされた種のレベル増加に基づいて凝集をモニターできる。

【００６６】

前記の遺伝子発現に関していくつかのタンパク質検出法について論じる。本発明の方法の態様において抗体またはそのフラグメントを用いる場合、慣用される技術により産生することができる。ポリクローナル抗体を、例えば対応するタウ抗原を動物、好ましくはウサギに注射し、イムノアフィニティ精製により抗血清を回収することにより集めることができ、ここでポリクローナル抗体は、抗原が結合するカラムに通され、次いで、慣用される様式で溶出される。好ましくは、本発明は、タウエピトープに選択的であり、KohlerおよびMilsteinの方法により調製できるモノクローナル抗体を使用する。タウエピトープに対する適当なモノクローナル抗体を公知の方法により修飾して、Ｆａｂフラグメントまたは（Ｆａｂ’）２フラグメント、キメラ、ヒト化または一本鎖抗体態様を提供することができる。

【００６７】

本発明による抗体を、多くの方法で修飾することができる。実際に、「抗体」なる用語は、結合ドメインを有するいずれかの結合物質を必要な特異性で補っていると解釈すべきである。このように本発明は、抗体フラグメント、誘導体、作動可能等価物および、その形状が抗原またはエピトープに結合するのを可能にする抗体の形状を擬似する合成分子および分子などの抗体の相同体に及ぶ。

【００６８】

概して、抗体が、検出に用いられる場合、抗体は、レポーター分子を担持できる。また別に、未標識のタウ特異的１次抗体に結合できる２次抗体の使用により結合の検出を実施できる。この場合、２次抗体は、レポーター分子に連結されている。

【００６９】

非限定例としてラジオイムノアッセイ、「サンドウィッチ」アッセイ、酵素結合イムノソルベントアッセイ（ＥＬＩＳＡ）；蛍光イムノアッセイ、プロテインＡイムノアッセイ等の技術分野で公知のいずれかのイムノアッセイにおいて抗体を用いることができる。典型的には、イムノブロット法を使用される。好ましくは、イムノアッセイは、技術者に公知であるように、固相で実施される。例えば、抗体を、例えばアッセイカラムに吸収させ、そして、目的のタンパク質のいずれかの凝集、例えばタウ－タウ凝集の固相抗体への結合を可能にするのに適した条件下で、細胞サンプルをカラムを通して洗浄することができる。過剰の試薬を洗い流し、例えば前記および後記で実例を示すようないずれか適当な手段により、凝集タンパク質のカラムへの結合を検出することができる。

【００７０】

好ましいモノクローナル抗体は、以下のとおりである：

・断端されたタウ種および全長のタウ種間の結合の測定を可能にするタウエピトープのＮ－末端またはＣ－末端を認識するもの。とりわけ有用なものは、ヒト特異的エピトープを認識する抗体である。かかるモノクローナル抗体の一つ（２７／４９９と称する）は、タウのＧｌｙ－１６およびＧｌｎ－２６間の領域に位置するヒト特異的エピトープを認識し、そしてヒト以外の供給源から誘導される場合、それにより全長のタウ種間の結合の測定が可能になる（「アルツハイマー病の神経原繊維病理の進行におけるタウタンパク質のリン酸化異常の役割」ＰｈＤ　Ｔｈｅｓｉｓ、ケンブリッジ大学）。

【００７１】
・Ｇｌｕ－３９１で断端されたコアタウフラグメントを認識するもの。実例は、ｍＡｂ４２３（Ｎｏｖａｋら、前出（１９９３））である。この抗体は、ｍＡｂ４２３により認識されない、Ｇｌｕ－３９１で終止する断端されたコアタウフラグメントの、Ａｌａ－３９０で終止する類似のフラグメントへの結合の検出を可能にする。このトランケーションは、アルツハイマー病のＰＨＦ集合の過程で自然に生じる（Ｍｅｎａら、前出（１９９５）、（１９９６）；Ｎｏｖａｋら、前出（１９９３）；Ｍｅｎａら、前出（１９９１））。更に、タウが、インビトロで反復ドメインを介して結合する場合、プロテアーゼ（例えばプロマーゼ）での消化によりｍＡｂ４２３で検出可能なフラグメントを生じる（Ｗｉｓｃｈｉｋら、前出（１９９６））。本発明の好ましい側面では、この抗体を用いて鋳型フラグメントの構成的発現（Ａｌａ－３９０）からタンパク質分解的に切断された産生物フラグメント（Ｇｌｕ－３９１終止）の作製を区別することができる。

【００７２】
・反復ドメインにおける一般的なタウエピトープを認識するもの。好ましい態様は、抗体（例えばＭＡｂ７．５１）を利用する。タウ－ＭＡＰ２またはＭＡＰ２－ＭＡＰ２凝集を検出すべき場合、一般的なＭＡＰ－２エピトープを検出する抗体を用いることができる。抗体７．５１は、終わりから３番目のタウの反復に位置する一般的なタウエピトープを認識し（Ｎｏｖａｋら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８８：５８３７～５８４１（１９９１））、これは、タウがＰＨＦ様免疫化学的立体配座で結合する場合に塞がれるが、ギ酸処理の後暴露され得る（Ｈａｒｒｉｎｇｔｏｎら、前出（１９９０）、（１９９１）；Ｗｉｓｃｈｉｋら、前出（１９９５ａ））。正常な可溶性タウまたは微小管に結合したタウを、ギ酸処理を行わずにｍＡｂ７．５１を用いて検出することができる（Ｈａｒｒｉｎｇｔｏｎら、前出（１９９１）；Ｗｉｓｃｈｉｋら、前出（１９９５ａ））。タウ－タウ結合アッセイにおける全長タウの結合は、ｍＡｂ７．５１エピトープの部分的閉塞に伴う。

【００７３】
抗体２７／３４２は、タウ－タウ相互作用の過程で部分的に塞がれるＳｅｒ－２０８およびＳｅｒ－２３８間に位置する種非特異的一般的タウエピトープを認識する（Ｌａｉ、前出）。
いくつかのモノクローナル抗体の結合部位を図６に示す。

【００７４】
モジュレーターおよびインヒビターに関するスクリーニング
前記したように、本発明は、好ましくは、モデル化および、本明細書にて論じた疾患の処置のための治療用物質を同定する方法における、本明細書で提供されるようなシステムの使用に関する。

【００７５】
鋳型フラグメントとの相互作用に応答して、凝集および／または前駆体タンパク質の産生物へのタンパク質分解性プロセシングを調整する作用物質の能力を評価するための典型的な方法は：
（ａ）前記で論じた安定した細胞または細胞系を提供することと、
（ｂ）細胞を刺激に供し、前駆体タンパク質が、細胞において発現されるようにし、そしてそれにより、鋳型フラグメントと前駆体タンパク質との相互作用がタンパク質における立体配座変化を引き起こし、例えば凝集および前駆体タンパク質の産生物フラグメントへのタンパク質分解性プロセシングを引き起こすことと、
（ｃ）作用物質の存在下、産生物フラグメントの産生をモニターすることと、
（ｄ）場合によって工程（ｃ）で得られた値を参考値と比較することと、
を含む。

【００７６】
基準値は歴史的観察に基づくか、または並行して実施した対照実験に基づいてよく、例えばここでアッセイのうちの一部（鋳型フラグメント、前駆体タンパク質、刺激、作用物質

）を修飾するかまたは欠如する。
前記した種々の方法は、更に工程（ｄ）の結果を作用物質の調整活性と相関させる工程を含むことができる。
【００７７】
したがって、タンパク質が誘起される立体配座相互作用を被る疾患に伴うタンパク質の凝集のモジュレーターを同定する方法は、凝集を調整（阻害または逆転）できることが推測される一つまたはそれ以上の作用物質の存在下で前記した凝集を誘起する方法を実施することを含むことができる。作用物質の存在下または不在下で凝集の程度（および場合によってはタンパク質分解性プロセシング）を観察でき、相対値は、モジュレーターとしてのその活性に相関した。
【００７８】
例えば被検物質を前記した細胞の系に添加し、そして結合を可能にし、そして結合の阻害を実証するのに十分な時間、細胞をインキュベートしてよい。次いで、例えば適当に標識した抗体、例えばＭＡｂ７．５１を全細胞抽出物のイムノブロットで用いることにより、またはいずれかその他の適当な検出方法を用いることにより結合タウ複合体を検出することができる。
【００７９】
スクリーニング法がこの目的で、すなわち、調整／阻害化合物の同定のために用いられる場合、非競合または競合アッセイを用いることができる。例えば、技術分野で公知の型の競合アッセイでは、公知のインヒビターまたはモジュレーターの影響を別の被検物質または作用物質の存在下または不在下で比較して、被検物質が目的のタンパク質に対する結合に関して公知のインヒビター／モジュレーターと競合する能力を決定することができる。
前記したモジュレーター（例えばインヒビター）を精製する方法もまた提供されるが、これは、更にこの同定されたモジュレーターを産生する工程を含む。
【００８０】
阻害の特異性
本発明のこの側面によるスクリーニング法を用いて、疾患関連タンパク質凝集（例えばタウ－タウ、タウ－ＭＡＰ２またはその他のタンパク質結合）の選択的競合阻害の特性を実証する化合物に関して、前駆体タンパク質が関わるいずれかの「正常な」（例えばタウまたはＭＡＰ２のチューブリンへの、または相似による、その他の前駆体タンパク質と解っている限りのその結合パートナーとの）結合と干渉することなく、スクリーニングすることができる。
【００８１】
とりわけタウの場合、可能性のあるインヒビター／モジュレーターにより、タウ、ＭＡＰ２またはその誘導体のチューブリンへの結合のいずれか可能な干渉を決定するための適当な方法は、脱重合化チューブリンまたはタクソール安定化微小管の調製物を作用物質と接触させ、続いてタウ－チューブリンまたはＭＡＰ２－チューブリン結合を検出することを含む。またタウ－チューブリン結合を、例えばＷＯ９６／３０７６６に記載されるように、例えば正常な細胞骨格分布により実証することもできる。チューブリンタンパク質またはそのフラグメントの調製方法を結合パートナーと組み合わせることが可能であり、これは、技術分野で公知であり、そして例えばＳｌｏｂａｄａら（Ｃｅｌｌ Ｍｏｂｉｌｉｔｙ（１９７６）（Ｒ．Ｇｏｌｄｍａｎ、Ｔ．ＰｏｌｌａｒｄおよびＪ．Ｒｏｓｅｎｂａｕｍ編）コールド・スプリング・ラボラトリー、コールド・スプリング・ハーバー、ニューヨーク、１１７１～１２１２頁）により記載されている。
「疾患」および「正常な」機能を有する別のタンパク質のための類似の方法は、本発明の開示に鑑みて当業者に想定されよう。
【００８２】
細胞生存性
所望する場合、本発明の方法は、更に、例えば乳酸デヒドロゲナーゼアッセイキット（シグマ）を用いることによる、鋳型タンパク質および場合によっては前駆体タンパク質を発

現する細胞の生存性を試験する工程を含むことができる。
タウ－タウ、タウ－ＭＡＰ２またはＭＡＰ２－ＭＡＰ２凝集が、研究される場合（前記の「特異性」の項目を参照)、タウ－チューブリンまたはＭＡＰ２－チューブリン結合の阻害または干渉が、細胞が分割する能力の低下、そしてしたがって細胞生存性の低下とある程度相関するので、この工程は、タウまたはＭＡＰ２のチューブリンへの結合の、被検物質によるいずれかの干渉の指数をも提供できる。
細胞生存性は、作用物質のＬＤ５０値を得るために使用されてもよい。
好ましい阻害は、少なくとも２、５、１０または２０の治療指数を有する（ＬＤ５０／ＢＥ５０、図９の論考を参照）。
【００８３】
被験物質の選択
試験される化合物は、適切な活性に関して評価するのが望ましいいずれかのものでよい。方法を新規インヒビター／モジュレーターを同定するための１次スクリーニングとして、または公知のインヒビター／モジュレーターを更に詳細に研究するための２次スクリーニングとして提供することができる。
【００８４】
作用物質は、天然または合成化学化合物でよい。アルツハイマー病様タンパク質凝集を認識する、および／またはアルツハイマー病様タンパク質凝集を調整する抗体は、凝集過程に関して、推定阻害または調整化合物の一つのクラスを形成する。より通常的には、相対的に小型の、好ましくは血液脳関門通過できる化学化合物が試験される。本発明の使用と組み合わせて（前に、同時に、または後に）確立するのが望ましいその他の特性には：骨髄に対する無有毒、血管活性の有害性の最小化；肝臓および腎臓の有毒の最小化；良好な経口吸収；不活性形態に代謝されないこと等がある。当業者は知っているように、これらの試験を、この方法で試験するのが望ましい化合物に関する十分に確立された方法によりコマーシャルベースで実施することができる。
【００８５】
典型的な被験物質および推定されるモジュレーターに関しては、可能な場合、例えばメルク・インデックスから溶解性を最初に決定する。物質が、水溶液中で可溶性である場合、濃縮された貯蔵溶液を、例えばＰＢＳ中５～２０mMで調製できる。使用の直前にこれを組織培養培地で希釈し、例えば１００μMの作業用貯蔵溶液を得ることができ、そして細胞に導入して、ほとんどの化合物に関して最終濃度を０～１０μMにすることができる。当然、被験化合物が１０μM以上の濃度であるのが望ましい場合、作業用貯蔵溶液の濃度を必要に応じて増加させることができる。
【００８６】
物質が水溶液中で可溶性でない場合、貯蔵溶液を（メルク・インデックスから、または実験的に決定される）適当な溶媒、例えばエタノールで５～２９nMに作製することができる。これを再度使用直前に組織培養培地で希釈し、例えば１００μMの作業用貯蔵溶液を得ることができ、そして細胞に導入して、ほとんどの被験化合物に関して最終濃度を０～１０μMにすることができる。前記のように、化合物を１０μM以上の濃度で試験すべきである場合、作業用貯蔵溶液の濃度を必要に応じて増加させることができる。
【００８７】
本発明のこの側面によるスクリーニングアッセイにおいて添加される被検物質または化合物の量、および実際に、それが導入される様式を、必要な場合、一連の試行を行うことにより当業者に決定できることは、技術者には理解されよう。投与された化合物および細胞系が矛盾する最適条件を有している（例えばｐＨ、またはイオン強度等に関して）場合、種々の条件を試みて最適な、妥協した、レベルを見出すべきである。初期濃度を選択して、治療の側面において現実的に用いることができる、すなわち患者に対して非致死的であるレベルにすることができる（以下の用量に関する論評を参照）。本発明の開示に鑑みて、かかる研究法は当業者になんら過度な負担を提示していない。
【００８８】

フェノチアジンのスクリーニング

本発明は別の側面で、本明細書にて提供されるスクリーニング法により同定される化合物にまで広げられ、そしてタンパク質の立体配座重合化の誘起のかかるインヒビター／モジュレーターを含む組成物にまで広げられる。

【００８９】

例えばＷＯ９６／３０７６６に記載されるように、病理学的に誘起されたタウのようなタンパク質の立体配座重合化を阻害できることが見出されている作用物質の中で、特定のジアミノフェノチアジンである。実例には、例えば、疾患例えばアルツハイマー病におけるタウ－タウ凝集の防御に使用するための強力な治療薬として特に興味深いチオニン、メチレンブルー（ＭＢ)、塩化トロニウム、およびジメチルメチレンブルー（ＤＭＭＢ）などが挙げられる。

【００９０】

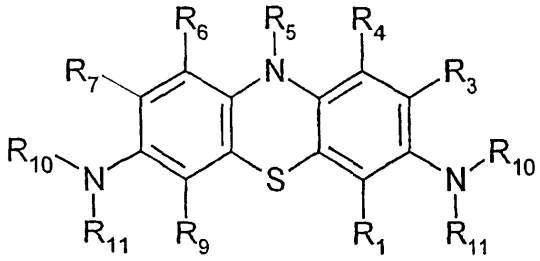
興味深いことに、実施例においてより詳細に記載するように、本発明者らは、本明細書に記載する方法を用いて、化合物、例えばＭＢの立体配座重合化の誘起、例えばタウ－タウ凝集に及ぼす作用メカニズムが、主に天然の立体構造であることを実証した。加えて、例えばタウ－タウ結合に及ぼすジアミノフェノチアジンの強力な立体構造的な阻害効果が化合物の拡散係数に依存することが示されている。化合物のスクリーニングおよび処方に関するこれらの観察の様々な意味を以下でより詳細に論じる。

【００９１】

先行技術におけるかかる化合物の使用に関する記載を考慮する場合、この知見はとりわけ予想外のものである。したがって、例えばかかる化合物はメトヘモグロビン血症の処置において、この場合その作用は、還元ピリジンヌクレオチドの細胞の内因性供給からの電子の移動による、酸化ヘモグロビンの触媒的還元により媒介されることが示されており（例えばＨａｕｓｃｈｉｌｄ，Ｆ．、Ａｒｃｈ．Ｅｘｐ．Ｐａｔｈｏｌ．Ｐｈａｒｍａｃｏｌ．１８２：１１８（１９３６）；「Ｐｈａｒｍａｃｏｌｏｇｉｃａｌ Ｂａｓｉｓ ｏｆ Ｔｈｅｒａｐｅｕｔｉｃｓ」第１版（１９４１）、ＧｏｏｄｍａｎおよびＧｉｌｍａｎ；Ｈｒｇｏｖｉｃ，Ｚ．、Ａｎａｓｔｈ．Ｉｎｔｅｎｓｉｖｔｈｅｒ．Ｎｏｔｆａｌｌｍｅｄ．２５：１７２（１９９０）；およびＣｕｄｄ，Ｌ．ら、Ｖｅｔ Ｈｕｍａｎ Ｔｏｘｉｃｏｌ．３８（５）：３２９（１９９６）参照）、そして躁鬱病の予防（Ｎａｒｓａｐｕｒ，Ｓ．Ｌ．、Ｊｏｕｒｎａｌ ｏｆ Ａｆｆｅｃｔｉｖｅ Ｄｉｓｏｒｄｅｒｓ ５：１５５（１９８３）；Ｎａｙｏｒ，Ｇ．Ｊ．、Ｂｉｏｌ．Ｐｓｙｃｈｉａｔｒｙ ２１：９１５（１９８６））において有用であることが以前から解っていた。これにもかかわらず、ＭＢ、チオニンおよび塩化トロニウムは実際に本来弱い酸化物質であり、そして還元ピリジンヌクレオチドを供給しない場合、これは、タンパク質、例えばヘモグロビンを酸化する（Ｍｏｒｓｅ，Ｅ．、Ａｎｎａｌｓ ｏｆ Ｃｌｉｎ．Ｌａｂ．Ｓｃｉ．１８（１）：１３（１９８８））。この有毒効果を用いてウイルスを不活性化することができ、そして血液製剤からＨＩＶおよび肝炎ウイルスを除去する方法においてＭＢを結果的に利用している（Ｃｈａｐｍａｎ，Ｊ．、Ｔｒａｎｓｆｕｓｉｏｎ Ｔｏｄａｙ ２０：２（１９９４）；Ｗａｇｎｅｒ，Ｓ．Ｊ．、Ｔｒａｎｓｆｕｓｉｏｎ ３５（５）：４０７（１９９５））。この効果の作用メカニズムは、ＭＢのＤＮＡへの介在に関係すると考えられている。化合物は、光活性化により更に高度な酸化還元状態に高められ、そしてそれがその基底状態に戻ったときにＤＮＡを酸化し、それを不活性化する一重項酸素を産生する（Ｂｅｎ－Ｈｕｒ，Ｅ．ら、Ｔｒａｎｓｆｕｓｉｏｎ Ｍｅｄｉｃｉｎｅ Ｒｅｖｉｅｗｓ Ｖｏｌ．Ｘ（１）：１５（１９９６）；Ｍａｒｇｏｌｉｓ－Ｎｕｎｎｏ，Ｈ．ら、Ｔｒａｎｓｆｕｓｉｏｎ ３４（９）：８０２（１９９４））。癌の処置に関する光活性化されたジアミノフェノチアジンの有毒効果の利用もまた示唆されている。細胞内では、酸化形態にまで光活性化された化合物は、ミトコンドリア（Ｄａｒｚｙｎｋｉｅｗｉｃｚ，Ｚ．ら、Ｃａｎｃｅｒ Ｒｅｓｅａｒｃｈ ４８：１２９５（１９８８））および／または微小管（Ｓｔｏｃｋｅｒｔ，Ｊ．ら、Ｃａｎｃｅｒ Ｃｈｅｍｏｔｈｅｒ．Ｐｈａｒｍａｃｏｌ．３９：１６７（１９９６））を損傷し得る。

【００９２】
したがって、先行技術に鑑みて、化合物、例えばＭＢおよびチオニンの実体、例えばＤＮＡまたはタンパク質に及ぼす作用の原因となる二つの可能なメカニズムが提示されていることは明らかである。第１は、細胞内の還元ピリジンヌクレオチドからの電子の移動による、例えば酸化されたタンパク質の触媒的還元である。第２の提示されたメカニズムは、酸化および結果的に、化合物例えば光活性化されたＭＢの酸化形態による例えばＤＮＡの不活性化である。したがって、これらの二つのメカニズムを考慮して、化合物、例えばＭＢ等の化合物のタウ－タウ会合の阻害効果もまた酸化還元活性に起因することが合理的に想定され得る。
【００９３】
すなわち、かかる化合物は、弱い酸化剤として、または触媒性還元剤として作用することにより立体配座重合化、例えばタウ－タウ会合の誘起を阻害すると想定されよう。
したがって本発明者らの研究は、作用のメカニズムが天然の本来の立体構造であることを実証することで、本明細書にて論じる疾患の側面においてかかる化合物の選択、評価、処方および使用のための予想外の意味を有している。
【００９４】
とりわけ、特定の化合物は、先行技術のアッセイの結果に基づいて却下されていた治療に可能性があると見なされた。具体的には、Ｗｉｓｃｈｉｋら（１９９６）（前出）は、阻害に必要なＭＢの濃度が臨床上達成され得る濃度よりも高かったことを１２１７頁で報告した。しかしながら、本明細書の結果は、ＭＢの還元によりそのスタッキング能力が、その阻害能力が、例えばタウ凝集随伴疾患の処置に関して臨床上適切になるレベルまで増強されるように調整されることを示している。これについては、拡散係数の測定に関連する本発明の態様に関連して以下でより詳細に論じている（これは、また部分的に化合物の「スタック」能力によって決定される）。
【００９５】
図８は、細胞ベースのアッセイにおいて試験された化合物のいくつかの部分のみの構造を示す。図９～１６は、還元形態の特定の化合物にいくつかの対照化合物を加えたものの可能性の増加を実証する。
【００９６】
したがって、本発明の一つの側面では、式：
（Ｉ）
【００９７】
【化２】

(I)



【００９８】
（式中、R_1、R_3、R_4、R_6、R_7およびR_9は、独立して水素、ハロゲン、ヒドロキシ、カルボキシ、置換されたまたは置換されていないアルキル、ハロアルキルまたはアルコキシから選択され；
R_5は、水素、ヒドロキシ、カルボキシ、置換されたまたは置換されていないアルキル、ハロアルキルまたはアルコキシから選択され；並びにR_{10}およびR_{11}は、独立して水素、

ヒドロキシ、カルボキシ、置換されたまたは置換されていないアルキル、ハロアルキルまたはアルコキシから選択される）
の還元（「ロイコ」）フェノチアジンまたは医薬的に許容されるその塩の、本明細書で開示された疾患の処置における使用が開示される。

【００９９】

好ましくは、R_1、R_3、R_4、R_6、R_7およびR_9は、独立して水素、－CH_3、－C_2H_5または－C_3H_7から選択され；
R_{10}およびR_{11}は、独立して水素、－CH_3、－C_2H_5または－C_3H_7から選択され；そして
R_5は、水素、－CH_3、－C_2H_5または－C_3H_7から選択される。

【０１００】

好ましくは、化合物は、ジアミノフェノチアジン核の周りに０、２、３または４個のメチル基を有しているジアミノフェノチアジンである。好ましくは、ジアミノフェノチアジンは、非対称的にメチル化されている（例えば塩化トロニウム、アズールＡ、アズールＢおよびチオニン）。
好ましくは、化合物はメチレンブルー、塩化トロニウム、チオニン、アズールＡ、アズールＢまたは１，９－ジメチルメチレンブルーから選択される。

【０１０１】

本発明で用いるためのフェノチアジンを標準的な参考書（例えばＭｅｒｃｋ　Ｍａｎｕａｌ、Ｈｏｕｂｅｎ－Ｗｅｙｌ、Ｂｅｉｌｓｔｅｉｎ，Ｅ．ＩＩＩ／ＩＶ　２７、１２１４　ｆｆ、Ｊ．Ｈｅｔｅｒｏｃｙｃｌ．Ｃｈｅｍ．２１：６１３（１９８４））から引用される方法により製造することができる。
これらの化合物を直接投与する代わりに、これらを前駆体の形態で投与して、処置される細胞で産生されるかまたはその細胞に標的化される活性化物質により活性化形態に変換することができる。例えばメチレンブルーを前駆体の形態で投与できるか、またはそれ自体を化合物アズールＡの前駆体として提供できる。

【０１０２】

<u>還元形態の安定化</u>

目的のこれらの化合物のいくつかは、還元形態で優先的に体内を循環することが知られている。例えばＭＢの薬物動態の論考に関しては、例えばＤｉＳａｎｔｏ，Ａ．ら、Ｊｏｕｒｎａｌ　Ｐｈａｒｍ．Ｓｃｉ．６１（７）：１０８６（１９７２）およびＤｉＳａｎｔｏ，Ａ．ら、Ｊｏｕｒｎａｌ　Ｐｈａｒｍ．Ｓｃｉ．６１（７）：１０９０（１９７２）を参照のこと。第３に、化合物例えばＭＢの還元形態のみが、血液脳関門を通過することが見出されている（Ｃｈａｐｍａｎ，Ｄ．Ｍ．、Ｔｉｓｓｕｅ　ａｎｄ　Ｃｅｌｌ　１４（３）：４７５（１９８２）；Ｍｕｌｌｅｒ，Ｔ．、Ａｃｔａ　Ａｎａｔ．１４４：３９（１９９２）；Ｍｕｌｌｅｒ，Ｔ．、Ｊ．Ａｎａｔ．１８４：４１９（１９９４）；Ｂｅｃｋｅｒ，Ｈ．ら、Ｚｅｉｔｓｃｈｒｉｆｔ　ｆｕｒ　Ｎａｔｕｒｆｏｒｓｃｈｕｎｇ　７：４９３（１９５２）；Ｍｕｌｌｅｒ，Ｔ．、Ｉｔ．Ｊ．Ａｎａｔ．Ｅｍｂｒｙｏｌ．１００（３）：１７９（１９９５）；Ｍｕｌｌｅｒ，Ｔ．、Ｈｉｓｔｏｌ．Ｈｉｓｔｏｐａｔｈｏｌ．１３：１０１９（１９９８））。

【０１０３】

このような参照文献は、化合物、例えばＭＢの還元形態が対象に投与するための可能な、そして医薬上許容される処方に相当することを説明している。ＭＢは、以前は臨床で経口用製剤に用いられていた。しかしながら、その臨床許容性が達成される前は、さらなる有毒試験が必要とされている。ＭＢおよび関連化合物（例えば塩化トロニウム）の血中の半減期は、およそ１００分である。このような比較的短い半減期を有する化合物の徐放性処方により、実質的に化合物の利用性、およびしたがって治療効率が改善されるのは明らかである。

【０１０４】

図１７は、化合物、例えば本明細書で論じた化合物は、アッセイ条件で還元の程度が大き

く異なることを示している（１２０分で、およそ５００：１　ＤＴＴ過剰）。この図は、これらの条件下でチオニンが完全に還元され、塩化トロニウムが中程度レベルで還元され、そしてＭＢおよびＤＭＭＢが相対的にほとんど還元されないことを示している。酸化形態のおよそ９０％の還元を１０分間で達成するのに必要な共通して用いられる還元剤の量は、投与／吸収の前には実行不能である（例えばＤＴＴのＭＢに対する比率は、２０００：１）。

【０１０５】

図１８で説明するように、生理学的条件下でのＭＢの還元の程度は、一晩還元させ、そして還元形態を凍結乾燥することにより大きく加速され得る。凍結乾燥物は、胃液酸度を擬似する条件（ＨＣｌ　５ｍＭ）で可溶化した後、１０分間で９０％還元されるようになる。１．５～２のmg比率で、アスコルビン酸で前還元されたジアミノフェノチアジンの形態を含有するカプセルは、最適でないとしても、治療用途に適した処方に相当する。

【０１０６】

同一の条件をその他の化合物、例えばチオニンおよび塩化トロニウムに適用し、これは、ＭＢよりも容易に還元されるが、その還元の程度は、例えば前記したような様式で加速できる。

したがって好ましい形態では、本発明のフェノチアジン物質は、場合によっては安定化剤の存在下、例えば凍結乾燥製剤で前還元化合物として提供される。

【０１０７】

活性化合物の好ましい形態（すなわち拡散係数が低い化合物の形態、例えば化合物の十分に還元された形態）を安定化するための作用物質は、還元剤または抗酸化剤でよい。阻害化合物の一つの形態（例えば酸化形態）をその好ましい形態（例えば還元形態）に変換するため、およびその好ましい（例えば還元）形態を安定化するために作用物質を提供できる。また別に、阻害化合物をその好ましい（例えば既に還元された）形態で組成物に加えることができ、作用物質は、単にこの形態の化合物を維持するために提供する。

【０１０８】

とりわけ本発明の処方に含まれる、活性物質（例えばジアミノフェノチアジン）の還元形態に変換する、および／またはそれを安定化するのに使用するのに特に適したものは、抗酸化アスコルビン酸塩である。アスコルビン酸塩は、以前はタンパク質の酸化的損傷を最低限にするために用いられていた（Ｐａｒｋｋｉｎｅｎ　Ｊ．「Ｔｈｒｏｍｂｏｓｉｓ　ａｎｄ　Ｈａｅｍｏｓｔａｓｉｓ」７５（２）：２９２（１９９６））。本明細書で提供される処方は、ジアミノフェノチアジン、とりわけＭＢ、塩化トロニウム、ＤＭＭＢまたはチオニンをアスコルビン酸塩と組み合わせて適当な比率、濃度および用量で含むのが有利である。

【０１０９】

別の態様では、適当な成分群の添加または選択は還元（ロイコ）形態に都合よい。

したがって、本発明の側面は更に前記した疾患の処置または予防に用いるための医薬品の調製方法を含み、この方法は化合物を還元し（これが少なくともおよそ５０、６０、７０、好ましくは８０、９０、９５、または９９％還元されるように)、そして同一物を必要とする患者に適当な用量を投与する前に、還元形態で凍結乾燥された組成物においてそれを安定化する工程を含む。

【０１１０】

治療用量

「予防的に有効な量」または「治療的に有効な量」で投与するのが好ましく（場合によっては、予防が治療であると考えられることもある)、これは、個体に利益を示すのに十分である。実際に投与された量および投与の割合および時間経過は、処置すべき疾患の特性および重篤度に依存する。処置の指示書、例えば投与量の決定等は、一般的な開業医およびその他の医師の責任範囲内であり、そして典型的には処置される障害、個々の患者の症状、分配部位、投与方法および開業医に公知のその他の因子を考慮に入れる。

【０１１１】

Ｍｕｌｌｅｒ（Ａｃｔａ Ａｎａｔ．１４４：３９（１９９２））は、全身投与後のＭＢのＣＮＳ透過について記載している。アズールＡおよびＢはＭＢの正常な代謝分解産生物として生じることが解っている（ＤｉｓａｎｔｏおよびＷａｇｎｅｒ、Ｊ．Ｐｈａｒｍ．Ｓｃｉ．６１：５９８（１９７２ａ）；ＤｉｓａｎｔｏおよびＷａｇｎｅｒ、Ｊ．Ｐｈａｒｍ．Ｓｃｉ．６１：１０８６（１９７２ｂ）。Ｃｕｄｄら、Ｖｅｔ Ｈｕｍａｎ Ｔｏｘｉｃ ３８（５）３２９～３３２（１９９６）は、ヒツジにおける塩化トロニウムの薬物動態および有毒について論じている。

【０１１２】

チオニンに関しては、本明細書にて具体例を示すように、一日量は、１～１０００mgが適当であり、好ましくはこれを１～８単位用量に分割し、例えば同量の単位用量にできる。しかしながら、必要な場合、より高いまたは低い活性または生物学的利用率を有する、チオニン以外の本発明の化合物に相応しいように、前記で示したこれらの制限から逸脱できることは理解されよう。

図１９は、ＭＢ対ＩＶ用量の組織レベルの変化を示す。

【０１１３】

メチレンブルーの薬物動態は、ＤｉＳａｎｔｏおよびＷａｇｎｅｒ、Ｊ．Ｐｈａｒｍ．Ｓｃｉ．６１：１０８６～１０９０（１９７２）および６１：１０９０～１０９４（１９７２）によりヒト、イヌおよびラットにおいて研究されている。ヒトにおける尿排泄に関するデータは、またＭｏｏｄｙら、Ｂｉｏｌ．Ｐｓｙｃｈ．２６：８４７～８５８（１９８９）からも利用可能である。ヒトにおけるＭＢの尿排泄に関するデータを組み合わせることにより、７０ｋｇの対象における一回で１００mg投与の後のＭＢの分配に関する全体モデルを誘導することができ、これは即時吸収と想定される（図１９Ｂ）。尿排泄は、摂取された用量の５４～９８％になる。この変動は、吸収における変動のためである可能性が最も高いが、代謝における変動は排除できない。尿排泄データから、全身クリアランスは、５６mg/kg／時間であると計算できる。したがって、有効標的組織濃度４μMを達成するのに必要な用量は、完全に吸収された場合、１．７３mg/kg/日（全溶解固形物０．５８mg/kg）である。しかしながら、Ｍｏｏｄｙらによると、全尿排泄、およびしたがって有効生物学的利用率は、それ自体用量の関数であることは明らかである。１．７３mg/kg/日を分配するのに必要な経口用量は、全身クリアランスに基づいて計算された用量のおよそ２倍である。したがって、実際に必要な用量は、３．２mg/kg/日のオーダーにのる。これは、ヒトにおいて臨床的に用いられる、例えば慢性尿路感染症の処置における最小の通常の経口用量に近い（３９０mg/日）。したがってヒトにおける経口維持用量は、およそ２２５mg/日または全溶解固形物７５mgである。およそ１時間で組織レベルのピークに到達し、組織半減期は、約１２時間である。

【０１１４】

メチレンブルーは、荷電した青色酸化形態、および非荷電性の無色の還元ロイコメチレンブルー形態で存在する。我々は、タウ凝集を５０％防御するのに必要な細胞における標的組織濃度（すなわちＥＣ５０）標的組織濃度が、還元型メチレンブルー形態では４μMであり、そして優先的に活性であるのはロイコ形態であることを細胞において実験的に示した。尿において回収されたメチレンブルーのおよそ７８％が還元形態であり、そして静脈内投与の後の解剖学的研究より、組織に結合している形態のみが無色の還元形態であり、これは、死後切開の後空気に暴露したときに青色に酸化されることがＤｉＳａｎｔｏおよびＷａｇｎｅｒ（１９７２）により示されている。静脈内投与後に血液脳関門を通過するメチレンブルーの形態のみが還元形態である（Ｍｕｌｌｅｒ、Ａｃｔａ Ａｎａｔ １４４：３９～４４（１９９２）並びにＢｅｃｋｅｒおよびＱｕａｄｂｅｃｋ（１９５２））。したがって、経口吸収されたメチレンブルーは、非常に急速に対内で還元され、排泄までそのまま維持され、ことによると還元形態でそれを安定化させる別の化学的修飾を被る。

【０１１５】

経口吸収における変動が、胃腸管における最初の還元の効率により大きく左右される可能

性が高い。したがって、より信頼性の高い吸収を達成するための一つの方法は、アスコルビン酸で前還元されたメチレンブルーにすることである。我々は、インビトロ研究から、この変換は、相当に時間がかかり、そしてアスコルビン酸２ｘ　mgの比率の存在下で水中のメチレンブルーの９０％の還元を達成するのに３時間かかることを示した。したがって、信頼できる吸収を確実にする可能性が最も高いメチレンブルーの用量は、アスコルビン酸７mg/kg/日の存在下、少なくとも３時間前還元したメチレンブルー３．５mg/kg/日である。

【０１１６】

ＭＢが、ヒトにおいて低い濃度で活性であり、したがって臨床上可能である用量範囲は、消化前に９０％以上の還元が達成されるような様式でアスコルビン酸の２ｘ　mg比率と組み合わせた、全溶解固形物２０mg、全溶解固形物５０mgまたは全溶解固形物１００mgである。

【０１１７】

治療の処方および投与

適当な化合物、例えば前記で示した式を有するもの、またはその薬学的に許容される塩を、更に有毒に関して試験した後、本発明のこの側面の組成物に組み込むことができる。組成物には、前記の構成成分に加えて、薬学的に許容される賦形剤、担体、バッファー、安定剤または当業者に公知のその他の材料を含むことができる。かかる材料は、無有毒であり、そして活性成分の有効性に干渉しないものでなければならない。担体またはその他の材料の正確な特性は、投与経路に依存し得る。

【０１１８】

組成物を医薬組成物に処方する場合、その投与を、例えば経口的に、粉末、錠剤、コーティング錠剤、糖衣錠、硬質および軟質ゼラチンカプセル、溶液、乳液または懸濁液、鼻用（例えば鼻用スプレイの形態で）または直腸用（例えば坐剤の形態で）で行うことができる。しかしながら、非経口的に、例えば筋肉内、静脈内、皮膚、皮下、または腹腔内で（例えば注射溶液の形態で）投与を行うこともできる。

【０１１９】

医薬用組成物が、錠剤の形態である場合、これは、固体担体、例えばゼラチンまたはアジュバントを含んでよい。錠剤、コーティング錠剤、糖衣錠および硬質ゼラチンカプセルの製造に関しては、活性化合物およびその薬学的に許容される酸添加塩を薬学的に不活性な無機または有機賦形剤で加工することができる。ラクトース、トウモロコシ、デンプンまたはその誘導体、タルク、ステアリン酸またはその塩等、例えば錠剤、糖衣錠および硬質ゼラチンカプセルを用いることができる。軟質ゼラチンカプセルに適した賦形剤は、例えば植物油、ワックス、脂肪、半固体および液体ポリオール等である。

【０１２０】

組成物が、液体医薬用処方の形態である場合、これは、一般に液体担体、例えば水、石油、動物または植物油、鉱油または合成油を含む。生理学的食塩水、デキストロースまたはその他の糖類溶液またはグリコール、例えばエチレングリコール、プロピレングリコールまたはポリエチレングリコールなどをも含んでよい。溶液およびシロップの製造に適したその他の適当な賦形剤は、例えば水、ポリオール、サッカロース、転化糖、グルコース、トリハロース、等である。注射溶液に適した賦形剤は、例えば水、アルコール、ポリオール、グリセロール、植物油等である。

坐剤に適した賦形剤は、例えば天然または硬化油、ワックス、脂肪、半固体または液体ポリオール等である。

【０１２１】

更に、医薬用製剤は保存剤、可溶化剤、粘性増強物質、安定剤、湿潤剤、乳化剤、甘味剤、着色剤、着香剤、浸透圧を変化させる塩、バッファー、またはコーティング剤を含有できる。

【０１２２】

静脈内、皮膚もしくは皮下注射、または脳へのカテーテル内注入用には、活性成分は、非

経口的に許容される、パイロジェン不含で、そして適当なｐＨ、等張性および安定性を有している水性溶液の形態である。適切な当業者は、例えば等張賦形剤、例えば塩化ナトリウム注射液、リンガー注射液、乳酸塩リンガー注射液を用いて適当な溶液を十分調製できる。保存剤、安定剤、バッファー、および／またはその他の添加剤を必要に応じて含むことができる。

【０１２３】

本発明による組成物を、処置すべき症状または疾患に依存して、単独で、またはその他の処置を同時に、または連続的のいずれかで組み合わせて投与することができる。

本発明に従って、本明細書で提供される処方を、アルツハイマー病、運動ニューロン疾患、レビ小体疾患、ピック病または進行性核上麻痺、またはタンパク質の立体配座重合の誘起が関係するいずれかその他の症状もしくは疾患の予防または処置に用いることができる（図５参照）。とりわけ、以下に詳細に記載するように、処方を、病理学的タウ－タウ会合の遮断、調整および阻害に用いることができる。

前記した技術およびプロトコルの実例は、「Ｒｅｍｉｎｇｔｏｎ’ｓ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｓｃｉｅｎｃｅｓ」第１６版、Ｏｓｏｌ，Ａ．（編）（１９８０）において見出すことができる。

【０１２４】

さらなる側面では、本発明は、タンパク質の立体配座重合の誘起が関係する症状の診断、予後または処置における先行の側面の組成物の使用に関する。症状は、疾患、例えばアルツハイマー病、または本明細書に記載した型のいずれかその他の症状でよい。

【０１２５】

スクリーニングとしての拡散定数の使用

前記するように、化合物をその還元形態に変換すること、および／またはその還元形態を安定化することにより、化合物の阻害可能性を最適化することができる。

しかしながら、本明細書後記の実施例にてより詳細に記載するように、驚くべきことに、化合物の酸化還元能力は、タンパク質の立体配座重合の誘起に関するその阻害活性を直接決定せず、そして、したがって酸化モデルも触媒還元モデルもタウ－タウ凝集インヒビターとしての化合物の活性の理解に関連しない。

【０１２６】

本発明者らは、化合物のタウ－タウ結合に対する阻害能力およびその拡散係数の２または３乗の間に強い逆相関があることを見出した。

拡散係数は、陰極での放電した分子のスタッキングの量により決定される。実験的には、還元電位で酸化還元セルでの電流を測定することによりこれを評価することができる。拡散係数は、陰極で形成するヘルムホルツ層内の放電した（すなわち還元）種の凝集の程度と逆相関する。これらの凝集は、フェノール環系を通ってパイ結合したスタッキング相互作用により形成される。

【０１２７】

一つのモデルでは、拡散係数が低いほど、スタックする傾向が高くなり、そして低いK_iを反映するようなタンパク質の立体配座重合の誘起の阻害、例えばタウ－タウ結合において化合物がより強力になる。

【０１２８】

酸化形態は荷電しており、そして分子と同様、他方と反発すると考えられ得るので、ジアミノフェノチアジンのスタッキングは、分子が酸化形態であるときにはあまり好都合でない。したがってこの現象により、タウ凝集の阻害におけるジアミノフェノチアジンの還元形態の大きな有効性が説明され得る（例えば図９参照）。

【０１２９】

したがって、拡散係数の評価（「スタッキング能力」に依存し、今度は形状および荷電に依存する）は、有効なモジュレーターの開発における有用な工程であり得る。かかる立体化学に関連するパラメーターの一つは、ジアミノフェノチアジンのその還元形態での提供により低減され得る拡散係数である。

【０１３０】
したがって、本発明者らは、本明細書にてタンパク質の立体配座重合の誘起の遮断、調整または阻害における化合物の有効性（本明細書では以後「阻害可能性」と称する）を、化合物の拡散係数を測定する工程を含むアッセイ方法において試験するできることを教示する。
【０１３１】
したがって、本発明は、その最も一般的な形態で、タンパク質の立体配座重合の誘起を遮断、調整または阻害する作用物質をスクリーニングする方法を提供し、この方法は、作用物質の拡散係数を測定する工程を含む。拡散係数値、および、とりわけその拡散係数の２乗または３乗の使用は、本明細書で記載するような疾患の処置に関するフェノチアジン（例えば前記したような）の阻害可能性の評価において、本発明のさらなる側面を成す。
【０１３２】
推定されるまたは確立されたモジュレーターを同定または最適化するための、拡張したスクリーニングプログラムのいずれかの段階で、被験物質の拡散係数を測定する工程を組み込むことができる。拡張した方法は、典型的には更に本明細書で記載するか、または先行技術（例えばＷＯ９６／３０７６６）のアッセイ工程を含む。したがって、例えば後者の場合、タウ－タウ凝集を遮断、調整または阻害する作用物質に関してスクリーニングしたい場合、方法は：
（ａ）タウタンパク質またはタウコアフラグメントを含有するその誘導体を；
（ｂ）タウ－タウ凝集を遮断、調整または阻害するその能力に関して試験すべき物質；並びに
（ｃ）工程（ａ）のタウタンパク質に結合できる標識されたタウタンパク質もしくは標識されたその誘導体、または工程（ａ）のタウタンパク質から区別され、そして工程（ａ）のタウタンパク質に結合することもできるタウタンパク質もしくはその誘導体；
と接触させる工程を含む。
【０１３３】
拡散係数をいずれか適当な手段、例えばＭｕｒｔｈｙおよびＲｅｄｄｙ（Ｊ．Ｃｈｅｍ．Ｓｏｃ．，Ｆａｒａｄａｙ　Ｔｒａｎｓ　Ｊ　８０：２７４５～２７５０（１９８４））により測定することができる。この文献は、またフェノチアジン染料に関する拡散係数のいくつかの測定値をも含み、そしてその内容を特に参照として本明細書に組み入れられる。
【０１３４】
したがって、拡散係数は、酸性水性溶媒におけるサイクリックボルタンメトリーにより適当に測定でき、それにより酸化還元セルの電流の大きさを化合物の還元電位で試験する。
【０１３５】
方法は、特定のタンパク質（例えばタウ）の立体配座重合の誘起のインヒビターもしくはモジュレーターとしてのその特異性を確認するため、または動物に投与するための作用物質としての薬学的許容性もしくは適性を決定するために作用物質に関してさらなる試験を実施する工程を含んでよい。
【０１３６】
本明細書にて提供される、タンパク質の立体配座重合の誘起の遮断、調整または阻害における作用物質の可能性が、少なくとも部分的に作用物質の拡散係数に依存するという驚くべき教示を作用物質の可能性の最適化に利用することができる。本発明者らは、タンパク質の立体配座重合の誘起に対する作用物質の阻害可能性がその拡散係数の２乗または３乗に逆相関することを確立した。換言すると、作用物質の阻害可能性は、その拡散係数が最小化された形態の作用物質を提供することにより最適化することができる。
【０１３７】
したがって、別の側面では、本発明は、タンパク質の立体配座重合の誘起の遮断、調整または阻害における作用物質の可能性を最適化する方法に関し、その方法は作用物質の拡散係数を最適化する工程を含む。

【０１３８】
別の側面では、本発明は、タンパク質の立体配座重合の誘起を生じる症状の予防または処置のための医薬用組成物を提供し、その組成物はその拡散係数が最小化された形態で提供されるか、またはその形態に変換される化合物を含む。
【０１３９】
本発明のこの、および別の側面は、以下の図および実施例によってのみ提供される実験データを参照することにより、よりよく理解されよう。
【０１４０】
実施例
一般的な材料および方法
３Ｔ６Ｈ細胞系の産生
３Ｔ６細胞は、ＥＣＡＣＣ番号：８６１２０８０１マウススイスアルビノ胚繊維芽細胞であった。
【０１４１】
誘導系に関しては、Ｌａｃレプレッサータンパク質を発現するｐ３’ＳＳベクターを用いてストラッタジーンのＬａｃスイッチ（商標）を実験で用いて、Ｌａｃレプレッサータンパク質を発現し、ｐＯＰＲＳＶＩＣＡＴを用いてＬａｃレプレッサーの制御下で全長タウを発現した。発現は、ＩＰＴＧの添加により誘導される。
【０１４２】
最初に３Ｔ６細胞をエレクトロポレーションによりｐ３’ＳＳプラスミドおよびヒグロマイシン抵抗産生により選択されたコロニーでトランスフェクトした。種々レベルのＬａｃレプレッサータンパク質（免疫細胞化学により決定）を発現した５個のクローンをとり、そしてまた非クローン化細胞を比較のために保持した。
【０１４３】
ｐＯＰＲＳＶＴ４０ベクターの産生
ＮｏｔＩ部位および必要に応じて出発または停止コドンを含むプライマー（以下に示す）を用いてベントポリメラーゼ（ＮＥＢ）でＰＣＲにより、ｐＯＰＲＳＶＩＣＡＴベクターへのクローニングのためのＴ４０インサートを調製した。ＰＣＲ産生物およびｐＯＰＲＳＶＩＣＡＴベクターをＮｏｔＩで切断し、精製した。ベクターを脱リン酸化して再ライゲーションを防御し、そしてインサートを標準的なプロトコルを用いてベクターにライゲートした。
【０１４４】
得られたライゲーションミックスをコンピテント大腸菌（Ｅ．ｃｏｌｉ）細胞にトランスフェクトし、そして細胞をａｍｐプレートに載せた。コロニーを取り、新たなａｍｐプレート上でグリッド化した。コロニーの盛り上がりをハイボンド－Ｎ０．４５μｍナイロン膜（アマシャム）に取り、そして（α－^{32}Ｐ）ｄＣＴＰ（アマシャム）で標識したｄＧＡを用いるコロニーハイブリダイゼーションにより可能な陽性を選択した（オリゴ標識キット（ファルマシア・バイオテック）を用い、そしてＮａｐ－１０カラム（ファルマシア・バイオテック）で精製した）。ハイブリダイゼーションをチャーチバッファー中６５℃で一晩実施し、続いてチャーチ洗浄液で２ｘ２０分間洗浄した。ブロットを－７０℃で一晩Ｘ線フィルムに暴露することにより放射活性プローブで標識した陽性コロニーを検出した。
【０１４５】
陽性コロニーを選択し、成長させ、次いでＰＣＲおよび制限消化により調べてインサートの存在を確認した。クローニング手段のための単一の制限部位の使用はＴ４０をいずれかの配向でベクターに挿入できることを意味する。インサートの配向を決定して、発現のために正確な配向でＴ４０を含有するベクターを伴うコロニーを選択した。
【０１４６】
使用したプライマー
【表１】

5'-3' T40-Not I

出発

5'-gtc gac tct aga ggc ggc cgc ATG GCT GAG CCC CGG CAG GAG-3'
Not I

3'-5' T40- Not I

停止

5'-act ctt aag ggt cgc ggc cgc TCA CAA CAA ACC CTG CTT GGC CAG -3'
Not I

【０１４７】
Ｔ４０配列に相補的な配列を大文字で示し、出発および停止コドンに印を付けた。加えられるＮｏｔＩ部位を下線で示す。小文字で示す残りの配列は、ＮｏｔＩ酵素に効果的に切断させることができる１３塩基対の張り出しである。これは、プライマーを効果的に結合させることができるｈＴａｕ４０プラスミドベクターの配列に相補的であった。
【０１４８】
インサート配向の決定
インサートを一回およびベクターを最大で数回切断し、そして各々の配向で異なる制限消化パターンが得られる制限酵素を用いて配向を決定した。ＨｉｎｄＩＩＩは、ｐＯＰＲＳＶＴ４０のためのこれらの基準に適合する。インサートが存在しない場合、二つの制限バンドが産生される。インサートが存在する場合、三つのバンドが産生され、そしてバンドの大きさは、以下に示すようなインサートの配向に依存する。

正（正しい）配向	５３８５ｂｐ	１０３０ｂｐ	３８１ｂｐ
逆配向	６１０１ｂｐ	３８１ｂｐ	３１４ｂｐ

【０１４９】
誘導プロモーターの制御化でＴ４０を発現する細胞の産生
ｐＯＰＲＳＶＴ４０プラスミドを産生し、ＣｓＣｌグラジエント遠心により精製した。これを前記のように産生した３Ｔ６Ｈ細胞（Ｌａｃレプレッサータンパク質を発現する）にトランスフェクトした（エレクトロポレーションによる）。陽性細胞をＧ４１８に対する抵抗性（５００μg/mlで）により選択した。抵抗性コロニーを取り、成長させた。ＩＰＴＧの添加を伴うおよび伴わないＴ４０全長の発現レベルを免疫細胞化学およびウェスタンブロットの双方により抗タウ抗体で決定した。
【０１５０】
ｐＺｅｏ２９５～３９１の産生
プラスミドｐＺｅｏ２９５～３９１をタウの断端されたフラグメント（２９５～３９１残基、以下参照）に対応するタンパク質を発現するように設計した。構成系（インビトローゲン、オランダのｐｃＤＮＡ３．１）を使用した（プラスミドは、抗生物質ゼオシンに対する抵抗性を付与する）。この領域に対するｃＤＮＡを特異的オリゴヌクレオチドプライマー（センスおよびアンチセンス；以下参照）を用いてポリメラーゼ連鎖反応（ＰＣＲ）により増幅した。センスプライマーは、ＥｃｏＲＩ部位およびアンチセンス、ＢａｍＨＩ部位を含有した。フラグメントをＥｃｏＲＩおよびＢａｍＨＩで消化したｐｃＤＮＡ３．１（－）ゼオ（インビトローゲン、オランダ）にサブクローニングした。挿入したＤＮＡは、サイトメガロウイルスプロモーター配列から下流で、そしてポリアデニル化シグナルの上流である。プラスミドは、細菌および真核細胞の各々で選択するためのアンピシリン

およびゼオシン抵抗産生を発現するためのＤＮＡ配列を含有する。挿入されたＤＮＡの確実性を、双方の鎖の全長シークエンシングにより確認した。

【０１５１】

断端されたタウフラグメント２９５～３９１のヌクレオチドおよびアミノ酸配列

【表２】

gataatatcaaacacgtcccgggaggcggcagtgtgcaaatagtctacaaaccagttgacctgagca
aggtgacctccaagtgtggctcattaggcaa

catccatcataaaccaggaggtggccaggtggaagtaaaatctgagaagcttgacttcaaggacaga
gtccagtcgaagattgggtccctggacaatat

cacccacgtccctggcggaggaaataaaaagattgaaacccacaagctgaccttccgcgagaacgcc
aaagccaagacagaccacggggcggag

DNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNIT
HVPGGGNKKIETHKLTFRENAKAKTDHGAE

295 sense primer

met asp[295]

5' - CGG AAT TCC ACC **ATG** GAT AAT ATC AAA CAC GTC CCG - 3'

EcoRI

391 anti-sense primer

stop glu [391]

5' - C GCG GGA TCC **TCA** CTC CGC CCC GTG GTC TGT CTT GGC - 3'

BamHI

【０１５２】

出発および停止コドンは、太字であり、そして加えられるＥｃｏＲＩおよびＢａｍＨＩ制限部位の下線を付す。

【０１５３】

アッセイのための細胞の組織培養

使用した培地は、ライフ・テクノロジーズ、スコットランドのＤＥＭ（グルタマックスＩ、ピルビンサン塩、グルコース４．５ｇ／ｌを伴う）であった。ＦＣＳ１０％（ヘレナ・バイオサイエンシズ)、ペニシリン５０U/ml、ストレプトマイシン５０μg/ml、および関連するプラスミドの選択および維持に適当な別の抗生物質でこれを補充した。抗生物質濃度は、ヒグロマイシン２００μg/ml（ｐ３’ＳＳ選択および維持)、Ｇ４１８　５００μg/ml（ｐＯＰＲＳＶＴ４０選択および維持)、ゼオシン４００または２００μg/ml（ｐＺｅｏ２９５～３９１選択および維持）であった。

【０１５４】
細胞を３７℃で、$CO_2$５％の加湿環境下で成長させる。細胞を１０cm皿中に保持し、全面成長に近づいたときに分ける。培地を除去し、細胞をＰＢＳで洗浄し、そして細胞は、トリプシン／ＥＤＴＡ溶液１ml／１０cm皿でトリプシン処理することにより細胞を遊離させる。新鮮培地中１：１０希釈で、または場合によっては１：５～１：２０（およそ５０００～２００００セル／cm^2）の希釈範囲で細胞を懸濁する。
【０１５５】
薬物を試験するために、細胞を２４時間以内に全面成長の５０～８０％まで成長できる初期密度で６ウェルまたは２４ウェルプレートに載せる。薬物を種々濃度でウェルに加えて、ＩＰＴＧ　０～５０μMの添加により全長タウの発現を誘起する。細胞を更に２４時間成長させ、次いで収集し、ＳＤＳ　ＰＡＧＥ／ウェスタンブロッティングにより分析する。
【０１５６】
タウタンパク質の調製
組換えタウ（クローンｈｔａｕ４０）並びに、ラットおよびヒトから抽出した過塩素酸可溶性タウを以前に記載されたように調製した（Ｇｏｅｄｅｒｔ，Ｍ．およびＪａｋｅｓ，Ｒ．、ＥＭＢＯ　Ｊ．９：４２２５；Ｇｏｅｄｅｒｔ，Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｄａｄ．Ｓｃｉ．　ＵＳＡ　９０：５０６６）。
【０１５７】
ゲル電気泳動およびブロッティング
前記で概要を示すように成長させた細胞をＰＢＳで一回洗浄し、次いでラエムリバッファー５０μl（２４ウェルプレート）または１００μl（６ウェルプレート）で溶解する。サンプルを－２０℃で貯蔵し、４分間煮沸してからバイオラッド・ミニプロティーンＩＩＩミニゲル系を用いてアクリルアミドゲル１５％上を走らせる。ＣＡＰバッファー系を用いてウェスタンブロッティングによりタンパク質をＰＶＤＦ膜に移す。膜を遮断バッファー（ＰＢＳ中脂肪粉乳５％（マーベル)、トゥイーン２０　０．１％）中１時間から一晩インキュベートする。遮断バッファーで１：５に希釈したｍＡｂ７．５１で膜をインキュベートすることによりタウタンパク質を検出し、ウェルをＰＢＳ／０．１％　トゥイーン２０で洗浄し、遮断バッファーで１：５０００希釈した抗マウスＨＲＰと共に１時間インキュベートし、そしてウェルをＰＢＳ／０．１％　トゥイーン２０で洗浄する。ＥＣＬハイパーフィルムに検出されるＥＣＬ反応により結合抗体を検出する（アマシャム）。
【０１５８】
ブロットをヒューレット・パッカード・スキャンゼット６１００Ｃフラットベッドスキャナーで６００dpiでコンピューターにスキャンし、ｔｉｆｆファイルとして保存する。アップルパワーマックＧ３でスキャナ分析プログラムでＴ４０およびｄＧＡＥバンドの濃度計測を実施する。
【０１５９】
薬物調製
チオニン、メチレンブルー、ＤＭＭＢ、および塩化トロニウムをすべてｄｄＨ２Ｏ中のストック１mMとして調製する。使用前に希釈ストック１００μMをＨＢＳＳで調製し、これを細胞の培地に直接添加する。
酸化薬物に関しては、ストック１mMをＨＢＳＳで希釈することにより、これを簡単に調製し、そして滅菌濾過する。
【０１６０】
還元薬物に関しては、１mMをアスコルビン酸およびＤＴＴで処理して薬物０．５mM、アスコルビン酸５０mM、ＤＴＴ５０mMを得、これを１５分間放置した（青色が無色に変化する）後、希釈ストックを作成する。これをＨＢＳＳで希釈して薬物１００μM、アスコルビン酸１０mM、ＤＴＴ１０mMを得、そして滅菌濾過する。還元ストック１００μM、酸化ストック１００μMおよびアスコルビン酸１０mMの適量を用いることにより、細胞を種々濃度の薬物で処理するが、還元薬物に関してはアスコルビン酸およびＤＴＴは、ずっと４０

０μＭを維持する。

【０１６１】

ＳＤＳゲル電気泳動およびイムノブロッティング

標準的な電気泳動およびイムノブロッティング手順を以前に記載されたように用いた（Ｗｉｓｃｈｉｋ，Ｃ．Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８５：４５０６（１９８８）；Ｎｏｖａｋ，Ｍ．ら、ＥＭＢＯ Ｊ．１２：３６５（１９９３）；Ｊａｋｅｓ，Ｒ．ら、ＥＭＢＯ Ｊ．１０：２７２５（１９９１））。イムノブロットをＡＢＣキットで展開した（ベクター・ラボラトリーズ）。モノクローナル抗体（ｍＡｂ）７．５１、２１．Ｄ１０、４９９および３４２を希釈していないハイブリドーマ培養上澄液として用いた。ｍＡｂ ＡＴ８（イノジェネティクス、ベルギー）を１／１０００希釈で用いた。抗タウｍＡｂ７．５１（これは、最後の反復のエピトープを認識する；Ｎｏｖａｋ，Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８８：５８３７（１９９１）参照)、４２３（これは、残基Ｇｌｕ－３９１で断端されたタウＣ－末端を認識する；Ｗｉｓｃｈｉｋ，Ｃ．Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８５：４５０６（１９８８）；Ｎｏｖａｋ，Ｍ．ら、ＥＭＢＯ Ｊ．１２：３６５（１９９３）参照)、４９９（これは、残基Ｇｌｙ－１４およびＧｌｎ－２６の間のヒト特異的タウセグメントを認識する；Ｗｉｓｃｈｉｋ，Ｃ．Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９３：１１２１３（１９９６）参照)、および３４２（これは、残基Ｓｅｒ－２０８およびＰｒｏ－２５１の間のセグメントを認識する）。ｍＡｂ２１．Ｄ１０は、Ａ６８－タウ脳抽出物に対して上昇した（Ｌｅｅ，Ｖ．Ｍ．－Ｙ．ら、Ｓｃｉｅｎｃｅ ２５１：６７５（１９９１））。

【０１６２】

タウ結合アッセイ

これは基本的にＷｉｓｃｈｉｋ，Ｃ．Ｍ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９３：１１２１３（１９９６）に記載されるように実施した。炭酸塩バッファー５０ｍＭ中３７℃で１時間、９６ウェルポリ（塩化ビニル）マイクロタイタープレート上で固相タウ（０～２０μｇ/ｍｌ）を被覆した。プレートをトゥイーン２０ ０．０５％で二回洗浄し、次いでＰＢＳＴ中マルベル２％で３７℃で１時間遮断した。再度洗浄した後、プレートを水相タウ（ゼラチン１％を含有するＰＢＳＴ中０～３００μｇ/ｍｌ）と共に３７℃で１時間インキュベートした。本出願では、ＤＴＴ１ｍＭをも添加した。

【０１６３】

プレートを二回洗浄し、ｍＡｂ４９９または３４２と共に３７℃で１時間インキュベートし、ＰＢＳＴ中マルベル２％と同容量で希釈した。洗浄後、西洋ワサビペルオキシダーゼ抱合ヤギ抗マウス抗体（ＰＢＳＴ中１／１０００）を３７℃で１時間インキュベートした。プレートを洗浄し、テトラメチルベンジジンおよびH_2O_2を含有する基質溶液と共にインキュベートし、そして吸収の変化の速度を以前に記載されるように、V_{max}プレートリーダー（モレキュラー・ディアグノースティックス、カリフォルニア州）を用いて測定した（Ｈａｒｒｉｎｇｔｏｎ，Ｃ．Ｒ．ら、Ｊ．Ｉｍｍｕｎｏｌ．Ｍｅｔｈ．１３４：２６１（１９９０））。各実験は3検体ずつ実施し、そして固相および水相タウの双方が存在しない対照、および二つの不在のうちのいずれか一つを伴う対照を含む。

【０１６４】

データ分析

これは、Ｗｉｓｃｈｉｋら、（前出）に記載されたように実施し、そしてクアシ－ニュートン近似値を用いてカレイダグラフ（シナージー、フィラデルフィア）またはシスタット（ＳＰＳＳ Ｉｎｃ．、シカゴ）プログラムでラングムイア等式曲線に従って曲線が適合された。曲線適合相関係数を図に示す。

【０１６５】

実施例１

全長、断端されたおよび突然変異タウの構成発現

真核細胞系におけるタウの発現は、リポフェクチンベースの研究法の制限を受けない生理

学的条件下でタウ凝集の細胞モデルを作製することが求められていた。これには、正常なタウおよび病原性突然変異を担持するタウの双方に関する全長タウおよび断端されたタウフラグメントの発現が関係する。

【０１６６】

全長タウ

正常な全長タウ（Ｔ４０）は、細胞（３Ｔ３およびＮＩＥ－１１５）にトランスフェクトされた場合、発現され、そして細胞内の微小管ネットワークの集合に関係した。

【０１６７】

断端されたタウ

最初に、フラグメント２９７～３９１に対応するＰＨＦのコアからの断端されたタウフラグメントのｃＤＮＡを非ニューロン性３Ｔ３繊維芽細胞にトランスフェクトした：この断端されたタウは：（ｉ）ＰＨＦ－コアに存在する；（ｉｉ）疾患の初期段階でＡＤ脳組織における沈着として検出される；（ｉｉｉ）触媒性捕捉およびインビトロでのタウ捕捉の伝播を支持することができる；ので、これを選択した。続いて、タウ分子の免疫化学特性に部分的に依存した、Ｎ－またはＣ－末端のいずれかでのトランケーションの程度が変化した一連のトランスフェクションを実施した。Ｎ－末端で（１８６～４４１；２９７～４４１)、Ｃ－およびＮ－末端で（１８６～３９１；２９７～３９１）およびＣ－末端（１～３９１）でのトランケーションを伴う６つの構築物を作製した。限定された抗体のパネルを伴う６つの構築物に関する免疫反応性のパターンにより、このように作製されたタウフラグメントのすべてを識別することができた。

【０１６８】

構築物を真核細胞において一過性（ベクターとしてｐＳＧ５を使用）および安定して（ベクターとしてｐＩＦ２およびｐＺｅｏを使用）の双方で発現した。ｐＩＦ２およびｐＺｅｏに関して各々抗生物質ゲンチシンおよびゼオシンに対する抵抗性に基づいて安定したトランスフェクト体を選択する。ベクターとしてｐＲＫ１７２を用いて細菌により発現されたタンパク質でエピトープ分析を実施した。図２０で３Ｔ３およびＣＯＳ－７細胞における種々フラグメントに関する結果を要約する。別の結果により、同一細胞におけるタウの二つの形態の発現が免疫反応性パターンに影響し得ることが示された。例えば、１～３９１および２９５～３９１の安定した発現が細胞内に異常な束の発現に至る。しかしながら、安定したおよび再現性のある状態でのかかる細胞の維持は困難であることが判明した。

【０１６９】

突然変異したタウ

全長タウの突然変異誘発を用いて臨床的に公知である突然変異を作製した。これらをｐＩＦ２にサブクローニングし、そしてタウの微小管集合特性に影響するもの（Ｇ２７２Ｖ、Ｖ３３７Ｍ、Ｐ３０１Ｓ、Ｒ４０６Ｗ)、およびタウ遺伝子のインビボ選択的スプライシングに影響するＳ３０５Ｎを含む多くの突然変異体に関して安定したトランスフェクト体を３Ｔ３およびＮＩＥ細胞において作製した。概して、突然変異を担持する全長タウを発現する細胞は、微小管ネットワークの標識化を呈し、野生型タウでトランスフェクトした細胞から区別できなかった。突然変異を含む、特定の断端されたタウフラグメントを発現する細胞系は、安定でないことが判明した。

【０１７０】

結論

要約すると、真核細胞内での断端されたタウの構成的発現は、達成するのが困難であると判明した。２９７－タウの開始コドンを取り囲むコザックコンセンサスを操作することにより、一過性のトランスフェクション系によるタウの発現の最適化が可能になったが、例えば２９７～３９１の発現は、依然わずかであり、これは、フラグメントのいくつかの固有の有毒特性を示唆している。安定したトランスフェクションによりこの結論が繰り返された。この後者の系は、Ｎ－またはＣ－末端のいずれかでのトランケーションにより、タウが微小管ネットワークにおいてよりもむしろ無晶形沈着において集合する傾向がわずかに大きくなることを実証した。またタウフラグメントの組み合わせの安定した発現により

細胞の細胞質内での凝集をも生じたが、この系は、容易に再現されなかった。

【０１７１】

実施例２

断端されたタウの誘導発現

構成的発現に伴う有毒を伴わない、安定した、再現性のある系を作製する別の試みでは、ＰＨＦのコアタウフラグメントの誘導発現（すなわち１２ｋＤの２９７～３９１）を試みた。

【０１７２】

真核細胞におけるタンパク質の発現のためのいくつかの誘導系を試みたが、好ましい系は、「ｌａｃスイッチ」系であった。この系では、典型的には内因性タウタンパク質はまったく発現しない３Ｔ３または３Ｔ６繊維芽細胞である二つのベクターを細胞に組み込む。第１のｐ３’ＳＳベクターは、ｌａｃ　Ｉ遺伝子の構成発現をコードし、そしてヒグロマイシン抵抗性に基づいてエクスプレッサーを選択する。第２のｐＯＰＲＳＶＩＣＡＴは、Ｌａｃオペロンからのオペレーター配列を含有する強力なＲＳＶプロモーターの制御下で、タウタンパク質フラグメントをコードするＤＮＡを組み込む。このベクターを組み込む細胞をネオマイシン抵抗性に基づいて選択する。双方のベクターを組み込んだ細胞は、ｌａｃ　Ｉの構成的発現がＬａｃオペロンにより制御される組み込まれたタンパク質（すなわちタウ）の発現を防御するという特性を有する。糖ＩＰＴＧの添加は、ｌａｃ　ＩのＬａｃオペロンへの結合と競合し、それでタウタンパク質の発現が可能になる。

【０１７３】

１２ｋＤフラグメントの誘導発現を二つの細胞系において実施した。これは、ＩＰＴＧでの処置の３日後までに、その段階で高レベルの１２ｋＤが突然現れ、細胞を即座に殺す細胞内凝集を形成し、タウタンパク質発現のレベルが適用可能でないことを示した。予想されるに、凝集の過程は、低レベルからなんらかの明確な漸次移行を伴わない有毒凝集の突然の蓄積まで非直線的に進行し、凝集および有毒を制御不能にした。この非直線的な進行は、系の適切な制御を防御した。

【０１７４】

実施例３

発明による安定した細胞系におけるタウの発現

前記の結果に鑑みて、以下のような別の系を用いた。組織培養細胞系ＤＨ１９．４．１．４．およびそのクローンは、全長タウ、誘導プロモーターの制御下の四つの反復ヒトタウおよび構成プロモーターの制御下の断端されたヒトタウ（２９５～３９１）を発現する３Ｔ６細胞（ＥＣＡＣＣ番号：８６１２０８０１　スイス・アルビノマウス胚繊維芽細胞）に基にした。

【０１７５】

誘導プロモーター、Ｔ４０．２２．１０の制御下でＴ４０を発現する細胞をｐＺｅｏ２９５～３９１プラスミドでトランスフェクトした（リポフェクションによる）。陽性細胞を４００μg/mlでのゼオシンに対する抵抗性に関して選択した。全長タウの誘導発現のバックグラウンドでの断端されたタウの発現を、Ｍａｂ７．５１でのウェスタンブロット分析により確認した。

【０１７６】

図２１は、二つの細胞系での３Ｔ６繊維芽細胞のみにおける全長ヒトタウの誘導発現を説明する。Ｔ４０．２２は、非誘導状態（「Ｕ」）およびＩＰＴＧの添加後の高レベル発現（すなわち誘導された「Ｉ」）での全長タウの低レベルバックグラウンド漏れを示す。Ｔ４０．３７は、同一であるが、より低レベルの誘導を行わない発現を示す。図２２は、３重ベクター系の結果を示す。ベクターにより非常に低レベルの１２ｋＤフラグメントの構成発現を可能にするベクターを、全長タウの誘導発現が既に達成されている細胞系に導入した（図２１で示されるＴ４０．２２）。図２２は、低レベルのＩＰＴＧを導入して全長タウの発現を誘導する場合、何が起こっているのかを示している。ＩＰＴＧ　０μＭでは、非常に低レベルの１２ｋＤバンドの発現および全長タウの低い「バックグラウンド漏れ

」発現がある。ＩＰＴＧの高レベルの導入によりより多くの全長タウが漸増的に誘導されるので、より多くの全長タウが１２ｋＤ種に変換されるほど、より多くの中間の高分子量フラグメント（図４３および４４により詳細に記載されている）が産生される。

【０１７７】

１２ｋＤフラグメントの構成発現のためのベクターを含有しなかった元のＴ４０誘導細胞系（Ｔ４０．２２．１０）の試験により、１２ｋＤ種が全長タウ誘導のトランケーション副産物として産生されないことが示される。Ｔ４０の誘導の後の１２ｋＤバンドの発現の増強は、１２ｋＤフラグメントの発現前に低レベルを伴った細胞においてのみ認められた（ＤＨ１９．４．１．４．６）。すなわち、既存の１２ｋＤにより全長タウの誘導後に、より多くの１２ｋＤの産生のための鋳型が提供される。細胞を非誘導状態にした場合（例えばＤＨ１９．４．１．４Ａ．Ｂ２と称する細胞系にて)、～２５／２７ｋＤの見かけのゲル可動性を伴う別のダブレットも現れ得る。ＩＰＴＧでの誘導の後、～３０／３２ｋＤ、～３６／３８ｋＤおよび～４２／４４ｋＤのゲル可動性を伴う別の一連のダブレットが現れ得る。

【０１７８】

これらの種を、誘導なし（「Ｕ」）および誘導後（「Ｉ」）の双方で図４０で示す。ｍＡｂ３４２および残基Ｓｅｒ４２２およびＬｅｕ４４１間に位置するエピトープを認識するＣ－末端ポリクローナル抗体Ｔ４６で認められるこれらのフラグメントの免疫反応性のパターンをも示す。

【０１７９】

非誘導状態で認められるフラグメントの派生（すなわち１２／１４ｋＤおよび２５／２７ｋＤ）を図４１を参照して説明できる。

図４１（ａ）は、矢頭で示されるおおよその位置で全長タウ分子の鋳型誘起タンパク質溶解性プロセシングによりどのように１２ｋＤフラグメントを生じるかを示している。

【０１８０】

２５／２７ｋＤ種の場合、これらのフラグメントは、Ｔ４６と免疫反応するので、これらのフラグメントは１２／１４ｋＤ種の二量体を呈することができない。したがって、図４１（ｂ）の矢頭で示されるおおよその位置で生じる切断により、全長凝集タウの別のタンパク質分解産生物を生じるはずである。

【０１８１】

誘導後（図４０、Ｉ)、別の一連のダブレットが認められる。これらの別のフラグメントの派生は、図４２～４４を参照にしてよりよく理解できる。

図４２は、これらのフラグメントの見かけのゲル可動性のプロットおよびアミノ酸残基におけるその長さを示し、これは、フラグメントの長さの特徴的なセットから見かけのゲル可動性を理解できることを示している。

【０１８２】

図４３で説明されるように、これらのフラグメントは、すべて～３４残基または～１７残基のいずれかの区間にあり、これは、単一のタウ反復またはその半分に等しい。したがって作製されたフラグメント、はすべて、図で示されるように形成された基本的な七量体凝集物から、図４３の矢頭で示される位置で生じるタンパク質分解性切断の単純なセットから生じると理解することができる。この概略図では、フラグメントは、凝集物のいずれかの末端で矢頭により示される三つの可能なおおよその位置で生じる提示された切断の全組み合わせのセットとして生じる。ｍＡｂ３４２およびこれらのフラグメントに伴うＴ４６で認められる対応する予測される免疫反応性パターンをも表にする。

【０１８３】

図４４は、長さが低下する順およびゲル可動性が増す順でこれらの同一のフラグメントを示す。七量体の凝集は、便宜上、全長タウ分子から専ら生じるとして示されるが、１２／１４ｋＤフラグメントが提示された凝集内に挿入されて、結合パートナーのいくつかと置換でき、そしてフルセットからの正確なフラグメントが示された実例において優勢である、凝集内のこれらの短いフラグメントの封入の正確なパターンが決定されることは理解さ

れよう。したがって、このタンパク質分解性フラグメントのファミリーは、細胞内で種々の方法で例示され得る可能なレパートリーとしてよりよく理解される。

【０１８４】

実施例４

タンパク質分解性フラグメントの産生における化合物の阻害効果

１２ｋＤのフラグメント（およびその他のもの）の産生が制御され得る安定した細胞系を達成したので、モデルを用いて低減されたチオニンの阻害効果を試験した。これを図２３に示す。レーンの各セットでは、より高レベルのＴ４０を誘導する漸増濃度のＩＰＴＧの存在下で１２ｋＤのバンドの産生が誘導される。チオニン濃度が増加するので、Ｔ４０からの１２ｋＤバンドの産生が、抑制される。これを図２４で定量的に示す。チオニンの不在下では、漸増濃度のＩＰＴＧでのＴ４０の誘導により、対応する１２ｋＤフラグメントの産生の増加が導かれる。チオニン２μＭの存在下では、Ｔ４０は、依然誘導されるが、１２ｋＤフラグメントに変換されない。

【０１８５】

還元チオニン自体が、有毒であるので、高レベルのチオニンで対応するＩＰＴＧ用量により誘導されるＴ４０のレベルでの還元に関して制御する必要がある。これは、１２ｋＤ：Ｔ４０の比率を決定することにより達成でき、これによりＩＰＴＧレベルと交わるデータを平均化することが可能になり、そして全長タウに相対して１２ｋＤのレベルの用量依存的低下が示される。

Ｔ４０／１２ｋＤアッセイにおける種々化合物の作用を図９～１６に示す。

【０１８６】

還元物質（ＤＴＴ／アスコルビン酸塩２００μＭ）の存在下、示される濃度で導入されたチオニンもしくは塩化トロニウム、または還元物質を伴わないで導入されたクロロルプロマジンもしくはタクリンの存在下、ＩＰＴＧ（０、１０、２５、５０μＭ）で細胞を処置することにより全長タウ（Ｔ４０）を誘導した後、１２ｋＤ：Ｔ４０の比率に関する結果を表す。認められるように、チオニンおよび塩化トロニウムは、実質的に同等な阻害を生み出し、一方クロルプロマジンおよびタクリンは同一の濃度範囲で阻害しない。還元剤単独の効果を対照実験で試験し、還元剤単独の存在下では１２ｋＤ：Ｔ４０比に著明な差異は認められなかった。

【０１８７】

高分子量分解産生物を産生する細胞系の特性もまたＭＢおよびＤＭＭＢ（ジメチルメチレンブルー）で試験した。

【０１８８】

図４５で認められるように、ＤＭＭＢは、細胞モデルにおいて驚くほど可能性があるであることが判明した。ＩＰＴＧ誘導の不在下および誘導後の双方でその阻害活性が認められ得る。ＤＭＭＢ１μＭでの処理により、細胞内のすべての分解産生物が、効果的に排除された。ＭＢおよびＤＭＭＢを用いる別の実験で、１２／１４ｋＤ種の見かけの基礎産生でさえ凝集により大きく決定されることが示されている。すなわち、２９５～３９１フラグメントの構成的産生自体は、イムノブロットによる検出レベル以下であるか、またはそうでなければイムノブロットにより検出され得る細胞内レベルに到達するように自発的な凝集により安定化される。また別に、ＩＰＴＧ誘導せずに、およびタウ凝集インヒビターでの処置を行わずに認められる１２／１４ｋＤフラグメントの見かけの基礎レベルは、それ自体、誘導なしで産生されたＴ４０の漏れレベルからの鋳型化凝集依存性産生により決定づけられ得る。基礎条件での１２／１４ｋＤフラグメントのレベルを決定する因子の組み合わせにかかわらず、強力な凝集インヒビター、例えばＤＭＭＢにより、その見かけの発現が、高分子量凝集産生物と共に実質的に排除され得る。これらの結果により更に、図４１、４３および４４に示すように、高分子量タンパク質分解性フラグメント（すなわち３０／３２、３６／３８、４２／４４ｋＤ）が、反復ドメインを介して生じる重大なタウ－タウ結合相互作用に依存することが確認される。

【０１８９】

図４６は、細胞内タウ濃度および図１０～１６で用いられるインビトロタウ－タウ結合親和性に関する同一の一連の仮説を用いて、１２／１４ｋＤ種の基礎発現に及ぼすＤＭＭＢの作用を示す。この場合、ＤＭＭＢは、細胞４．４nM内での見かけのＫＩを有することが見出され、そして細胞性Ｂ５０値は～１００nMである。これは、ＤＭＭＢが細胞環境内で非常に強力であることを示している。

【０１９０】

実施例５

還元および酸化化合物の阻害効果の比較

インビトロデータに関して使用するための数学的モデルを用いて、Ｔ４０：１２ｋＤ細胞アッセイにおける被験物質の効果を分析した。インビトロデータからのＫｄおよびＫＩに関する公知の値を用いて、示された発現を用いて全長タウの細胞内濃度を解釈した（例えば図１０参照）。

【０１９１】

これは約５００nMであることが見出されたが、これは、脳および細胞系におけるタウの実験から予測される範囲内である。実験データに対する良好な適合性が得られ、これは、いくつかの化合物に関して、細胞内での断端されたタウ産生の阻害が、実験的にインビトロで決定されたおよそのＫｄおよびＫＩ値から予測され得ることを意味している。

【０１９２】

実施例６

ジアミノフェノチアジンの阻害特性の試験

インビトロ研究では、同定された最も活性なタウ－タウ結合インヒビターは、０、２、または３個のメチル基を有するジアミノフェノチアジンの還元形態であった。図２５は、かかる化合物の還元形態を示す。図２６および２７で対応するタウ－タウ結合曲線は、タウに関するモル比の関数として示される。示したように、「脱メチルシリーズ」（０、２または３個のメチル基）化合物は、化合物：タウ「ＡＭＲ」の３：１～４：１のモル比（横軸で対数目盛りで示す）でタウ－タウ結合（縦軸で示す）のおよそ５０％阻害を生み出す。この群の化合物に関するタウ－タウ結合の５０％阻害の平均モル比は、４：１である。

【０１９３】

４または６個のメチル基（メチル化基）を有するジアミノフェノチアジンは、２相性作用を有し、低濃度でタウ－タウ結合を増強し、高濃度でタウ－タウ結合を阻害する（図２７）。したがってこれらの化合物は、タウ－タウ結合を５０％阻害するのにより多くのモル比を必要とする。

【０１９４】

ジアミノフェノチアジン化合物の別の特徴の実験をも行った。ヘテロ環式窒素またはイオウ原子の置換は、化合物の阻害可能性に重篤に干渉することが見出された。同様に、ジアミノ基の除去は、阻害活性に不利益であることが見出された。したがってジアミノおよびヘテロ環式ＮＢおよびＳ－構造はタウ－タウ結合の阻害における分子の活性に重要であると思われる。

【０１９５】

比較のために、二つの方法を用いてタウ－タウアッセイにおける阻害活性を決定した：ＳＴＢは、標準的なタウ濃度４４８nMで、化合物１および１０μg/mlで観察される平均タウ－タウ結合である；ＬＢ５０は、タウ－タウ結合の５０％阻害を生み出す化合物：タウのｌｏｇ１０　モル比である（図２８）。図２９に示すように、様々な化合物に関するＳＴＢおよびＬＢ５０値間に強力な相関性があり、クロルプロマジンおよびリボフラビンは二つの外れ値である（図３０および３１をも参照）。

【０１９６】

実施例７

阻害活性および拡散能力

図３２は、被験化合物におけるメチル基の数（ＮＭＥＴＨ）並びに還元電位（Ｅ）および拡散係数（ＤＩＦ）の双方の間に相関性があることを示している。全比較でスピアマンの

順位相関を用いた。図３２に示すように、メチレンブルーを排除する場合のみ、メチル基の数（ＮＭＥＴＨ）および還元電位の間に強力な逆関係が認められる（標準体：メチレンブルーを含む相関値；イタリック体：メチレンブルーを排除した相関値）

【０１９７】

これはメチレンブルーが、このシリーズのメチル基の数（ＮＭＥＴＨ）に相対して不釣合いに高い還元電位を有していることを示している。メチル基の数および拡散係数（ＤＩＦ、図３２）間にも強力な正相関が存在する。

【０１９８】

メチル基の数および還元電位間に相関性が観察されない（図３３）のと同様に、還元電位および阻害電位間に相関性が観察されなかった（図３４ｂ）が、アッセイ条件におけるジアミノフェノチアジンの還元の程度は、還元電位に高度に相関する（図３３）。そして実際に、これらの化合物の還元の程度および阻害可能性の間に相関性は存在しない（図３４ａ）。一方、化合物の阻害可能性およびその拡散係数間に強力な逆相関が存在し、そしてより大きな重量が拡散係数に与えられる場合、還元係数および拡散係数の１次関数として推定ＬＢ５０およびＳＴＢ値を予測することが可能である（図３５、３６および３７）。ＬＢ５０およびＳＴＢ値の双方は、３を含んで３までのＮＭＥＴＨ値に関して一様に低いことが見出されるが、より高いＮＭＥＴＨ値に関しては（とりわけメチレンブルー、ＮＭＥＴＨ＝４)、メチル基の数に相対して阻害可能性は不釣合いに低い。これは、拡散係数により測定されるように、分子のスタッキング能力に干渉するメチル基の対称的な配置に関係し得る。これは、例えばメチレンブルーの結晶性構造において認められる（図３８参照）。ジアミノフェノチアジン分子は、実質的に平面的でスタッキングアレイを形成する。分子における荷電の存在は、酸化形態と同様に、かかるスタッキングアレイの形成を防御し、そしてこの化合物の還元形態が、シリーズの阻害可能性を決定するかようなスタッキング関係を形成する傾向があるように思われる。

【０１９９】

本発明者らにより実施された実験は、ＷＯ９６／３０７６６に更に詳細に記載されるように、水相における全長タウの、固相におけるタウの断端された反復ドメインフラグメントに対する結合を試験した。ｍＡｂ３４２またはｍＡｂ４９９のいずれかで結合を検出した。図３９に示すように、大過量の標準的な還元剤ジチオスレイトール（ＤＴＴ、１ｍＭ）の存在下、典型的なタウ濃度依存的タウ－タウ結合が存在する。しかしながら、前記したアッセイの標準的な形態において、フェノチアジンの阻害活性もまたＤＴＴ（１ｍＭ）の存在下で実証される（すなわちＳＴＢおよびＬＢ５０に関するデータ）。本発明者らは、阻害活性は、ＤＴＴそのものに起因し得るものではなく、むしろ過剰のＤＴＴによる還元形態でのフェノチアジンの存在に起因すると結論付ける。

【０２００】

要約すると、本発明者らは本明細書にて、タンパク質の立体配座重合が誘起されるアルツハイマー病のごとき、例えばアルツハイマー病の場合で病理学的なタウ－タウ結合により説明されるような疾患の処置および予防のための強力で著明に改善された系を提供する。本出願の重要な教示、すなわち化合物の拡散係数が、この立体配座タンパク質重合の誘起に対するその阻害可能性の決定において重要であり得るということは、アルツハイマー病のごとき疾患の理解の進歩、およびその治療を提供する能力において大きな利益がある可能性がある。最後に、ＭＢの還元形態の嗜好性に関する知見、および単にインビトロデータに基づいて予測された値よりも実質的に低い濃度での細胞ベースのアッセイにおけるその活性の実証を組み合わせることにより、本発明者らはこの化合物および同様のその他のものをＡＤおよび関連障害の予防または処置に適当な還元処方として使用できることを示している。

【０２０１】

【表３】

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【図面の簡単な説明】

【０２０２】

【図１】対になったらせんフィラメントの構造（上）およびアルツハイマー病の進行中の神経原繊維濃縮体の免疫化学（下）の概略図を示す。

【図２】重要な核形成因子がタウ捕捉を開始し、次いで自己触媒性になる「種」を提供する概念図を示す。

【図３】アルツハイマー病の推定される病原モデルを示す。タウ凝集は、軸索輸送の失敗およびその結果であるニューロン死に先行する最も近い過程である。タウ凝集カスケードは、タウ遺伝子の上流の変化から、または１次突然変異から生じる種まき／核形成事象のいずれかにより誘発される。

【図４】細胞中にいくつかの既存の１２ｋＤタウがある場合、全長タウの誘導がどのようにその１２ｋＤフラグメントへの変換に至り得るかを示す。

【図５ａ】タンパク質凝集の疾患において役割を果たすタンパク質を列挙する表を示す。疾患そのもの、関与すると考えられる凝集ドメインおよび／または突然変異、および推定（最大）フィブリルサブユニットの大きさをも列挙している。各タンパク質に関する一つまたはそれ以上の文献参照を提示する。

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（最大）フィブリルサブユニットの大きさをも列挙している。各タンパク質に関する一つまたはそれ以上の文献参照を提示する。
【図６】Ｎ－およびＣ－断端されたタウの異なる形態に対する種々モノクローナル抗体の結合部位の概略図を示す。
【図７ａ】ヒトタウタンパク質アイソフォームのヌクレオチドおよび予測されるアミノ酸配列を示す。配列はｃＤＮＡクローンｈｔａｕ４０から導いた。
【図７ｂ】ヒトタウタンパク質アイソフォームのヌクレオチドおよび予測されるアミノ酸配列を示す。配列はｃＤＮＡクローンｈｔａｕ４０から導いた。
【図８】チオニン、塩化トロニウム、クロロプロマジンおよびタクリンの構造を示す。
【図９】ジアミノフェノチアジンに関する細胞アッセイデータ、および構造的に関連するアンスロキノンを本明細書で記載するように決定した見かけのＫＩ値と共に提示する。本明細書の図および実施例では、別のパラメーターＢ５０を計算して細胞ベースのアッセイの条件に直接関連する様式で活性を発現し、そしてしたがって対応するインビボ活性を達成するのに必要な組織濃度の指数を提供した。Ｂ５０値は、細胞アッセイで用いられた被験化合物の濃度であり、そのアッセイでは、全長タウからの１２ｋＤバンドの相対的産生が、化合物不在で観察された値の５０％まで低減した。見かけのＫＩ値およびＢ５０値間には以下のような単純な直線的関係がある：　　細胞性Ｂ５０＝０．０２１７　ｘ　ＫＩ　治療用化合物の相対的な有用性を比較するために、ＬＤ５０値を計算するのが望ましい。阻害特性が、類似する場合、臨床使用に好ましい化合物は、ＬＤ５０値が最も高い化合物でよい。治療指数（Ｒｘインデックス）を以下のように細胞アッセイにおいて試験された各化合物に関して計算できる：　　Ｒｘインデックス＝ＬＤ５０／Ｂ５０　残存細胞の溶解の後、乳酸塩デヒドロゲナーゼアッセイキットＴＯＸ－７（シグマ・バイオサイエンシズ）を用いて製造者の指示書に従って、化合物に２４時間暴露した後の細胞数により化合物の有毒を測定できる。また別に、プロメガＵＫ（ＣｙｔｏＴｏｘ　９６）のキットを製造者の指示書に従って用いることができる。
【図１０】７つの実験の一連のデータに基づく、本発明の還元チオニンを用いた結果を示す。１２ｋＤバンドの産生に関して観察された細胞データを、インビトロのタウ－タウ結合の阻害を記載する標準関数に密接に適合させることができる（すなわち観察された相関係数対予測された相関係数＞０．９）。この適合を得るために、別の細胞ベースの研究およびインビトロ研究からの結果と一致する二つの仮説：　１）タウの細胞内濃度は、およそ５００nMである；　２）タウ－タウ結合親和性は、２２nMである。を作る必要があり、これらの仮説を用いて標準阻害モデルにより予測される細胞活性に関する関数：
活性＝［タウ］／［タウ］Ｋｄ＊（１＋［チオニン］／ＫＩ）を見かけのＫＩの値を誘導する標準的な数的方法により解くことができる。示したように、チオニンの還元形態の値は、１００nMであり、これはタウ濃度５００nMでインビトロのタウ－タウ結合に関して観察された値と実質的に同一であり、この場合、タウ－タウ結合に関するＫｄ値は、２２nMであることが解っている。したがって、チオニンの活性は、この場合読み出しは全長タウからの１２ｋＤトランケーション産生物の産生であり、細胞内の反復ドメインを通って生じるタウ－タウ結合の阻害の程度に基づいて定量的に説明できる。これによりタウ－タウ結合の程度が、細胞内ＰＨＦのタンパク質分解的に安定したコアタウユニットの産生を決定することが確認される。　タウ細胞内濃度（５００nM）および反復ドメインを介するタウ－タウ結合親和性（２２nM）に関する同一の仮説を用いて、その他の化合物の活性の後続のすべての細胞分析を同一の標準化された様式で報告する。
【図１１】還元剤を省略した条件に関する結果を示す（すなわち酸化チオニン、図１０参照）。　再度細胞活性を標準阻害モデルにより予測する：　　活性＝［タウ］／［タウ］Ｋｄ＊（１＋［酸化チオニン］／ＫＩ）　この場合、チオニンは、ここで１２００nMの見かけのＫＩ値を有する。これにより、ジアミノフェノチアジンが、活性のために還元形態を必要とすることが確認される。同様の結論が、インビトロ結合データかの分析から誘導された（結果は示していない）。
【図１２】還元または部分的還元条件を用いることにより、メチレンブルーが、細胞ベー

スアッセイにおいて、アッセイの時間経過（１～２時間）が、還元を達成するのに十分ではないインビトロ研究から予測されるものよりも更に活性なようであることを示している。　細胞活性は、再度、標準阻害モデルにより予測される：　　活性＝［タウ］／［タウ］Ｋｄ＊（１＋［ＭＢ］／ＫＩ）　細胞活性では、メチレンブルーの見かけのＫＩ値は、１２３nMであり、これはチオニンおよび塩化トロニウムと同一の範囲内である。図９で示すように、タウ凝集を阻害するのに必要とされる対応する脳組織濃度（すなわちＢ５０値）は２～３μMである。

【図１３】還元塩化トロニウムの対応する細胞ベースの活性データを示し、これは、再度、インビトロ研究から誘導された予測されるＫＩ値を用いて、細胞内の全長タウからの１２ｋＤフラグメントの産生について記載することができる。　細胞活性は、標準阻害モデルにより予測される：　　活性＝［タウ］／［タウ］Ｋｄ＊（１＋［ＴＣ］／ＫＩ）　これにより用いた数学的分析手順の正当性が更に確認される。

【図１４】構造的にジアミノフェノチアジンに関連するＤＨ１２が、アッセイ条件において不活性であることを示している。

【図１５】クロルプロマジンおよびタクリンは各々別にして、前記の図９～１４で提示されたものに対する類似の分析を示す。同一の仮説（タウ濃度４１５nM、およびタウ－タウ結合Ｋｄ　２２nM)、および標準阻害モデルにより予測される細胞活性：　　活性＝［タウ］／［タウ］Ｋｄ＊（１＋［ｃｐｚ］／ＫＩ）を用いると、クロロプロマジンおよびタクリンの見かけのＫＩ値（各々２１１７nMおよび８０２nM）は、インビトロ研究から予期されたものよりも大きい。

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【図１７】ＤＴＴ存在下での種々化合物の還元の程度を示す。

【図１８】ＭＢ：ビタミンＣの比率に対してプロットされたＭＢの還元パーセントを示す。

【図１９ａ】標的組織濃度を４μＭ（すなわち１．５μｇ／ｇ）と想定することにより、このオーダーの組織濃度が、ＩＶ投与量０．１１mg/kgで達成されるというＤｉＳａｎｔｏおよびＷａｇｎｅｒ（１９７２）のデータから決定することが可能であることを示している。

【図１９ｂ】７０kgの対象に一回で１００mg投与の後のＭＢの分布に関するモデルを示し、即時的吸収が想定される。

【図２０】顕微鏡的および生化学的実験の双方のデータに基づいて、３Ｔ３およびＣＯＳ－７細胞におけるタウフラグメントの一過性発現の結果を要約する。　真核細胞における組換えタウフラグメントの発現を以下のように実施した。３Ｔ３細胞およびＣＯＳ－７細胞において一過性に発現された８個のタウ構築物を、免疫細胞化学およびイムノブロットにより試験した。各細胞型における発現の程度を、双方の一連の結果に基づいて半定量的に提示した：－、発現検出不能；±、非常に弱い免疫反応性；＋から＋＋＋＋、免疫反応陽性の増加レベル。すべての場合で、ｍＡｂ７．５１を各構築物と共に用いて結果を得た。加えて、タウタンパク質の異なるドメインに対する抗体パネルを用いることにより特異性を各構築物に関して確認した（ｍＡｂ　４９９、Ｔ１４、タウ１、３４２、７．５１、４２３およびＴ４６）。コザック配列は、最初の６個の構築物には存在しなかったが、ｃＤＮＡ構築物７および８には存在した。

【図２１】二つの細胞系の３Ｔ６繊維芽細胞における全長ヒトタウの発現誘導を説明する。Ｔ４０．２２は、非誘導状態の全長タウ（「Ｕ」）の低レベルバックグラウンド漏れおよび、ＩＰＴＧ添加後の高レベル発現（すなわち誘導「Ｉ」）を示す。Ｔ４０．３７は、同一であるが、誘導なしでは発現レベルが低いことを示している。

【図２２】３重ベクター系の結果を示す。１２ｋＤフラグメントの非常に低レベルの構成発現が可能なベクターを、全長タウの発現誘導が既に達成されている細胞系に導入した（実際に、細胞系Ｔ４０．２２は、前記の図２１に示される）。低レベルのＩＰＴＧを導入して全長タウの発現を誘導する。ＩＰＴＧ　０μＭでは、１２ｋＤバンドの非常に低レベルの発現、および全長タウの低い「バックグラウンド漏れ」発現がある。より高レベルのＩＰＴＧを導入することにより、累進的に多くの全長タウが誘導され、より多くの全長タウが１２ｋＤ種に変換される。

【図２３】還元チオニンの阻害効果を示す。各々のレーンのセットで、漸増濃度のＩＰＴＧの存在下、１２ｋＤのバンドの産生が誘導され、より高レベルのＴ４０が誘導される。チオニンの濃度が増加するので、Ｔ４０からの１２ｋＤバンドの産生が抑制される。

【図２４】図２３ｍの定量的な結果を示す。チオニンの不在下では、漸増濃度のＩＰＴＧでのＴ４０の誘導により１２ｋＤフラグメントの対応する産生増加が導かれる。チオニン２μＭの存在下では、Ｔ４０の誘導が依然存在するが、１２ｋＤフラグメントには変換されない。

【図２５】種々化合物に関するインビトロＫＩ値をnMで比較として示す。ＫＩ値は、用いた特定のアッセイ条件に関連する（５００：１　ＤＴＴ：化合物。１２０分、図１７参照）。

【図２６】各々０、２、３または０、４、６個のメチル基を有するフェノチアジンのタウ－タウ結合に及ぼす阻害効果を示す。

【図２７】各々０、２、３または０、４、６個のメチル基を有するフェノチアジンのタウ－タウ結合に及ぼす阻害効果を示す。

【図２８】被験化合物によるタウ－タウ会合の阻害を測定するのに有用な二つのパラメーターの導出を示す。ＳＴＢは、化合物の不在下で認められるものに相対して標準化された結合であり、１および１０μg/mlで観察された平均と見なす。ＷＯ９６／３０７６６に記載されるように、１．０のＳＴＢ値は、化合物の不在下で観察される値に等価の結合を示すが、一方０．２の値は、被験化合物濃度１および１０μg/mlで結合が平均２０％まで低減したことを示している。ＬＢ５０は、化合物不在下で認められる値と比較して５０％タウ－タウ結合を産生する化合物：タウのモル比（Ｂ５０）のｌｏｇ１０である。

【図２９】ＳＴＢおよびＬＢ５０パラメーター間の関係を示す。ＳＴＢは、ＬＢ５０の一次関数であることを示すことができる。　ＳＴＢは、タウ－タウ結合が、５０％低減している化合物：タウのモル比の対数関数である。　ＬＢ５０は、タウ－タウ結合が化合物の不在下で観察された値の５０％であるタウに関する化合物のモル比のｌｏｇである：

　　ＬＢ５０＝０．０５＋（２．６５　ｘ　ＳＴＢ）　　ｒ＝０．９５　インビトロＢ５０の決定には、ある程度のタウ－タウ結合の阻害が存在し、そして５０％の値が外挿法により得られることが必要である。ＳＴＢの決定には、かかる外挿手順を必要としない。

【図３０】ＳＴＢおよびＢ５０値の双方が決定された化合物を示す。細胞における全タウ濃度がおよそ５００nMである（すなわちアッセイで用いたタウの濃度）と仮定すると、Ｂ５０値は、インビトロアッセイにおいて細胞系で活性が予測され得る濃度（すなわち［５００　ｘ　Ｂ５０］nM）に対する近似値を提供する。

【図３１】一連のジアミノフェノチアジンに関するインビトロＬＢ５０値およびｌｏｇＫＩ値間の形式的な関係を示す。

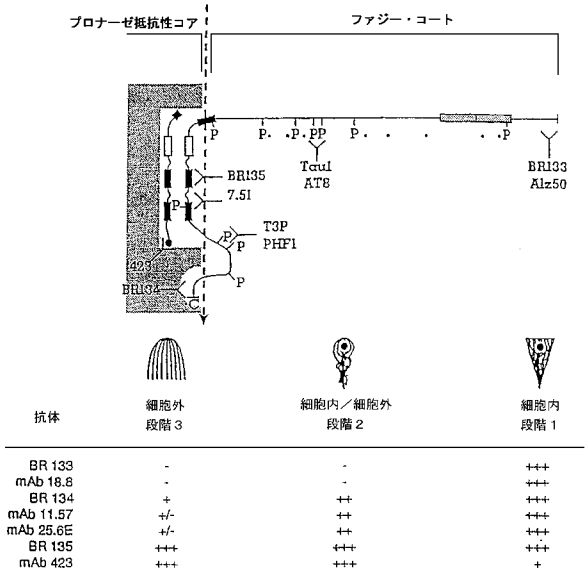
【図３２】ジアミノフェノチアジンにおけるメチル基の数（ＮＭＥＴＨ）並びに酸化還元可能性（Ｅ）および拡散係数（ＤＩＦ）間の関係を示す。イタリックの文字は、ＭＢを排除した後の相関係数（Ｒ）およびｐ値を示す。

【図３３】実験的に決定された還元されている化合物のパーセント、および化合物の公知の還元可能性間の関係を示す。還元電位により、観察されたジアミノフェノチアジンの還元の程度が予測される。

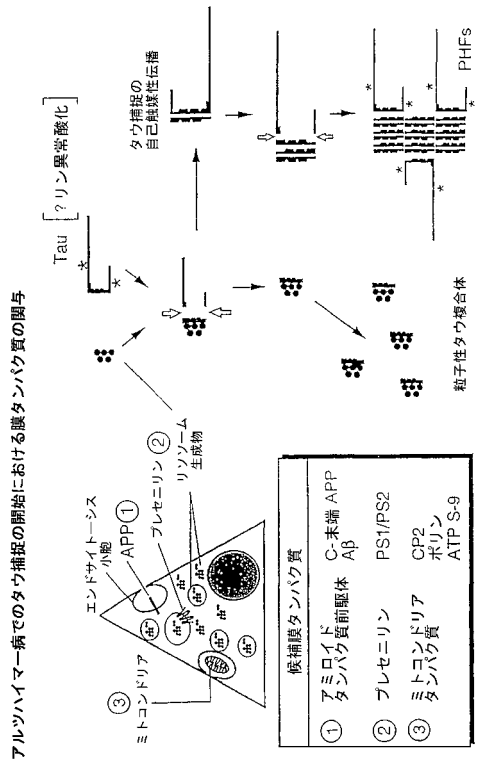
【図３４ａ】阻害可能性および化合物の還元の程度の間に明白な関係がないことを示している。

【図３４ｂ】阻害可能性が単純に還元電位により決定されないことを示している。
【図３５】阻害可能性が直接、拡散係数に関係し得ることを示している（これは、還元形態のスタックおよび凝集の傾向を測定する）。
【図３６】各々推測されたＬＢ５０（「ＥＳＴＬＢ５０」）およびＳＴＢ（「ＥＳＴＳＴＢ」）値、並びに還元電位および拡散係数間の予測された関係を示し、ここで拡散係数は、大きく増量されている。
【図３７】各々推測されたＬＢ５０（「ＥＳＴＬＢ５０」）およびＳＴＢ（「ＥＳＴＳＴＢ」）値、並びに還元電位および拡散係数間の予測された関係を示し、ここで拡散係数は、大きく増量されている。
【図３８】メチレンブルーの結晶構造を示す。
【図３９】ＷＯ９６／３０７６６の固相アッセイで測定される１ｍＭ　ＤＴＴの存在下のタウ－タウ結合を示す。二つの異なる抗体、すなわちｍＡｂ　３４２（上）および４９９（下）を用いてタウ－タウ結合を検出した。縦軸は、タウ－タウ結合を表し、横軸は、水層における全長タウの濃度を示し、そして記号表は、固相タウの濃度変化を示す。見られるように、タウ－タウ結合は、ＤＴＴ存在下で依然生じている。
【図４０】本発明の細胞系で誘導なし（「Ｕ」）および誘導後（「Ｉ」）に存在する様々な種のタウフラグメントおよびダブレットを示す。これには１２／１４ｋＤ、～２５／２７ｋＤ、～３０／３２ｋＤ、～３６／３８ｋＤおよび～４２／４４ｋＤに等価な可動性を有する種が含まれる（実施例３参照）。
【図４１】Ａは、どのように矢頭で示されるおおよその位置でタウ分子全長の鋳型誘起のタンパク質分解性プロセシングにより１２ｋＤフラグメントを生じるかを示している。Ｂは、どのように矢頭で示されるおおよその位置でタウ分子全長の鋳型誘起のタンパク質分解性プロセシングにより２５／２７ｋＤ種を生じるかを示している。
【図４２】図４０～４１の種の見かけのゲル可動性のプロットおよびアミノ酸残基におけるその長さを示す。
【図４３】図４０～４２のフラグメントが一回のタウ反復またはその半分と等価である～３４残基または～１７残基のいずれかの間隔であることを示している。矢頭で示す位置で生じたタンパク質分解性切断の単純なセットとして基本的な七量体凝集からすべてのフラグメントを生じることができる。
【図４４】これらの同一のフラグメントを長さが下降する順およびゲル可動性が増加する順で示している。
【図４５】ＤＭＭＢが細胞モデルにおいて驚くほど強力であることを示している。その阻害活性はＩＰＴＧ誘導の不在下、および誘導後の双方で認められた（実施例４参照）。
【図４６】図１０～１６で用いた細胞内タウ濃度およびインビトロタウ－タウ結合親和性に関する同一の一連の仮説を用いて、１２／１４ｋＤ種の基底発現におけるＤＭＭＢ活性を示す。　細胞活性は、標準的な阻害モデルにより予測される：　　活性＝［タウ］／（［タウ］Ｋｄ＊（１＋［ＤＭＭＢ］／Ｋｉ））　ＤＭＭＢは、細胞内で４．４nMの見かけのＫＩを有し、細胞Ｂ５０値は～１００nMである。

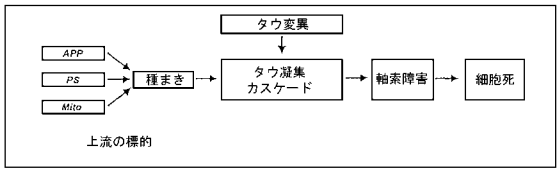
【図１】



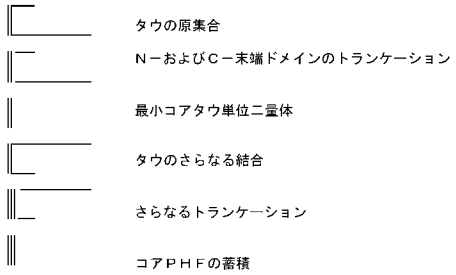
【図２】



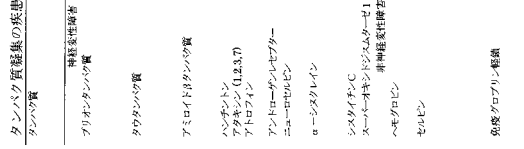
【図３】



【図４】



【図５ａ】



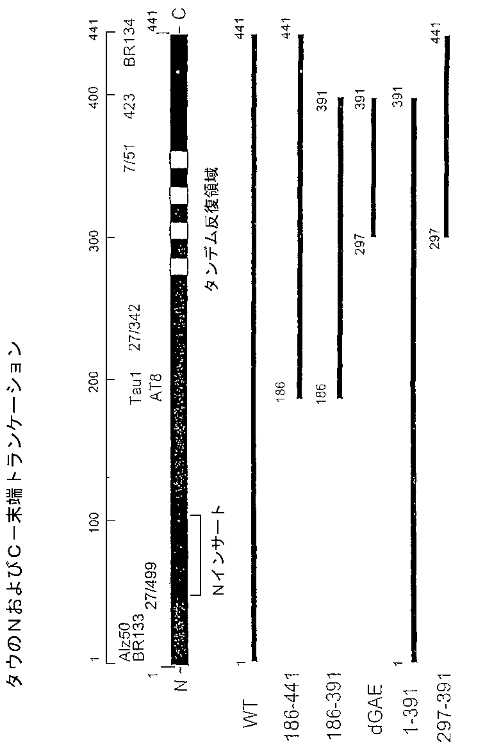
タンパク質凝集の疾患

タンパク質	疾患	凝集ドメインおよび／または変異	フィブリルのユニット大きさ (kDa)	参照
神経変性疾患				
プリオンタンパク質	プリオン病 (CJD、vCJD、発症性家族性予期症、グルストマン・ストラスラー・シェインカー症候群、クールー)	潜伏および疾患性形態 PrP 27～30、多くの変異原媒導形成性ドメイン：113～120、178～191、202～218	27	Prusiner (1998)
タウタンパク質	アルツハイマー病、ダウン症候群、FTDP-17、CBD、脳卒後パーキンソン病候群、ピック病、グアムのパーキンソン痴呆症候群	潜伏性および疾患性形態 トランケートされたタウ（チューブリン結合ドメイン）297～391 FTDP-17におけるタウの変異 プレセニリンタンパク質における多くの変異	10-12	Wischik et al. (1988)
アミロイドβタンパク質	アルツハイマー病、ダウン症候群	潜伏性および疾患性形態 アミロイドβタンパク質：1～42 (3)、痴呆家族性のAPPにおける11の変異	4	Glenner & Wong. (1984)
ハンチントン	ハンチントン病	グルタミン反復の伸長を伴うタンパク質のN－末端	40	DiFiglia et al. (1997)
アタキシン(1,2,3,7)	脊髄小脳性運動失調(SCA1,2,3,7)	グルタミン反復の伸長を伴うタンパク質		Paulson et al. (2000)
アトロフィン	Dentarubropallidoluysian 萎縮症 (DRPLA)	グルタミン反復の伸長を伴うタンパク質		Paulson et al. (2000)
アンドロゲンレセプター	脊髄および延髄筋肉萎縮症	グルタミン反復の伸長を伴うタンパク質		Paulson et al. (2000)
ニューロセルピン	ニューロン封入体を伴う家族性痴呆症 (FENIB)	ニューロセルピン：S49P、S52R	57	Davis et al. (1999)
α－シヌクレイン	パーキンソン病、レビ小体を伴う痴呆症、多発性萎縮症	潜伏および疾患性形態 稀な家族性の常染色体優性 PD における A53T、A30P、	19	Spillantini et al. (1998) Baba et al. (1998) Hasegawa et al. (1999)
シスタチンC	遺伝性小脳性血管障害（アイスランド）	10 残基末端のシスタチンC：L68Q	12-13	Abrahamson et al. (1992)
スーパーオキシドジスムターゼ1	筋萎縮性側索硬化症	SOD1 変異		Shibata et al. (1996)
ヘモグロビン	非神経変性障害			
	鎌状赤血球貧血	ヘモグロビンβ鎖（S）		Carrell & Gooptu (1998)
	封入体筋炎	多くの変異		
セルピン	α1アンチトリプシン欠乏症（気腫、硬変）	変異		Lomas et al. (1992)
	アンチトロンビン欠乏症（血栓素因体症）	変異		Carrell & Gooptu (1998)
	C1－インヒビター欠乏症（血管性浮腫）	変異		Carrell & Gooptu (1998)
免疫グロブリン軽鎖	プラスマ細胞疾患（1次全身性ALアミロイドーシス）	軽鎖またはフラグメント	0.5-2.5	Westermark et al. (1995)

【図５ｂ】

血清アミロイドA	反応性2次全身性AAアミロイドーシス 慢性炎症疾患	SAA の可変性N　末端フラグメント	4.5-10	Westemark et al. (1985)
トランスチレチン	家族性アミロイド多発性神経炎（全身性；FAP I）	立体配座的に単量体変種に分離した四量体 多くの変異（いくつかはアミロイドに随伴されない；いくつかは異なる型の疾患）	10-14	Gustavsson et al. (1991)
	老人性心臓性アミロイドーシス	正常なトランスチレチン	10-14	Gustavvson et al. (1991)
ゲルソリン	家族性アミロイドーシス・フィンランド型（FAP IV）	D187Q がトランケートされた 173～225/243 に至る（必須残基 182～192）	9.5	Maury & Baumann (1990)
β２－マイクログロブリン	血液透析アミロイドーシス 前立腺アミロイド	β２－マイクログロブリン	12-25	Gorevic et al. (1985)
アポリポプロテインＡＩ	家族性アミロイド多発性神経炎（全身性；FAP III）	N　末端 83～93 残基；G26R、W50R、L60R	9	Booth et al. (1995)
ライソゾーム	家族性内臓性アミロイドーシス	ライソゾームまたはフラグメント（156T、D67H を伴うかまたは伴わない）	14	Pepys et al. (1993)
アミリン（島アミロイドポリペプチド）	II 型糖尿病（NIDDM）	フラグメント（20～29 の必須コア）；変異なし	3.9	Westemark (1990)
フィブリノーゲンα鎖	遺伝性腎アミロイドーシス	フィブリノーゲンフラグメント	7-10	Uemichi et al. (1994)
プロカルシトニン	甲状腺髄様癌	カルシトニンフラグメント	3.4	Sletten et al. (1976)
心房性ナトリウム利尿因子	心臓性アミロイドーシス	ANF、変異なし	3.5	Johansson et al. (1987)
インスリン	注射局所アミロイドーシス	インスリン		Dische et al. (1988)
アミロイドを形成するその他のタンパク質	（インビボ）	その他のタンパク質		Chlti et al. (1999)

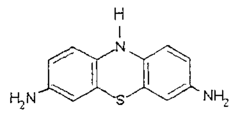
【図６】



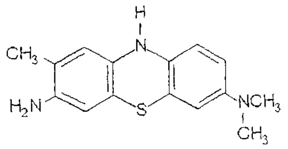
【図８】

細胞基盤アッセイで試験された化合物

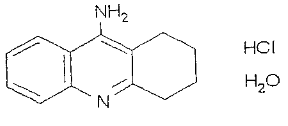
チオニン



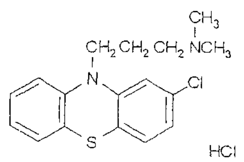
塩化トロニウム



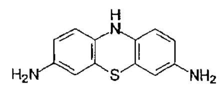
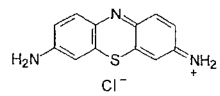
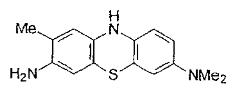
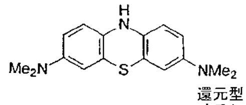
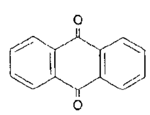
タクリン



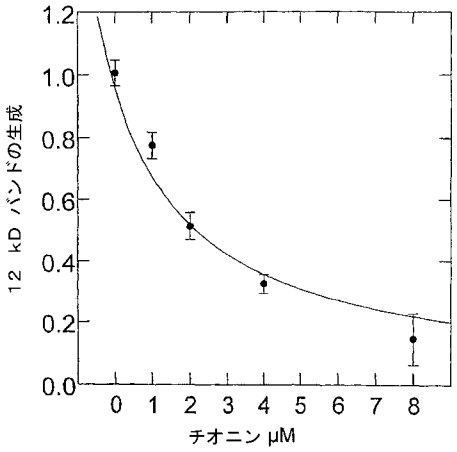
クロルプロマジン



【図９】

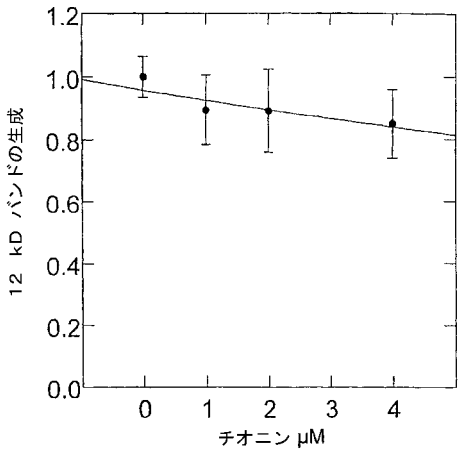
	KI (nM)	B_{50} (μM)
I		
 還元チオニン	100	2.17
 酸化チオニン	1200	26.07
 還元塩化トロニウム	105	2.28
 還元型メチレンブルー	123	2.67
 DH12	—	—

【図１０】



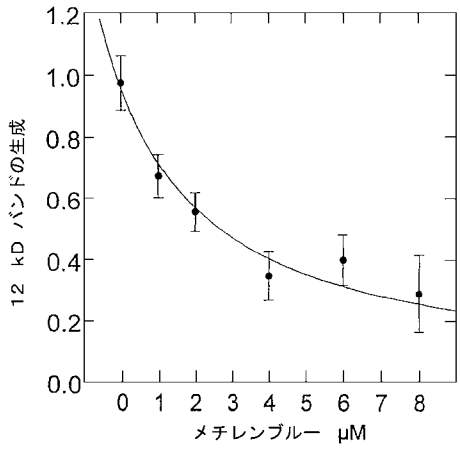
観察された活性対予測された活性	r = 0.986
細胞内タウ濃度	500 nM
タウータウ結合親和性	22 nM
チオニン KI	100 nM

【図１１】



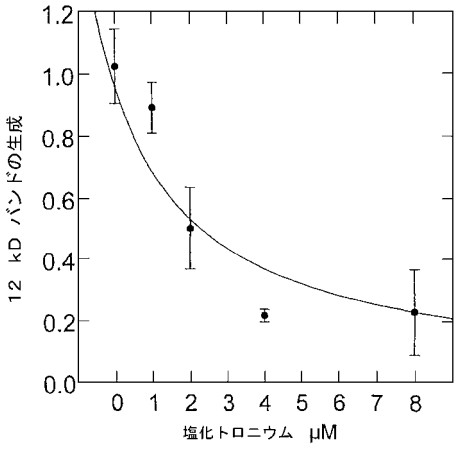
観察された活性対予測された活性	r = 0.784
細胞内タウ濃度	500 nM
タウータウ結合親和性	22 nM
酸化チオニン KI	1200 nm

【図１２】



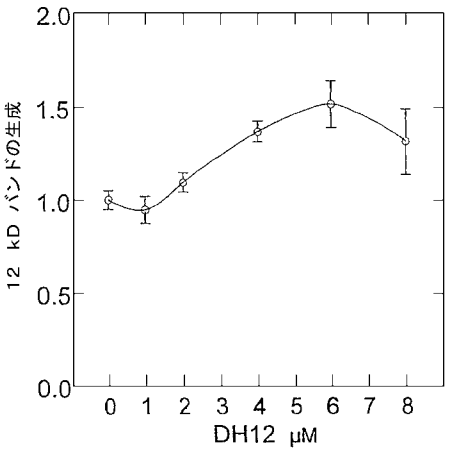
観察された活性対予測された活性	r = 0.962
細胞内タウ濃度	500 nM
タウータウ結合親和性	22 nM
メチレンブルー KI	123 nM

【図１３】

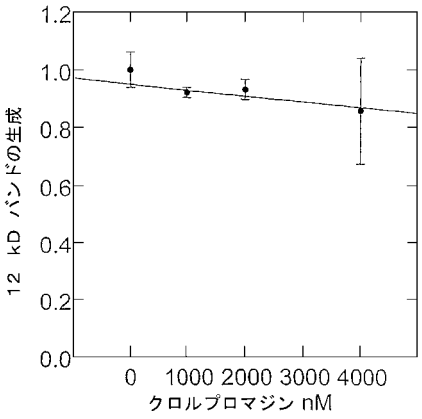


観察された活性対予測された活性	r = 0.913
細胞内タウ濃度	500 nM
タウータウ結合親和性	22 nM
塩化トロニウム KI	105 nM

【図１４】

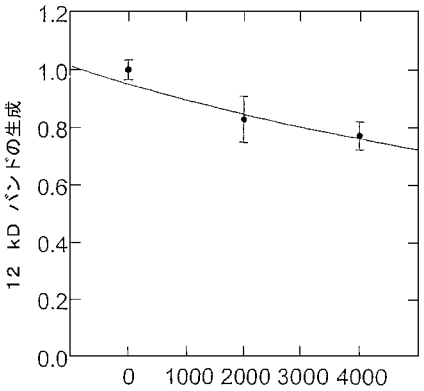


【図１５】



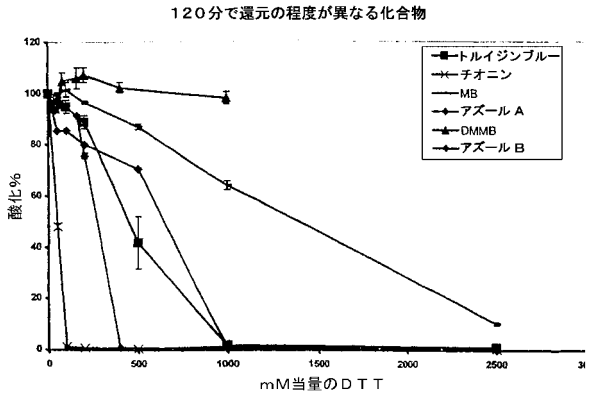
観察された活性対予測された活性	r = 0.937
細胞内タウ濃度	415 nM
タウ－タウ結合親和性	22 nM
クロルプロマジン KI	2117 nm

【図１６】

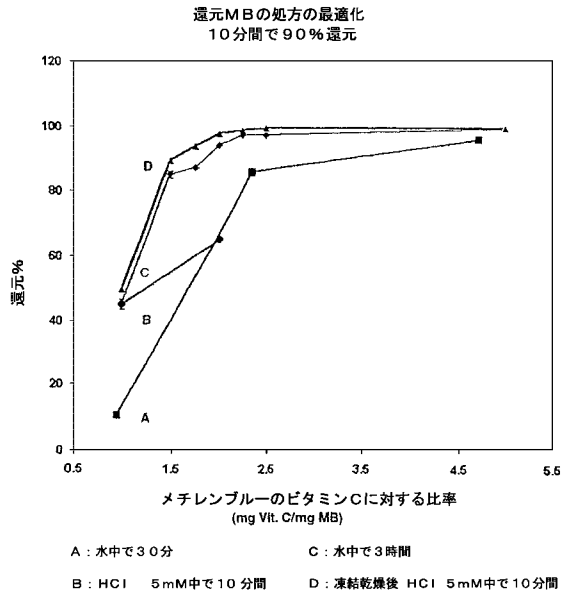


観察された活性対予測された活性	r = 0.976
細胞内タウ濃度	415 nM
タウ－タウ結合親和性	22 nM
タクリン KI	802 nm

【図１７】



【図１８】



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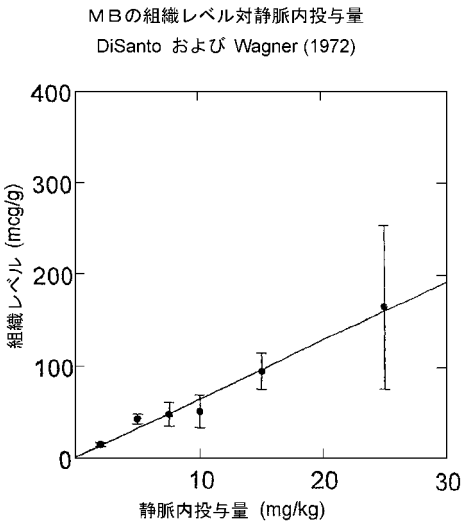
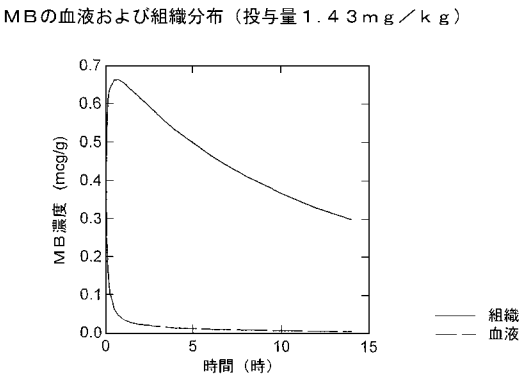


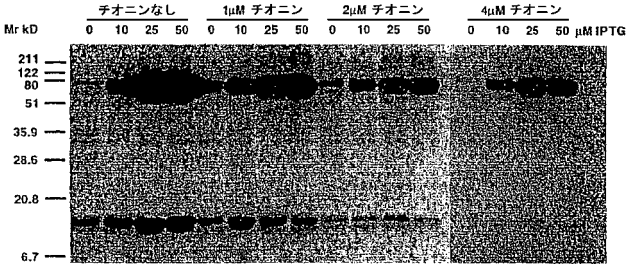
【図１９ｂ】



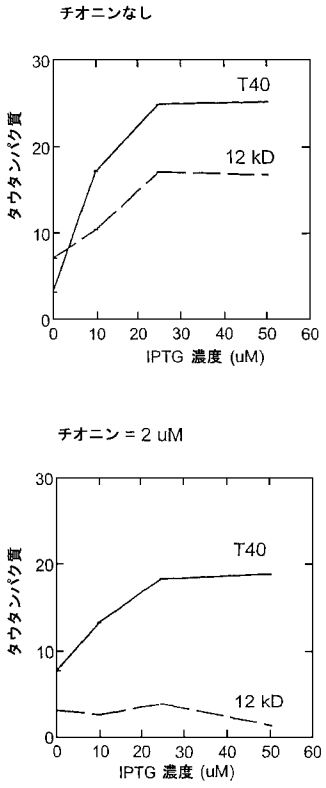
【図２０】

タウの長さ	見かけの Mr (kDa)	発現	
		3T3	COS-7
1) 1-391	55	++	++++
2) m186-391	26	++	++++
3) m297-391	12	+/-	+
4) m186-441	32	++	+++
5) m297-441	18	+	+
6) 1-441	67	++	++++
7) [kozak]m295-391	12	+	+++
8) [kozak]m297-391	12	+/-	++

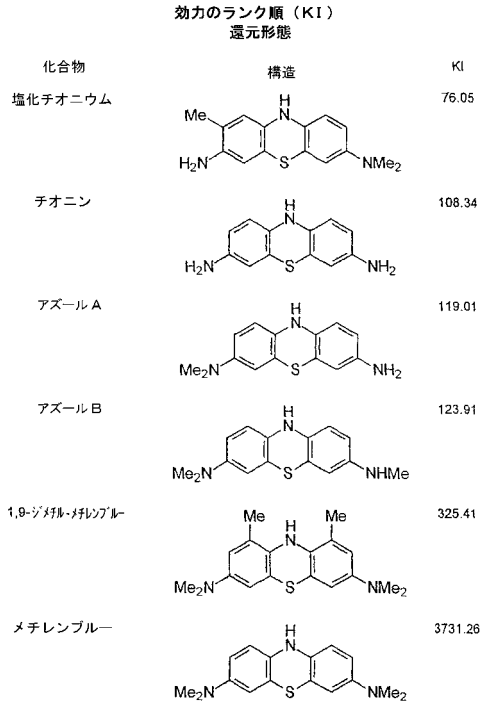
【図２３】



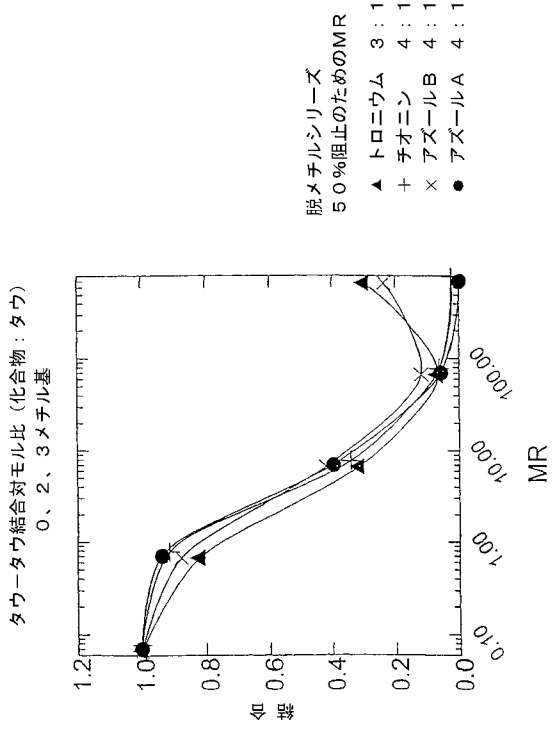
【図２４】



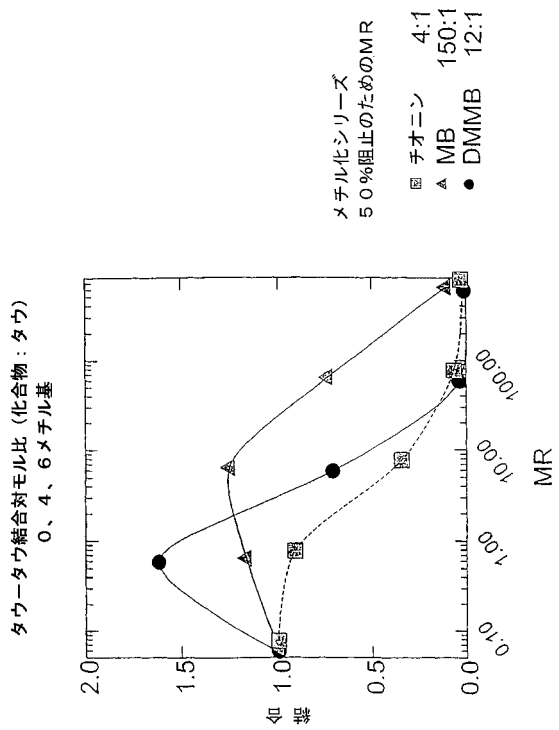
【図２５】



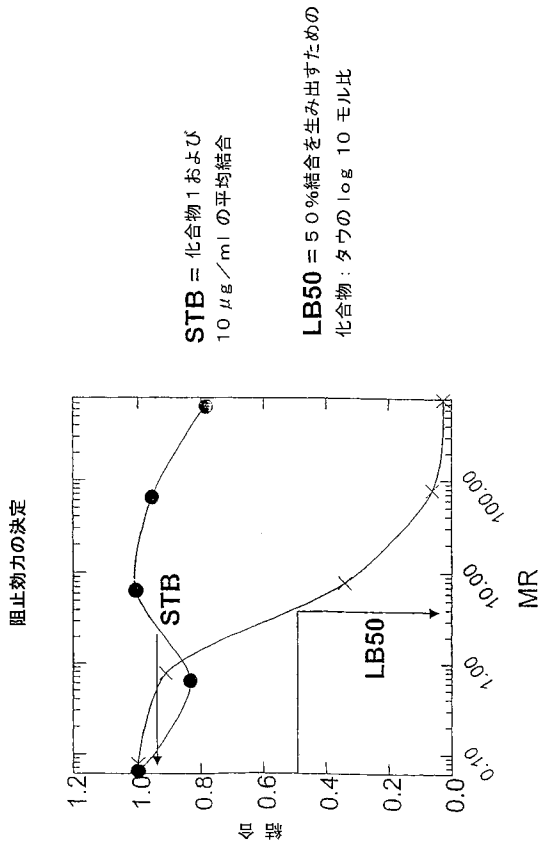
【図２６】



【図２７】



【図２８】



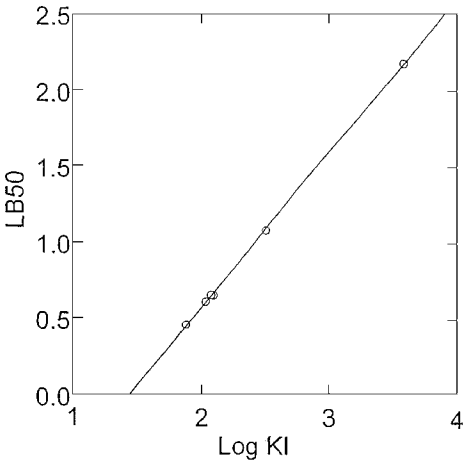
【図３０】

ＳＴＢおよびＢ５０値の間の関係
（タウータウ結合が５０%低減される化合物：タウのモル比）

化合物	STB	B50
塩化トロニウム	0.190	2.86
チオニン	0.201	4.06
アズールＡ	0.227	4.49
アズールＢ	0.269	4.46
ジメチルＭＢ	0.372	12
ビタミンＫ	0.674	48
ニュートラルレッド	0.787	56
ピロニンＹ	0.783	104
プリムリン	0.788	109
アクラフラビン	0.583	132
メチレンブルー（ＭＢ）	0.992	150
フェノチアジン	1.040	508
ガロシアニン	0.997	608
チアジンレッド	0.929	1419

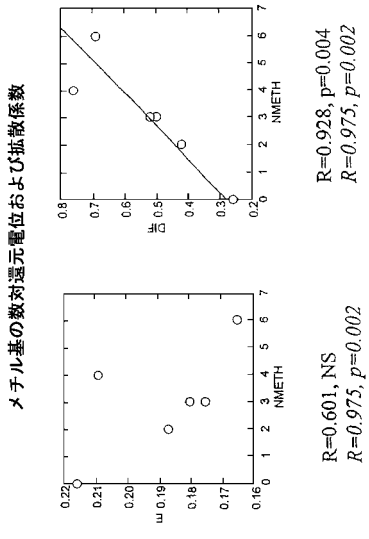
【図３１】

ＬＢ５０値は、ＫＩ値の別の表現であり、ここでジアミノフェノチアジンに関してＬＢ５０値を決定できる。

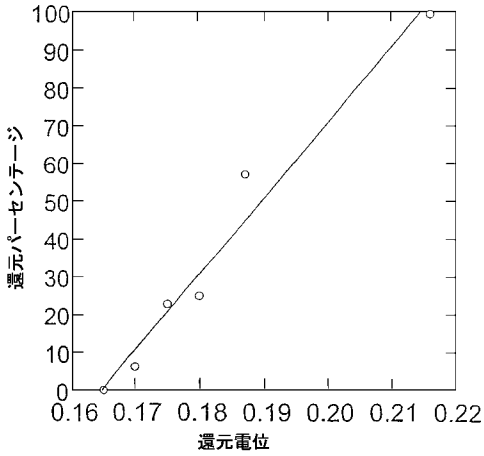


LB50 = (1.019 * Log(KI)) - 1.471

【図３２】

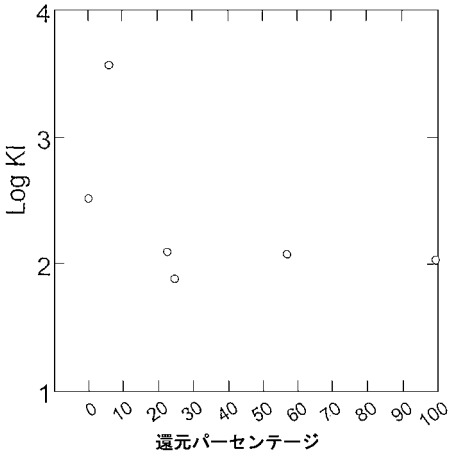


【図３３】

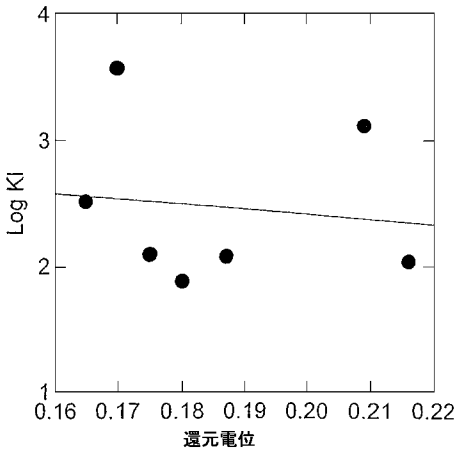


R = 0.947

【図３４ａ】

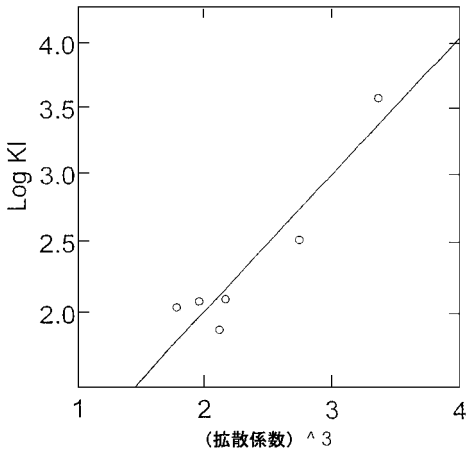


【図３４ｂ】



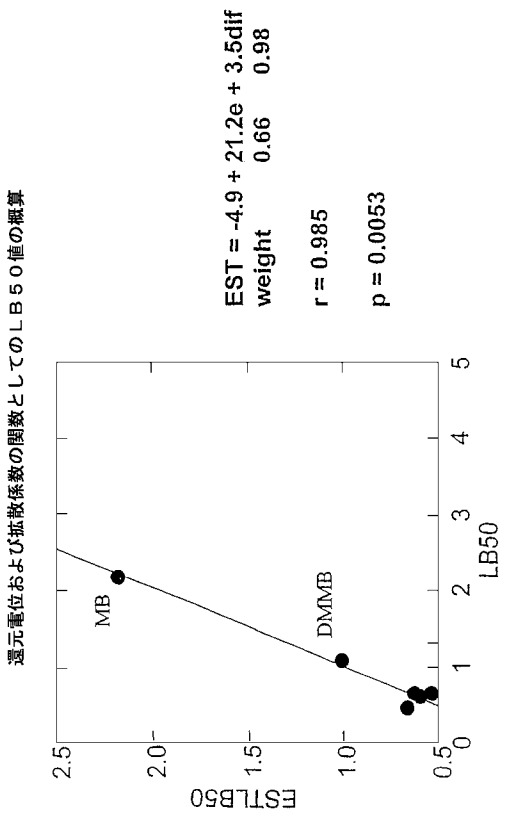
阻止効力は、還元電位により決定されない

【図３５】

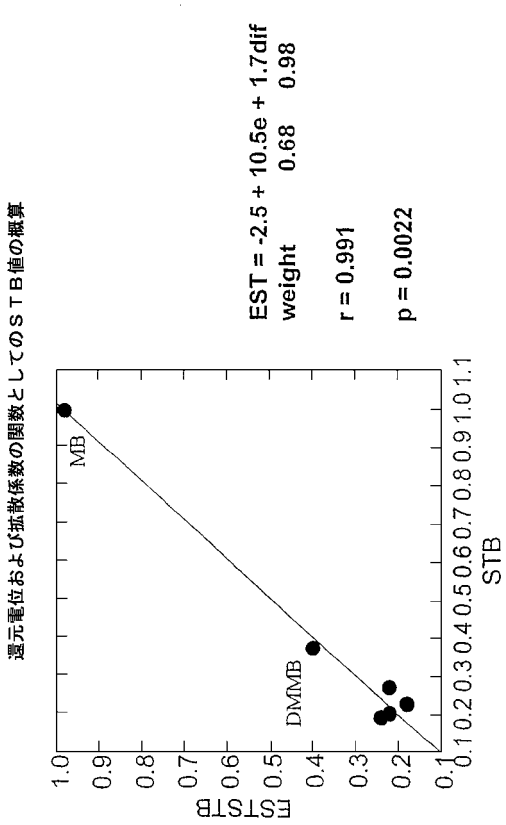


効力は、還元形態の凝集効率に伴うと思われる

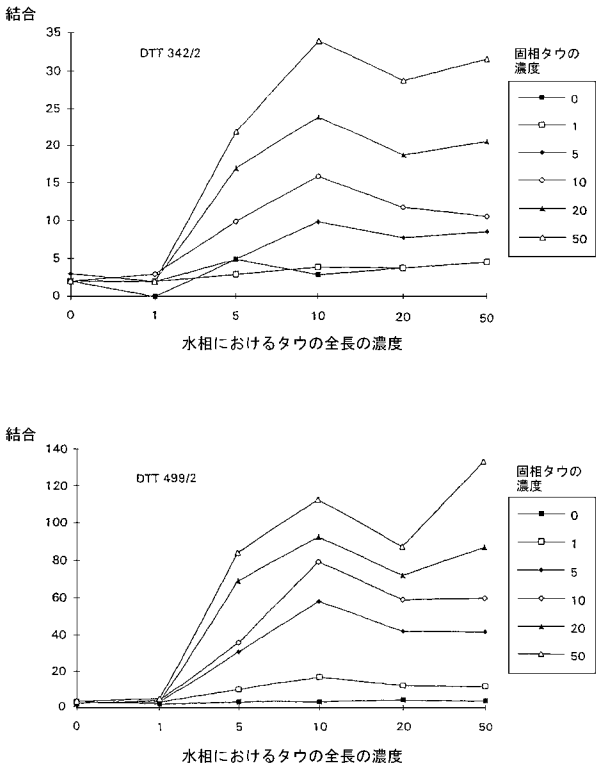
【図３６】



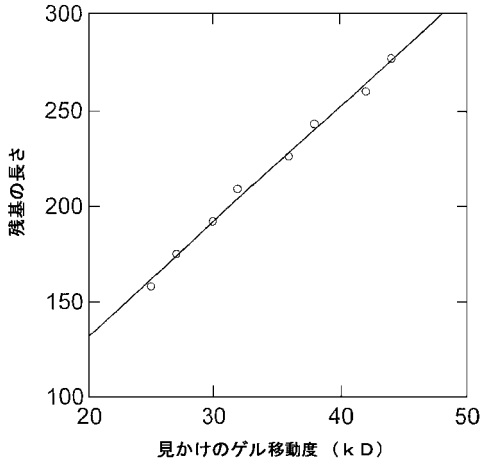
【図３７】



【図３９】

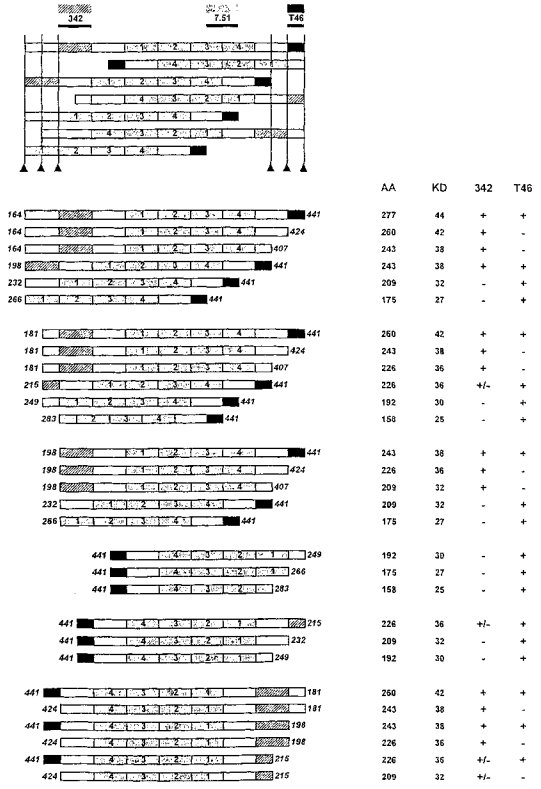


【図４２】



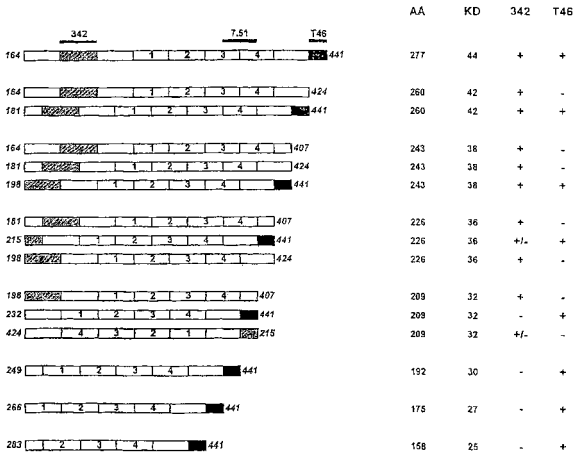
【図４３】

七量体凝集のタンパク質分解性プロセシングから観察されたフラグメントの派生



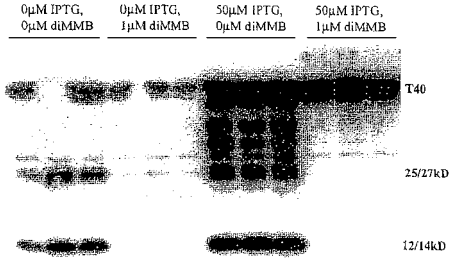
【図４４】

七量体凝集のタンパク質分解性プロセシングから誘導されたフラグメント

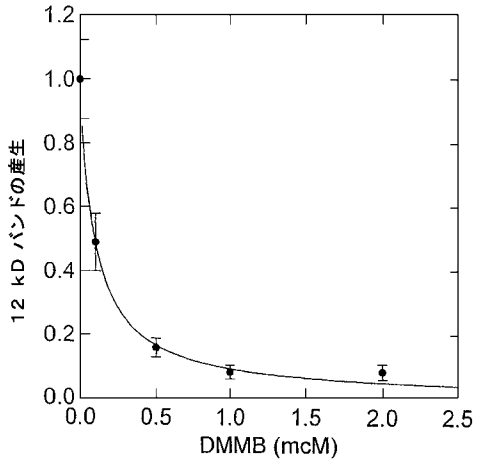


【図４５】

ジメチルメチレンブルーによるタウ凝集の阻止



【図４６】



観察された活性対予測された活性	r = 1.00
細胞内タウ濃度	500 nM
タウ－タウ結合親和性	22 nM
DMMB KI	4.4 nM
DMMB B50	100 nM

【国際公開パンフレット】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 July 2002 (18.07.2002)

PCT

(10) International Publication Number
WO 02/055720 A2

(51) International Patent Classification⁷: C12N 15/85, A61P 25/28, G01N 33/68

(21) International Application Number: PCT/GB02/00153

(22) International Filing Date: 15 January 2002 (15.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0101049.5 15 January 2001 (15.01.2001) GB

(71) Applicant *(for all designated States except US)*: **THE UNIVERSITY COURT OF THE UNIVERSITY OF ABERDEEN** [GB/GB]; Regent Walk, Aberdeen, Grampian AB24 3FX (GB).

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/055720 A2

(54) **Title:** MATERIALS AND METHODS RELATING TO PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASE

(57) **Abstract:** Disclosed are methods of proteolytically converting a precursor protein (e.g. tau) to a product fragment (e.g. a 12 kd fragment) in a stable cell line, wherein the precursor protein is associated with a disease state in which the precursor protein aggregates pathologically (e.g. a tauopathy), and the methods comprise:(a) providing a stable cell line transfected with nucleic acid encoding: (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein, which protein is inducibly expressed in the cell in response to a stimulus, whereby interaction of the template fragment with the precursor protein causes a conformational change in the precursor protein such as to cause aggregation and proteolytic processing of the precursor protein to the product fragment. The method is preferably used to screen for modulators of the aggregation process by monitoring production (or modulation of production) of the product band or bands. Also provided are materials for used in the assays, plus medicaments, and related uses and processes, based on compounds which show high activity in the assay of the invention e.g. reduced diaminophenothiazines.

MATERIALS AND METHODS RELATING TO PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASE

Technical field

The present invention concerns cell-based models and other test systems for modelling the aggregation of proteins associated with neurodegenerative disease. It further relates to compounds capable of modulating such aggregation.

Background art

Conditions of dementia such as Alzheimer's disease (AD) are frequently characterised by a progressive accumulation of intracellular and/or extracellular deposits of proteinaceous structures such as β-amyloid plaques and neurofibrillary tangles in the brains of affected patients. The appearance of these lesions largely correlates with pathological neurofibrillary degeneration and brain atrophy, as well as with cognitive impairment (Mukaetova-Ladinska, E.B. *et al.* (2000) Am. J. Pathol. Vol. 157, No. 2, 623-636).

Both neuritic plaques and neurofibrillary tangles contain paired helical filaments (PHFs), of which a major constituent is the microtubule-associated protein tau (Wischik *et al.* (1988) PNAS USA 85, 4506). Plaques also contain extracellular β-amyloid fibrils derived from the abnormal processing of amyloid precursor protein (APP; Kang *et al.* (1987) Nature 325, 733). An article by Wischik *et al.* (in 'Neurobiology of Alzheimer's Disease', 2nd Edition (2000) Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford) discusses in detail the putative role of tau protein in the pathogenesis of neurodegenerative dementias.

Studies of Alzheimer's disease indicate that the loss of the normal form of tau (Mukaetova-Ladinska *et al.* (1993) Am. J. Pathol., 143, 565; Wischik *et al.* (1995a) Neurobiol. Ageing, 16: 409; Lai *et al.*

(1995b) Neurobiol. Ageing, 16: 433), accumulation of pathological PHFs (Mukaetova-Ladinska *et al.* (1993), *loc. cit.*; Harrington *et al.* (1994a) Dementia, 5, 215; Harrington *et al.* (1994b) Am. J. Pathol., 145, 1472; Wischik *et al.*,(1995a), *loc. cit.*) and loss of synapses in the mid-frontal cortex (Terry et al. (1991) Ann. Neurol., 30, 572) correlate with associated cognitive impairment. Furthermore, loss of synapses (Terry *et al.*, *loc. cit.*) and loss of pyramidal cells (Bondareff *et al.* (1993) Arch. Gen. Psychiatry, 50: 350) both correlate with morphometric measures of tau-reactive neurofibrillary pathology, which parallels, at a molecular level, an almost total redistribution of the tau protein pool from a soluble to a polymerised form (PHFs) in Alzheimer's disease (Mukaetova-Ladinska et al. (1993), *loc. cit.*; Lai *et al.* (1995), *loc. cit.*).

Tau exists in alternatively-spliced isoforms, which contain three or four copies of a repeat sequence corresponding to the microtubule-binding domain (Goedert, M., *et al.* (1989) *EMBO J.* 8, 393-399; Goedert, M., *et al.* (1989) *Neuron* 3, 519-526). Tau in PHFs is proteolytically processed to a core domain (Wischik, C.M., *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85, 4884-4888; Wischik *et al.* PNAS USA 1988, 85:4506-4510); Novak, M., *et al.* (1993) *EMBO J.* 12, 365-370) which is composed of a phase-shifted version of the repeat domain; only three repeats are involved in the stable tau-tau interaction (Jakes, R., *et al.* (1991) *EMBO J.* 10, 2725-2729). Once formed, PHF-like tau aggregates act as seeds for the further capture and provide a template for proteolytic processing of full-length tau protein (Wischik *et al.* 1996 Proc Natl Acad Sci USA 93, 11213-11218).

In the course of their formation and accumulation, paired helical filaments (PHFs) first assemble to form amorphous aggregates within the cytoplasm, probably from early tau oligomers which become truncated prior to, or in the course of, PHF assembly (Mena, R., *et al.* (1995) *Acta Neuropathol.* 89, 50-56; Mena, R., *et al.* (1996) *Acta Neuropathol.* 91, 633-641). These filaments then go on to form classical intracellular neurofibrillary tangles. In this state,

the PHFs consist of a core of truncated tau and a fuzzy outer coat containing full-length tau (Wischik., C. M., *et al*, (1996) loc. cit.). The assembly process is exponential, consuming the cellular pool of normal functional tau and inducing new tau synthesis to make up the deficit (Lai, R. Y. K., et al., (1995), *Neurobiology of Ageing*, Vol. 16, No. 3, 433-445). Eventually, functional impairment of the neurone progresses to the point of cell death, leaving behind an extracellular tangle. Cell death is highly correlated with the number of extracellular tangles (Wischik *et al.* 2000, loc.cit). As tangles are extruded into the extracellular space, there is progressive loss of the fuzzy outer coat of the neurone-PHF with corresponding loss of N-terminal tau immunoreactivity, but preservation of tau immunoreactivity associated with the PHF core (Figure 1; also Bondareff, W. *et al.*, (1994) *J. Neuropath. Exper. Neurol.*, Vol. 53, No. 2, 158-164).

The phase shift which is observed in the repeat domain of tau incorporated into PHFs suggests that the repeat domain undergoes an induced conformational change during incorporation into the filament. During the onset of Alzheimer's disease, it is envisaged that this conformational change could be initiated by the binding of tau to a pathological substrate, such as damaged or mutated membrane proteins (see Figure 2 - also Wischik, C.M., *et al.* (1997) in "*Microtubule-associated proteins: modifications in disease*", eds. Avila, J., Brandt, R. and Kosik, K. S. (Harwood Academic Publishers, Amsterdam) pp.185-241).

In the case of Alzheimer's disease, current pharmaceutical therapies are focused on symptomatic treatment of the loss of cholinergic transmission which results from neurodegeneration (Mayeux, R., *et al.* (1999) *New Eng. J. Med.* 341, 1670-1679). However, although the available treatments delay progression of the disease for up to six to twelve months, they do not prevent it. The discovery of drugs that could prevent the aggregation of tau which leads to neurodegeneration would provide a more effective strategy for prophylaxis or for inhibiting the progression of the disease, which would not require an immediate knowledge of the diverse upstream events that initiate the aggregation (see Figure

3).

Models and assays

WO 96/30766 describes an *in vitro* assay for tau aggregation in which a fragment of tau corresponding to the core repeat domain, which has been adsorbed to a solid phase substrate, is able to capture soluble full-length tau and bind tau with high affinity (see Figure 4). This association confers stability against digestion of proteases on the tau molecules on the repeat domains of tau molecules which have aggregated. The process is self-propagating, and can be blocked selectively by prototype pharmaceutical agents ((Wischik *et al.* 1996 Proc Natl Acad Sci USA 93, 11213-11218).

Although the *in vitro* assay described in WO 96/30766 enables the identification of inhibitors or modulators of tau-tau association, the present inventors have also recognized that cell-based models of Alzheimer's disease-like protein aggregation would be useful. Such cellular models could be used both in the primary screening of candidate modulators of tau-tau aggregation, and in the secondary screening of compounds already identified in the *in vitro* assay of WO 96/30766. Furthermore, the demonstration of tau aggregation in cells could also aid in the identification of normal cellular substrates which are involved in the initiation of pathological tau aggregation, which substrates could themselves be targets for pharmaceutical intervention.

However, numerous papers reporting the expression of various tau constructs in tissue culture models have failed to demonstrate aggregation (see e.g. Baum, L. *et al.*, (1995) Mol. Brain Res. 34:1-17). For instance, 3T3 mouse fibroblasts do not possess tau protein and thus present a cellular environment in which recombinant tau can be expressed independent of endogenous mouse tau. Transfection of various cell lines has been reported previously (Kanai *et al.* , 1989; Goedert and Jakes, 1990; Knops *et al*, 1991; Lee and Rook, 1992; Gallo *et al.*, 1992; Lo *et al.*, 1993; Montejo de Garcini *et al.*, 1994; Fasulo *et al.*, 1996). However the

stable long term expression of truncated tau in such cell lines was not achieved. For example, tau constructs for residues 164 or 173 to 338 or 352 did not express protein (Lee and Rook, 1992).

Although Fasulo *et al.* (Alzheimer's Research 1996, 2, 195-200) reported transient expression of truncated tau in COS cells, data for stable long term expression of this tau was not shown. These workers concluded from the use of the transient transfection system that expression of truncated tau by itself was not sufficient to induce tau aggregation in a manner suitable for testing drugs.

Thus far, the aggregation of soluble tau *in vitro* has only been achieved under non-physiological conditions and at high concentrations (reviewed in Wischik (2000), loc. cit).

WO 96/30766 describes two approaches for studying tau aggregation in a cellular environment. In the first approach, full-length tau or fragments of tau were stably expressed in cells. In the second approach, aggregated tau was transiently transfected into cells by use of lipofectin.

Although both of these approaches are useful for the study of tau-tau aggregation, they have some limitations. Transfection of aggregated tau into cells using lipofection is of variable efficiency, as is the production *in vitro* of aggregated tau itself. Moreover, the core tau fragment, which is the most efficient seed for tau aggregation, is found to be toxic when stably expressed in cells, leading to low expression levels. Thus, constitutive expression of the truncated tau fragment of the PHF core in eukaryotic cells is difficult to achieve. Transient expression systems permit the optimization of expression of tau, but the inherent toxicity of the fragments renders even these systems unreliable. Longer fragments of tau are less toxic, but these do not reliably aggregate when expressed in cells.

Thus it would be desirable for an alternative model system to be developed, in which the interaction between e.g. tau molecules and the like could be investigated under physiological conditions, in a

stable and controllable cell line, and which could be used to screen for potential diagnostic, prognostic or therapeutic agents of conditions such as Alzheimer's disease.

Disclosure of the invention

The present inventors have devised a stable cellular test system which can be used to model the template-driven proteolytic processing of a protein, the aggregation of which is associated with neurodegenerative disease. In one test system, exemplified with the tau protein, very low level constitutive expression of a fragment of the tau protein was combined with inducible expression of full-length tau. Induction of the full-length tau lead to its proteolytic conversion to a processed fragment, confirming that "templated proteolytic processing" of the tau was occurring. The system readily permits the demonstration of the effects of tau aggregation inhibitors through their inhibition of production of the processed, 12 kD, fragment from induced full-length tau.

That such a stable system can be achieved notwithstanding the inherent toxic properties of the 12 kD fragment is particularly surprising. For instance, as demonstrated in the Examples below, although partial truncation at either N- or C-termini of full-length tau results in cell lines in which stable expression can be maintained, these longer constructs show only a weak propensity to aggregate, rather than binding to the microtubular network. Stable expression of combinations of tau fragments generates aggregates within the cytoplasm of cells, but this system cannot be maintained reproducibly. Systems based on the inducible expression of the 12 kD fragment lead to toxicity as a result of unpredictable intracellular aggregation of the fragment.

Thus there would appear to be a trade-off in stable expression cell systems between inducing aggregation and hence toxicity on the one hand, which produces cell lines which are either variable or non-viable, and maintaining viable cell lines in which tau has a low propensity to aggregate. Notwithstanding this, the inducible tau expression system of the present invention is both stable, and yet

able to provide controlled aggregation of protein for use in screens and the like.

Additionally, use of the assay has provided evidence that the mechanism of action of certain inhibitors (e.g. phenothiazines) of protein aggregation is primarily steric in nature, rather than essentially redox, as may have been suspected on the basis of the prior art. This discovery has unexpected implications for the choice, assessment, formulation and use of such compounds in the context of the diseases discussed herein. In particular, it shows that assessment of diffusion coefficients can provide a valuable screen for identifying putative inhibitors, or optimising the structure or state of known ones, because the parameters inherently assessed by measuring the diffusion coefficient may be highly relevant to the inhibitors' potency.

The assay further shows that use of phenothiazines in their reduced form can be advantageous for enhancing their inhibitory properties. These observations form the basis of further aspects of the present invention.

In general the present invention provides a method for converting, through proteolytic processing, a precursor protein to a product fragment of the precursor protein, in a stable cell line, which method comprises the steps of: (a) providing a stable cell line transfected with nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein, which protein is inducibly expressed in the cell in response to a stimulus, whereby interaction of the template fragment with the precursor protein causes a conformational change in the precursor protein such as to cause aggregation and proteolytic processing of the precursor protein to the product fragment.

The method may include subjecting the cell to the stimulus such that the precursor protein is expressed in the cell. However in embodiments where an inducible promoter is used which causes low,

but detectable levels of expression even in the absence of the stimulus, then the stimulus step may be omitted.

Generally speaking, the precursor protein will be one which, *in vivo*, is capable of undergoing an induced conformational polymerisation interaction (in a self-propagating manner) leading ultimately to the formation of aggregates comprised of the product fragment, and associated with the disease state. The product fragment obtained in the method provided herein is a measure of the pathological aggregation and proteolysis process which *in vivo* leads to the production of one or more toxic products and the disease state. The product fragment (or one or more of the fragments) of the present method may be toxic, or may simply be used as an indicator of the pathological aggregation process.

The proteins and interactions upon which the method is based are discussed in more detail below.

The present inventors have demonstrated that it is unexpectedly possible to constitutively express the template fragment at a (first) concentration which is not toxic to the cell line i.e. the cell line is viable. Nor does it show cellular abnormalities of the sort shown e.g. in WO 96/30766 at Fig 29.

Nevertheless (e.g. at a time predetermined by addition of the stimulus) it is possible to seed the processing of the precursor protein to a product fragment (which may be the same, broadly equivalent, or quite different to the template fragment) which can thus accumulate to a (second, higher) concentration which is toxic to the cell and which corresponds to the disease state. This in turn provides convenient methods for modeling the disease state associated with the effects of the product fragment, and assessing the effect of modulators on the generation of the product fragment.

In various other, discrete, embodiments the invention provides corresponding methods for any of initiating, seeding, or controlling the proteolytic processing and optionally aggregation of the precursor protein to the product fragment.

In each case the method may involve monitoring (directly or indirectly) the level of proteolytic processing of the precursor protein.

In one embodiment of the present invention fibroblast cells (3T6) express full-length tau ("T40") under the control of an inducible promotor and low constitutive levels of the PHF-core tau fragment (12 kD fragment). When T40 expression is induced in this system, it undergoes aggregation-dependent truncation within the cell, N-terminally at ~a.a.295 and C-terminally at ~a.a.390, thereby producing higher levels of the 12 kD PHF-core domain fragment. Production of the 12 kD fragment can be blocked in a dose-dependent manner by tau-aggregation inhibitors. Indeed the quantitation of inhibitory activity of compounds with respect to proteolytic generation of the 12 kD fragment within cells can be described entirely in terms of the same parameters which describe inhibition of tau-tau binding in vitro. That is, extent of proteolytic generation of the 12 kD fragment within cells is determined entirely by the extent to tau-tau binding through the repeat domain. The availability of the relevant proteases within the cell is non-limiting.

Precursor proteins and diseases (including tauopathies)

As stated above, the invention may be based around the use of any protein which is associated with a disease in which the protein undergoes an induced conformational polymerisation interaction i.e. one in which a conformational change of the protein, or in a fragment thereof, gives rise to templated binding and aggregation of further (precursor) protein molecules in a self-propagating manner.

Once nucleation is initiated, an aggregation cascade may ensue which involves the induced conformational polymerisation of further protein molecules, leading to the formation of toxic product fragments in aggregates which are substantially resistant to further proteolysis. The protein aggregates thus formed are

thought to be a proximal cause of neurodegeneration, clinical dementia, and other pathological symptoms of this group of diseases.

Preferred embodiments of the invention are based on tau protein. Where used herein, the term "tau protein" refers generally to any protein of the tau protein family. Tau proteins are characterised as being one among a larger number of protein families which co-purify with microtubules during repeated cycles of assembly and disassembly (Shelanski *et al.* (1973) Proc. Natl. Acad. Sci. USA, 70., 765-768), and are known as microtubule-associated-proteins (MAPs). Members of the tau family share the common features of having a characteristic N-terminal segment, sequences of approximately 50 amino acids inserted in the N-terminal segment, which are developmentally regulated in the brain, a characteristic tandem repeat region consisting of 3 or 4 tandem repeats of 31-32 amino acids, and a C-terminal tail.

MAP2 is the predominant microtubule-associated protein in the somatodendritic compartment (Matus, A., in "*Microtubules*" [Hyams and Lloyd, eds.] pp 155-166, John Wiley and Sons, NY). MAP2 isoforms are almost identical to tau protein in the tandem repeat region, but differ substantially both in the sequence and extent of the N-terminal domain (Kindler and Garner (1994) Mol. Brain Res. 26, 218-224). Nevertheless, aggregation in the tandem-repeat region is not selective for the tau repeat domain. Thus it will be appreciated that any discussion herein in relation to tau protein or tau-tau aggregation should be taken as relating also to tau-MAP2 aggregation, MAP2-MAP2 aggregation and so on.

Figure 5 shows a Table listing various other disease-associated aggregating proteins which may be used in the present invention. In each case the disease or diseases in which the initiation of aggregation and\or mutation of the protein(s) may play a role is also listed. The domain or mutation responsible for the disease activity is listed, and at least all or part of this minimal portion of the protein would preferably be encompassed by the template fragment used in the present invention.

As can be seen from the table, example diseases which are characterised by pathological protein aggregation include motor neurone disease and Lewy body disease.

Notably it is not only Alzheimer's Disease in which tau protein (and aberrant function or processing thereof) may play a role. The pathogenesis of neurodegenerative disorders such as Pick's disease and Progressive Supranuclear Palsy (PSP) appears to correlate with an accumulation of pathological truncated tau aggregates in the dentate gyrus and stellate pyramidal cells of the neocortex, respectively. Other dementias include fronto-temporal dementia (FTD); parkinsonism linked to chromosome 17 (FTDP-17); disinhibition-dementia-parkinsonism-amyotrophy complex (DDPAC); pallido-ponto-nigral degeneration (PPND); Guam-ALS syndrome; pallido-nigro-luysian degeneration (PNLD); cortico-basal degeneration (CBD) and others (see Wischik *et al.* 2000, loc. cit, for detailed discussion - especially Table 5.1). All of these diseases, which are characterized primarily or partially by abnormal tau aggregation, are referred to herein as "tauopathies".

Thus it will be appreciated, in the light of the above discussion, (and except where context requires otherwise) where the embodiments of the invention are described with respect to tau protein or tau-like proteins (e.g. MAP2) the description should be taken as applying equally to the other proteins discussed above (e.g. β-amyloid, synuclein, prion etc.) or other proteins which may initiate or undergo a similar pathological aggregation by virtue of conformational change in a domain critical for propagation of the aggregation, or which imparts proteolytic stability to the aggregate this formed (article by Wischik *et al.* (in "Neurobiology of Alzheimer's Disease", 2nd Edition (2000) Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford). All such proteins may be referred to herein as "aggregating disease proteins."

Likewise, where mention is made herein of "tau-tau aggregation", or the like, this may also be taken to be applicable to other

"aggregating-protein aggregation", such as β-amyloid aggregation, prion aggregation and synuclein aggregation etc. Likewise "tau proteolytic degradation" and so on.

Template Fragments

In preferred embodiments of the present invention, the template fragment, comprises, consists essentially of, or consists of a "core fragment" of the precursor protein, which term refers to that part of the protein that is able to bind to the precursor protein to initiate or propagate proteolysis and aggregation as described above.

In the case of disease proteins which aggregate, such core fragments are also likely to be those which contribute to the proteolytic stability of the aggregate.

Thus, for example, a "tau core fragment" is a tau fragment comprising a truncated tau protein sequence derived from the tandem repeat region and, which, in the appropriate conditions, is capable of binding to the tandem repeat region of a further tau protein or a MAP2 protein with high affinity. In the case of tau, the preferred fragment is thus exemplified by, but not limited to, the tau fragments present in PHFs (and, ultimately, neurofibrillary tangles) in Alzheimer's disease brains.

A preferred tau fragment may thus be from about (say) between 295-297 extending to about 390-391 (see 'dGAE' in Figure 6) although variants of such fragments may also be used, as discussed below.

In the case of APP (amyloid precursor protein), for instance, expression of a fragment of the APP that encompasses the Aβ domain of 1-40 or 1-42 amino acids as a fusion protein, may be preferred.

Other core fragments may be based e.g. on the domains discussed with reference to Figure 5. Template fragments may include domains from two, or more than two, of these proteins (e.g. as fusions).

The total length of the template fragment may be any which is appropriate to the assay and aggregation disease protein core fragment being used, but will generally be greater than or equal to about 20, 30, 40, 50, 60, 70, 80, 90, or so amino acids in length. However in some embodiments it may be greater than 100, 200 or even 500, if this is desired.

Derivatives

In all instances herein where a named protein (e.g. precursor protein, template or core fragment) or a recited nucleic acid sequence is discussed, a derivative or other variant of the corresponding reference protein (or nucleic acid) may be used as appropriate, provided that it retains appropriate characteristics of the reference sequence. Such derivatives will also share sequence identity with the reference sequence.

For instance the protein used may include an extended N- or C-terminus, which extension may be heterologous to the protein sequence. Equally, the derivative will be one by way of amino acid insertion, deletion, or addition of the reference sequence. For example, a tau protein, or tau core fragment, derivative will comprise at least a partial amino acid sequence resembling the tandem repeat region of the tau proteins, but in which one or more of the amino acids of the natural tau or its fragments have been replaced or deleted, or into which other amino acids have been inserted.

Such changes may be made to enhance or ablate binding activity (the latter case being useful for control experiments). Controls may contain deletions of sequences or domains to see what effect on aggregation these may have.

Preferred derivatives may be those which incorporate mutations corresponding to those known or suspected to be associated with the disease state. These may include changes corresponding to P301S within the tau sequence (see Figure 7). Other mutations include G272V, G389R, P301L, N279K, S305N, V337M, G272V, K280Δ, R406W (see

also Wischik et al, 2000, supra).

Other preferred derivatives may include tandem repeats of the core-fragments discussed above, or binding domains within those fragments.

Yet further derivatives may be based on chimeric products based on multiple, related, disease proteins in which their sequences are mixed or combined. For example restriction enzyme fragments of tau could be ligated together with fragments of MAP2 or even of an unrelated gene to generate recombinant derivatives. An alternative strategy for modifying the core fragments would employ PCR as described by Ho et al., 1989, Gene 77, 51-59 or DNA shuffling (Crameri et al., 1998 Nature *391*).

Use of nucleic acid constructs

Nucleic acids of, or for use in, the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities. Nucleic acids e.g. encoding the template fragment, will be at least partially synthetic in that it will comprise nucleic acid sequences which are not found together in nature (do not run contiguously) but which have been ligated or otherwise combined artificially.

Nucleic acid according to the present invention may be in the form of, or derived from, cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs. Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

As described above, the nucleic acids may encode derivatives or other variants sharing homology with the reference sequences in question. Preferably, the nucleic acid and/or amino acid sequence in question would share about 50%, or 60%, or 70%, or 80% identity,

most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% of the sequence upon which the variant is based. Similarity or homology may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711) using the default parameters. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is: T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex.

Nucleic acid sequences which encode the appropriate proteins or polypeptides can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989, and Ausubel *et al.*, Short Protocols in Molecular Biology, John Wiley and Sons, 1992). These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of the relevant nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparation of cDNA sequences.

DNA encoding e.g. tau core fragments may be generated and used in any suitable way known to those of skilled in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. Modifications to the protein (e.g. tau)-encoding sequences can be made, e.g. using site directed mutagenesis.

Constructs

Thus the invention also relates, in a further aspect, to nucleic acid molecules encoding the appropriate precursor and template fragment proteins. As discussed below, these may be present on the

same or different constructs, and in the latter case, compositions comprising two or more types of construct are also provided.

Nucleic acid sequences which enable a vector to replicate in one or more selected host cells are well known for a variety of bacteria, yeast, and viruses. For Example, various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Expression vectors comprising a nucleic acid as described herein may, for example, be in the form of a plasmid, cosmid, viral particle, phage, or any other suitable vector or construct which can be taken up by a cell and expressed appropriately.

Expression vectors will contain a promoter which is operably linked to the protein-encoding nucleic acid sequence of interest, so as to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional control" of the promoter. Transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems. Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA.

The promoter used for the template fragment will be "constitutive". This promoter may be sufficiently weak that the level of template fragment expressed in the cell is not itself (directly) detectable using conventional techniques, other than (indirectly) by its

affect on precursor protein, leading to aggregation and proteolytic processing thereof (i.e. effectively undetectable when said aggregation is inhibited). Such promoters may be selected by those skilled in the art in the light of the present disclosure without undue burden such as those listed above.

In the case of the precursor protein, the promoter is "inducible" - which is to say, and as is well understood by those skilled in the art, expression is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. In experiments below, a Lac inducible promoter has been used.

Expression vectors of the invention may also contain one or more selection genes. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*. An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the desired protein-encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell, when wild-type DHFR is employed, is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al.*, *Proc. Natl. Acad Sci USA* 77:4216 (1980). A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid Rp7 [Stinchcomb *et al.*, Nature, 282:39 (1979); Kingsman et *al.*, Gene, 7:141 (1979); Tschemper *et al.*, Gene, 10:157 (1980)]. The trp1 gene provides a selection marker for a mutant strain of yeast which lacks the ability to grow in tryptophan, for example, ATCC: No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

Thus a typical vector for use in the present invention may include an origin of replication, one or more protein sequence(s) operably linked to a constitutive or inducible promoter as appropriate, a transcription termination sequence, an enhancer element, a marker gene. Construction of suitable vectors containing various of these components employs standard ligation techniques which are known to the skilled artisan.

Transformation

Also provided by the present invention is a process for producing a stable cell for use in a method as described above, which process comprises the steps of: (a) introducing into a cell nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly expressed in the cell in response to a stimulus.

The introduction, which may be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. The calcium treatment employing calcium chloride, as described in Sambrook *et al., supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens is* used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out

according to the method of Van Solingen *et al.*, J. Bact., 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185:527 537 (1990) and Mansour et al., Nature 336:348-352 (1988).

Host cells

Suitable host cells for use in the invention may include bacteria, eukaryotic cells such as mammalian and yeast cells, and baculovirus systems.

Mammalian cell lines available in the art for expression of a heterologous polypeptide include fibroblast 3T6 cells, HeLa cells, baby hamster kidney cells, COS cells, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651), Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumour cells (MMT 060562, ATCC CCL51); and many others.

Suitable prokaryotic hosts include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *E. coli.* Various E. *coli* strains are publicly available, such as E. *coli* K12 strain MM294 (ATCC 31,446); E. *coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635). Eukaryotic microbes such as filamentous fungi or yeast are also suitable cloning or expression hosts for vectors. *Saccharomyces cerevisiae is* a commonly used lower eukaryotic host microorganism. The selection of the appropriate host cell is deemed to be within the skill in the art.

In a further aspect, the present invention provides a host cell containing heterologous nucleic acid of the invention as described above. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. Alternatively, the nucleic acid may be on an extrachromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

The cell may be produced by a method described above (introduction of nucleic acid construct) or be the ancestor of such a cell. Corresponding cell-lines are also provided. Preferred cell-lines may be based on the fibroblast cell line, e.g. 3T6.

Host cells transfected or transformed with expression or cloning vectors described herein may be cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in "Mammalian Cell Biotechnology: a Practical Approach", M. Butler, ed. JRL Press, (1991) and Sambrook *et al, supra*.

Gene expression can be confirmed in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl Acad Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequence of the aggregating disease protein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes.

Gene expression, alternatively, may be measured by immunological methods such as immunohistochemical staining of cells or tissue

sections, and assay of cell culture, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence of the aggregating disease polypeptide.

Thus one aspect of the present invention entails causing or allowing expression from the nucleic acids discussed herein, e.g. by culturing host cells under conditions for expression of the gene (presence of stimulus) so that the product fragment is produced. The present invention also encompasses a method of producing the product fragment, the method including expression from nucleic acid as described above.

Another aspect of the present invention is a kit comprising a transformed cell or cell line as described herein, plus at least one further component e.g. an agent for stimulating production of the precursor protein, or an agent for detecting the interaction of the precursor protein with the template fragment, as described in the following section.

Detection of aggregation and\or proteolytic processing and\or toxic fragment

In various embodiments, the progress of proteolytic processing or aggregation (or modulation thereof - see below) may be detected directly or indirectly by monitoring the concentration or level any one or more of the following species: the precursor protein; the product fragment; any by-product fragments formed during the process; an aggregate of any of these (e.g. based on sedimentation coefficients).

Thus, as exemplified with particular tau proteins and fragments (based on 297-351 fragment and T40), aggregation can be monitored on the basis of increasing levels of a 12kDa processed species, derived primarily from the precursor protein.

Some protein detection methods are discussed in relation to gene expression above. Where antibodies or fragments thereof are used in embodiments of the method of the present invention may be produced by conventional techniques. Polyclonal antibodies may raised e.g. by injecting the corresponding tau antigen into an animal, preferably a rabbit, and recovering the antiserum by immunoaffinity purification, in which the polyclonal antibody is passed over a column to which the antigen is bound and is then eluted in a conventional manner. Preferably the invention will use monoclonal antibodies which are selective to tau epitopes may be prepared by the method of Kohler and Milstein. Suitable monoclonal antibodies to tau epitopes can be modified by known methods to provide Fab fragments or (Fab')2 fragments, chimeric, humanised or single chain antibody embodiments.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Generally speaking, where antibodies are employed for detection, the antibody may carry a reporter molecule. Alternatively, detection of binding may be performed by use of a second antibody capable of binding to a first unlabelled, tau-specific antibody. In this case, the second antibody is linked to a reporter molecule.

Antibodies may be used in any immunoassay system known in the art, including, but not limited to: radioimmunoassays, "sandwich" assays, enzyme-linked immunosorbent assays (ELISA); fluorescent immuno-assays, protein A immunoassays, etc. Typically, an immunoblot method is used. Preferably the immunoassay is performed in the solid phase, as would be well known to the skilled person. For instance, an antibody may be adsorbed to e.g. an assay column, and the cellular sample may then be washed through the column under

conditions suitable for enabling binding to the solid-phase antibody of any aggregate of the protein of interest, e.g. a tau-tau aggregate. Excess reagent is washed away, and the binding of aggregated protein to the column can then be detected by any suitable means, e.g. as exemplified above and below.

Preferred monoclonal antibodies are as follows:

- Those which recognise the N-terminal or C-terminal of the tau epitope permit measuring of binding between truncated and full-length tau species. Especially useful are antibodies recognising human-specific epitopes. One such monoclonal antibody (designated 27/499) recognises a human-specific epitope located in the region between Gly-16 and Gln-26 of tau, and thereby permits measurement of binding between full-length tau species, provided one is derived from a non-human source (Lai (1995); "The role of abnormal phosphorylation of tau protein in the development of neurofibrillary pathology in Alzheimer's disease", PhD Thesis, University of Cambridge).

- Those which recognise the core tau fragment truncated at Glu-391. An example is mAb 423 (Novak et *al.* (1993), loc. cit.). This antibody enables detection of the binding of a truncated core tau fragment terminating at Glu-391 to a similar fragment terminating at Ala-390, which is not recognised by mAb 423. This truncation occurs naturally in the course of PHF assembly in Alzheimer's disease (Mena et *al.* (1995), (1996), *loc.* cit.; Novak et *al.* (1993), *loc.* cit.; Mena et *al.* (1991), *loc. cit.*). Additionally, when tau is bound via the repeat domain *in vitro*, digestion with a protease (e.g. pronase) generates a fragment detectable by mAb 423 (see Wischik et al, 1996, *loc cit*). In the preferred aspects of the present invention, as it relates to tau protein, this antibody may be used to distinguish the generation of proteolytically cleaved product fragment (Glu-391 termination) from constitutive expression of template fragment (Ala-390).

- Those which recognise a generic tau epitope in the repeat domain. A preferred embodiment utilises an antibody (e.g. MAb 7.51). Where

tau-MAP2 or MAP2-MAP2 aggregation is to be detected, an antibody which detects a generic MAP2 epitope could be used. Antibody 7.51 recognises a generic tau epitope located in the antepenultimate repeat of tau (Novak et al. (1991) Proc. Natl. Acad. Sci. USA, 88, 5837-5841), which is occluded when tau is bound in a PHF-like immunochemical configuration but can be exposed after formic acid treatment (Harrington et al. (1990), (1991), *loc. cit.*; Wischik et *al.* (1995a), *loc. cit.*). Normal soluble tau, or tau bound to microtubules, can be detected using mAb 7.51 without formic acid treatment (Harrington *et al.* (1991), *loc.* cit.; Wischik et *al.* (1995a), *loc. cit.*). Binding of full-length tau in the tau-tau binding assay is associated with partial occlusion of the mAb 7.51 epitope.

Antibody 27/342 recognises a non-species specific generic tau epitope located between Ser-208 and Ser-238 which is partially occluded in the course of the tau-tau interaction (Lai, *loc. cit.*).

The binding sites of some monoclonal antibodies are shown in Figure 6.

Screening for modulators and inhibitors

As described above, the invention is preferably concerned with use of a system as provided herein, in a method of modeling, and identifying therapeutic agents for treatment of, the diseases discussed herein.

A typical method for assessing the ability of an agent to modulate the aggregation and\or proteolytic processing of a precursor protein to a product in response to interaction with a template fragment, may comprise:

(a) providing a stable cell or cell line as discussed above,

(b) subjecting the cell to the stimulus such that the precursor protein is expressed in the cell and whereby interaction of the template fragment with the precursor protein causes a conformational change in the protein such as to cause aggregation and proteolytic processing of the precursor protein to a product

fragment,
(c) monitoring the production of the product fragment in the presence of the agent,
(d) optionally comparing the value obtained in step (c) with a reference value.

The reference value may be based on historical observation, or may be based on control experiments carried out in parallel e.g. in which one integer of the assay (template fragment, precursor protein, stimulus, agent) is modified or absent.

The various methods described above may comprise the further step of correlating the result of step (d) with the modulatory activity of the agent(s).

Thus a method of identifying a modulator of aggregation of a protein associated with a disease in which the protein undergoes an induced conformational interaction, may comprise performing a method for inducing aggregation as described above in the presence of one or more agents suspected of being capable of modulating (e.g. inhibiting or reversing) the aggregation. The degree of aggregation (and optionally proteolytic processing) may be observed in the presence or absence of the agent, and the relative values correlated with its activity as a modulator.

For example, a test substance may be added to a cellular system as described above, and the cells incubated for a period of time sufficient to allow binding and to demonstrate inhibition of binding. The bound tau complex can then be detected, e.g. using a suitably-labeled antibody such as MAb 7.51 in an immunoblot of total cell extract, or any other suitable detection method.

Where a screening method is employed for this purpose, i.e. for the identification of modulatory/inhibitory compounds, a non-competitive or competitive assay may be used. For instance, in a competitive assay of the type well known in the art, the effect of a known inhibitor or modulator can be compared in the presence or absence of further test substances or agents, to determine the

ability of the test substance to compete with the known inhibitor/modulator for binding to the protein of interest.

Also provided are methods of producing modulators (e.g. inhibitors) which are as described above, but which further comprise the step of producing the modulator this identified.

Specificity of inhibition

Screening methods according to this aspect of the present invention may be used to screen for compounds which demonstrate the properties of selective competitive inhibition of disease-related protein aggregation (e.g. tau-tau, tau-MAP2, or other protein, binding), without interference with any 'normal' binding in which the precursor protein participates (e.g. tau or MAP2 to tubulin, or by analogy, other precursor proteins with their binding partners insofar as these are known).

Specifically in the case of tau, a method for determining any possible interference of the binding of tau, MAP2 or a derivative thereof to tubulin by potential inhibitors/modulators, comprises contacting a preparation of depolymerised tubulin or taxol-stabilised microtubules with the agent, followed by detection of the tau-tubulin or MAP2-tubulin binding. Tau-tubulin binding could also, for example, be demonstrated by a normal cytoskeletal distribution, as described in e.g. WO 96/30766. Methods for the preparation of tubulin proteins or fragments thereof, possibly in combination with binding partners, are known in the art and are described e.g. by Slobada *et al.* (1976, in: Cell Mobility (R. Goldman, T. Pollard and J. Rosenbaum, eds.), Cold Spring Laboratory, Cold Spring Harbor, New York, pp 1171-1212).

Analogous methods for other proteins having 'disease' and 'normal' functions will occur to those skilled in the art in the light of the present disclosure.

Cell viability

Where desired, methods of the present invention may further include the step of testing the viability of the cells expressing the template protein and optionally precursor protein e.g. by use of a lactate dehydrogenase assay kit (Sigma).

In the case where tau-tau, tau-MAP2 or MAP2-MAP2 aggregation is being investigated (see above, under 'specificity'), this step may also provide an indication of any interference by the test agent of the binding of tau or MAP2 to tubulin, since inhibition or interference of tau-tubulin or MAP2-tubulin binding will correlate to some extent with a decreased ability of the cells to divide, and thus with decreased cell viability.

Cell viability may be used to derive an LD50 value for the agent.

Preferred inhibitors will have a therapeutic index (LD50/B50 - see discussion of Figure 9) of at least 2, 5, 10, or 20.

Choice of test agent

Compounds which are tested may be any which it is desired to assess for the relevant activity.

The methods can serve either as primary screens, in order to identify new inhibitors/modulators, or as secondary screens in order to study known inhibitors/modulators in further detail.

Agents may be natural or synthetic chemical compounds. Antibodies which recognise an Alzheimer's disease-like protein aggregate and/or which modulate Alzheimer's disease-like protein aggregation form one class of putative inhibitory or modulatory compounds with respect to the aggregation process. More usually, relatively small chemical compounds, preferably which are capable of crossing the blood-brain barrier, will be tested. Other qualities which it may be desirable to establish in conjunction with (before, simulataneously with, or after) use of the present invention, include: non-toxic to bone marrow, minimal deleterious cardiovascular activity; minimal liver and renal toxicity; good

oral absorption; non-metabolised to inactive form, and so on. As those skilled in the art are aware, these tests can be performed on a commercial basis by well established methods for compounds which it is desired to test in this way.

For a typical test substance and putative modulator, where possible, the solubility will first be determined e.g. from The Merck Index. Where the substance is soluble in aqueous solution, a concentrated stock solution may be prepared e.g. at 5-20mM in PBS. Immediately prior to use this can be diluted with tissue culture medium to give a working stock solution e.g. at 100μM and introduced to cells to give a final concentration of between 0-10μM for most compounds. Naturally, if it is desired to test compounds at a concentration greater than 10μM, the concentration of the working stock solution may be increased appropriately.

Where the substance is not soluble in aqueous solution, stock solutions may be made in an appropriate solvent (determined from The Merck Index or experimentally) e.g. ethanol at 5-29 nM. This can again be diluted with tissue culture medium immediately prior to use to give a working solution e.g. at 100μM concentration, and added to cells to yield a final concentration of e.g. 0-10μM for most test compounds. As above, if compounds are to be tested at a concentration greater than 10μM the concentration of the working solution will be increased as appropriate.

The skilled person will appreciate that the amount of test substance or compound which is added in a screening assay according to this aspect of the invention, and indeed the manner in which it is introduced, can be determined by those skilled in the art, if necessary by use of a series of trials. Where the administered compound and the cell line have conflicting optimal conditions (e.g. in terms of pH, or ionic strength etc.) a variety of conditions should be tried to find an optimal, compromise, level. Initial concentrations may be selected to be a level which could realistically be used in therapeutic context i.e. would be non-lethal to a patient (see comments on dosages below). In the light of the present disclosure, such an approach will not present any

undue burden to one skilled in the art.

Screening phenothiazines

The present invention extends, in further aspects, to compounds identified by a screening method as provided herein, and to compositions comprising such inhibitors/modulators of induced conformational polymerisation of a protein.

As described in e.g. WO 96/30766, amongst the agents found to be able to inhibit pathological induced conformational polymerisation of proteins such as tau are certain diaminophenothiazines. Examples include such as thionine, methylene blue (MB), tolonium chloride, and dimethyl-methylene blue (DMMB) which are of particular interest as potential therapeutic agents for use in the prevention of tau-tau aggregation in diseases such as Alzheimer's Disease.

Interestingly, as described in more detail in the Examples, the present inventors have used the methods described herein to demonstrate that the mechanism of action of compounds such as MB on induced conformational polymerisation such as tau-tau aggregation is primarily steric in nature. Additionally, it has been shown that the potent steric inhibitory effect, e.g. of the diaminophenothiazines on tau-tau binding, is dependent on the diffusion coefficient of the compound. The various implications of these observations in terms of screening and formulating compounds are discussed in more detail below.

This finding is particularly unexpected when considering the description of the use of the such compounds in the prior art. Thus, for example, such compounds were previously known to be useful in the treatment of methaemoglobinaemia, where their action has been shown to be mediated by the catalytic reduction of oxidised haemoglobin by transfer of electrons from the cell's intrinsic supply of reduced pyridine nucleotides (see, e.g. Hauschild, F. (1936) *Arch. Exp. Pathol. Pharmacol.* 182:118; "*Pharmacological Basis of Therapeutics*", First Edition (1941), Goodman and Gilman; Hrgovic, Z. (1990) *Anästh. Intensivther.*

Notfallmed. 25: 172; and Cudd, L. *et al.* (1996) *Vet Human Toxicol.* 38(5): 329) and in the prophylaxis of manic depressive psychosis (Narsapur, S.L. (1983) *Journal of Affective Disorders* 5:155; Naylor, G.J. (1986) *Biol. Psychiatry* 21:915). Notwithstanding this, MB, thionine and tolonium chloride are actually intrinsically weak oxidising agents and, in the absence of a supply of reduced pyridine nucleotides, they oxidise proteins such as haemoglobin (Morse, E. (1988) *Annals of Clin. Lab. Sci.* 18(1):13). This toxic effect can be used to inactivate viruses, and MB has consequently been exploited therapeutically in a process for removing HIV and hepatitis virus from blood products (Chapman, J. (1994), *Transfusion Today* 20:2; Wagner, S. J. (1995) *Transfusion* 35(5):407). The mechanism of action of this effect is thought to involve intercalation of MB into DNA. The compound is boosted to a higher redox state by photoactivation and, when it drops back down to its ground state, produces singlet oxygen which oxidises the DNA and inactivates it (Ben-Hur, E. *et al.* (1996) *Transfusion Medicine Reviews*, Vol. X, No. 1: 15; Margolis-Nunno, H. et al. (1994), *Transfusion* 34(9): 802). Exploitation of the toxic effect of photoactivated diaminophenothiazines has also been suggested for the treatment of cancer. Within cells, compounds which have been photoactivated to the oxidised form can damage mitochondria (Darzynkiewicz, Z. et al. (1988), *Cancer Research* 48: 1295) and/or microtubules (Stockert, J. et al. (1996) *Cancer Chemother. Pharmacol.* 39: 167).

Thus, on reviewing the prior art, it is apparent that two possible mechanisms have been proposed to account for the action of compounds such as MB and thionine on entities such as DNA or proteins. The first is the catalytic reduction of e.g. oxidised proteins by means of transfer of electrons from reduced pyridine nucleotides in the cell. The second proposed mechanism is the oxidation, and consequent inactivation of e.g. DNA by a photoactivated, oxidised form of compounds such as MB. In the light of these two mechanisms, it could therefore reasonably have been assumed that the inhibitory effect on tau-tau association of compounds such as MB was also attributable to a redox activity.

That is, it might be assumed that such compounds inhibit induced conformational polymerisation such as tau-tau association by acting as weak oxidising agents or as catalytic reducing agents.

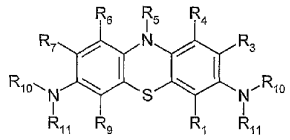
Thus the work of the present inventors, in demonstrating that the mechanism of action is primarily steric in nature, has unexpected implications for the choice, assessment, formulation and use of such compounds in the context of the diseases discussed herein.

In particular, certain compounds have been identified as feasible therapeutics which would have been dismissed based on the result of prior art assays. Specifically, Wischik *et al.* 1996 (*loc cit*) reported on page 1217 that the concentration of MB required for inhibition was higher than could be achieved clinically. However the results herein show that the reduction of MB modifies its stacking ability in such a way as to enhance its inhibitory potential to a level at which it becomes clinically relevant for the treatment of e.g. tau aggregation associated disease. This is discussed in more detail below in relation to the embodiments of the invention concerned with measurement of diffusion coefficients (which are also determined, in part, by the compound's ability to 'stack').

Figure 8 shows the structure of only some of the compounds which have been tested in the cell based assay. Figures 9-16 demonstrate the increased potency of certain compounds in the reduced form, plus some control compounds.

Thus in one aspect of the present invention there is disclosed use, in the treatment of a disease disclosed herein, of a reduced ('leuco') phenothiazine of the formula:

(I)



wherein R_1, R_3, R_4, R_6, R_7 and R_9 are independently selected from hydrogen, halogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;
R_5 is selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy; and each R_{10} and R_{11} are independently selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;
or a pharmaceutically acceptable salt thereof.

Preferably, R_1, R_3, R_4, R_6, R_7 and R_9 are independently selected from -hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$;
each R_{10} and R_{11} are independently selected from hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$; and
R_5 is hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$.

Preferably, the compound is a diaminophenothiazine which has 0, 2, 3 or 4 methyl groups around the diaminophenothiazine nucleus. Preferably, the diaminophenothiazine is asymmetrically methylated (e.g., tolonium chloride, azure A, azure B and thionine).

Preferably the compound is selected from Methylene Blue, Tolonium chloride, Thionine, Azure A, Azure B or 1,9-Dimethylmethylene Blue.

Phenothiazines for use in the present invention may be manufactured by the processes referred to in standard texts (e.g. *Merck Manual*, Houben-Weyl, Beilstein, E. III/IV 27, 1214 ff, *J. Heterocycl. Chem.* 21, 613 (1984)).

Instead of administering these compounds directly, they could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be

treated. For instance, methylene blue may be administered in a precursor form, or it may itself serve as a precursor of the compound Azure A.

Stabilisation of reduced form

Some of these compounds of interest are known to circulate in the body predominantly in the reduced form. For example, for a discussion of the pharmacokinetics of MB, see e.g. DiSanto, A. *et al.* (1972) *Journal Pharm. Sci.* 61(7):1086 and DiSanto, A. *et al.* (1972) *Journal Pharm. Sci* 61(7):1090. Thirdly, only the reduced form of compounds such as MB is found to cross the blood-brain barrier (Chapman, D.M. (1982) *Tissue and Cell* 14(3):475; Müller, T. (1992) *Acta Anat.* 144:39; Müller, T. (1994) *J. Anat.* 184:419; Becker, H. *et al.* (1952) *Zeitschrift für Naturforschung* 7:493; Müller, T. (1995) *It. J. Anat. Embryol.* 100(3):179; Müller, T. (1998) *Histol. Histopathol.* 13:1019).

Such references as these illustrate that the reduced form of compounds such as MB represents a feasible and pharmaceutically-acceptable formulation for administration to subjects. MB has previously been used clinically in an oral preparation. Further toxicological tests are, however, required before its clinical acceptability is achieved. The half live of MB and related compounds (e.g. tolonium chloride) in blood is approximately 100 minutes. It is evident that slow release formulations of compounds with such, relatively short, half lives can substantially improve compound availability and hence therapeutic efficacy.

Figure 17 shows that compounds such as those discussed herein differ greatly in their extent of reduction in the conditions of the assay (approx. 500:1 DTT excess, at 120 minutes). As this figure shows, thionine is completely reduced under these conditions, tolonium chloride is reduced at an intermediate level, and MB and DMMB are relatively little reduced. The amounts of commonly used reductant required to achieve, say, 90% reduction of the oxidized form in 10 minutes, prior to administration\absorption may not be feasible (e.g. 2000:1 ratio of DTT to MB).

As Figure 18 illustrates, the extent of reduction of MB under physiological conditions can be greatly accelerated by allowing reduction over night and then lyophilising the reduced form. The lyophilisate becomes reduced by 90% in 10 minutes, after solubilisation in conditions mimicking gastric acidity (5mM HCl). Capsules containing a form of the diaminophenothiazine pre-reduced with ascorbic acid at a mg ratio of 1.5-2 represent a suitable, if not optimal, formulation for therapeutic use.

The same considerations apply to other compounds, such as thionine and tolonium chloride, which are more readily reduced than MB, but the extent of reduction of which can be accelerated in a manner such as that described above.

Thus in preferred forms the phenothiazine agents of the present invention are provided as pre-reduced compounds e.g. in lyophilised preparations, optionally in the presence of a stabilising agent.

An agent for stabilising the preferred form of the active compound (i.e. a form of the compound having a low diffusion coefficient, e.g. the fully-reduced form of the compound) may be a reducing agent or antioxidant. The agent may serve both to convert one form of the inhibitory compound (e.g. the oxidised form) to the preferred form thereof (e.g. the reduced form), and to stabilise that preferred (e.g. reduced) form. Alternatively, the inhibitory compound may be added to the composition in its preferred (e.g. already-reduced) form, so that the agent merely serves to maintain the compound in this form.

Particularly suitable for use in converting to, and/or stabilising, the reduced form of the active agent (e.g. the diaminophenothiazine) comprised in the formulations of the present invention is the antioxidant ascorbate. Ascorbate has previously been used to minimise oxidative damage of proteins (Parkkinen J. (1996), "Thrombosis and Haemostasis" 75(2): 292). A formulation as provided herein could thus advantageously comprise a diaminophenothiazine, especially MB, tolonium chloride, DMMB or

thionine, in combination with ascorbate, in suitable proportions, concentrations and dosages.

In other embodiments the reduced (leuco) form may be favoured by the addition or selection of appropriate constituent groups.

Thus aspects of the invention further include a method of preparing a medicament for use in the treatment or prophylaxis of a disease as described above, which method comprises the step of reducing the compound (such that it is, say, at least 50, 60, 70, preferably 80, 90, 95, or 99% reduced) and stabilizing it in a lyophilized composition in the reduced form, prior to administration of an appropriate dose to a patient in need of the same.

Dosage of therapeutics

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the disease being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.

CNS penetration of MB following systemic administration has been described by Müller (1992; *Acta Anat.* 144:39). Azure A and B are known to occur as normal metabolic degradation products of MB (Disanto and Wagner (1972a) *J. Pharm. Sci.* 61: 598; Disanto and Wagner (1972b) *J. Pharm. Sci.* 61: 1086). The pharmacokinetics and toxicity of tolonium chloride in sheep is discussed by Cudd et al (1996) Vet Human Toxic 38 (5) 329-332.

For thionine, which is specifically exemplified herein, a daily dosage of between 1 and 1000 mg may be suitable, preferably divided

into 1 to 8 unit doses, which can, for example, be of the same amount. It will, however, be appreciated that these limits given above can be departed from when required, as may be appropriate with the compounds of the invention other than thionine, which have higher or lower activity or bioavailability.

Figure 19 shows the variation of tissue levels of MB vs IV dose.

The pharmacokinetics of methylene blue have been studied in humans, dogs and rats by DiSanto and Wagner, J Pharm Sci 1972, 61:1086-1090 and 1972, 61:1090-1094. Further data on urinary excretion in humans is also available from Moody et al., Biol Psych 1989, 26: 847-858. Combining data on urinary excretion of MB in humans, it is possible to derive an overall model for distribution of MB following single 100 mg dose in a 70 kg subject, assuming instantaneous absorbtion (Fig 19B). Urinary excretion accounts for 54 - 98% of the ingested dose. This variability is most likely due to variability in absorbtion, although variability in metabolism cannot be excluded. From urinary excretion data, it is possible to calculate that whole body clearance is 56 mg/kg/hr. Therefore, the dosage required to achieve an effective target tissue concentration of 4 μM is 1.73 mg/kg/day (0.58 mg/kg tds) if there were complete absorbtion. However, from Moody et al., it is clear that total urinary excretion, and hence effective bioavailability, is itself a function of dose. The oral dose required to deliver 1.73 mg/kg/day is approximately 2x the dosage calculated on the basis of whole-body clearance. Therefore the actual required dosage is on the order of 3.2 mg/kg/day. This is close to the minimum routine oral dosage used clinically in humans, eg in the treatment of chronic urinary tract infection (390 mg/day). The maintenance oral dosage in humans is therefore approximately 225 mg/day, or 75 mg tds. Peak tissue levels are reached at approximately 1 hr and the tissue half-life is about 12 hours.

Methylene blue exists in the charged blue oxidised form, and the uncharged colourless reduced leukomethylene blue form. We have shown experimentally in cells that the target tissue concentration in cells required to prevent tau aggregation by 50% (ie the EC50)

is 4 μM for reduced methylene blue, and that it is the leuko- form which is preferentially active. It is shown by DiSanto and Wagner (1972) that approximately 78% of the methylene blue recovered in urine is in the reduced form, and from anatomical studies following iv administration, the only form which is bound to tissues is the colourless reduced form, which becomes oxidised to the blue colour on exposure to air after post-mortem dissection. The only form of methylene blue which crosses the blood-brain barrier after iv administration is the reduced form (Muller, Acta Anat 1992, 144:39-44 and Becker and Quadbeck, 1952). Therefore, orally absorbed methylene blue is very rapidly reduced in the body, and remains so until excretion, possibly undergoing further chemical modification which stabilises it in a reduced form.

It is highly likely that variability in oral absorbtion is determined largely by the efficiency of initial reduction in the GI tract. One way to achieve more reliable absorbtion is therefore be to pre-reduce methylene blue with ascorbic acid. We have shown from in vitro studies that this conversion is rather slow, so that it takes 3 hours to achieve 90% reduction of methylene blue in water in the presence of 2x mg ratio of ascorbic acid. Therefore, the dosage of methylene blue which is most likely to ensure reliable absorbtion will be 3.5mg/kg/day of methylene blue pre-reduced for at least 3 hours in the presence of 7 mg/kg/day of ascorbic acid.

It is also possible that MB may be active at lower concentrations in man, and that a range of clinically feasible doses would be therefore 20 mg tds, 50 mg tds or 100 mg tds, combined with 2x mg ratio of ascorbic acid in such a manner as to achieve more than 90% reduction prior to ingestion.

Formulation and administration of therapeutics

Suitable compounds, such as those with a formula as shown above or their pharmaceutically-acceptable salts, may be incorporated into compositions of this aspect of the present invention after further testing for toxicity. The compositions may include, in addition to the above constituents, pharmaceutically-acceptable excipients,

carriers, buffers, stabilisers or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration.

Where the composition is formulated into a pharmaceutical composition, the administration thereof can be effected parentally such as orally, in the form of powders, tablets, coated tablets, dragees, hard and soft gelatine capsules, solutions, emulsions or suspensions, nasally (e.g. in the form of nasal sprays) or rectally (e.g. in the form of suppositories). However, the administration can also be effected parentally such as intramuscularly, intravenously, cutaneously, subcutaneously, or intraperitoneally (e.g. in the form of injection solutions).

Where the pharmaceutical composition is in the form of a tablet, it may include a solid carrier such as gelatine or an adjuvant. For the manufacture of tablets, coated tablets, dragees and hard gelatine capsules, the active compounds and their pharmaceutically-acceptable acid addition salts can be processed with pharmaceutically inert, inorganic or organic excipients. Lactose, maize, starch or derivatives thereof, talc, stearic acid or its salts etc. can be used, for example, as such excipients for tablets, dragees and hard gelatine capsules. Suitable excipients for soft gelatine capsules are, for example, vegetable oils, waxes, fats, semi-solid and liquid polyols etc.

Where the composition is in the form of a liquid pharmaceutical formulation, it will generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may also be included. Other suitable excipients for the manufacture of solutions and syrups are, for example, water, polyols, saccharose, invert sugar, glucose, trihalose, etc. Suitable excipients for injection solutions are, for example, water, alcohols, polyols, glycerol, vegetable oils,

etc.

Suitable excipients for suppositories are, for example, natural or hardened oils, waxes, fats, semi-liquid or liquid polyols etc.

Moreover, the pharmaceutical preparations may contain preserving agents, solubilizers, viscosity-increasing substances, stabilising agents, wetting agents, emulsifying agents, sweetening agents, colouring agents, flavouring agents, salts for varying the osmotic pressure, buffers, or coating agents.

For intravenous, cutaneous or subcutaneous injection, or intracatheter infusion into the brain, the active ingredient will be in the form of a parenterally-acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers and/or other additives may be included, as required.

A composition according to the present invention may be administered alone, or in combination with other treatments, either simultaneously or sequentially, dependent upon the condition or disease to be treated.

In accordance with the present invention, the formulations provided herein may be used for the prophylaxis or treatment of Alzheimer's disease, motor neuron disease, Lewy body disease, Pick's disease or Progressive Supranuclear Palsy, or any other condition or disease in which induced conformational polymerisation of a protein is implicated (see Figure 5). In particular, as described in detail below, the formulation may be used for the blocking, modulation and inhibition of pathological tau-tau association.

Examples of the techniques and protocols mentioned above can be found in "*Remington's Pharmaceutical Sciences*", 16th edition, Osol, A. (ed.), 1980.

In a further aspect, the present invention relates to the use of a composition of the preceding aspect, in the diagnosis, prognosis or treatment of a condition in which induced conformational polymerisation of a protein is implicated. The condition may be a disease such as Alzheimer's disease, or any other condition of the type described herein.

Use of diffusion constant as a screen

As stated above, by converting a compound into, and/or stabilising its reduced form, the inhibitory potency of the compound can be optimised.

However, as described in more detail in the examples hereinafter, surprisingly, the redox potential of a compound does not directly determine its inhibitory activity with respect to induced conformational polymerisation of proteins, and that, therefore, neither the oxidation model nor a catalytic reduction model are relevant to an understanding of the activity of compounds as tau-tau aggregation inhibitors.

The inventors have found that there is a strong inverse correlation between the inhibitory potential of a compound towards tau-tau binding and the square or third power of its diffusion coefficient.

The diffusion coefficient is determined by the amount of stacking of discharged molecules at a cathode. Experimentally, this can be evaluated by measuring the current flow in a redox cell at the reduction potential. The diffusion coefficient is inversely correlated with the degree of aggregation of the discharged (i.e. reduced) species within the Helmholtz layer forming at the cathode. These aggregates form by pi-bonded stacking interactions across the phenol ring systems.

In one model, the lower the diffusion coefficient, the higher the tendency to stack, and the more potent the compound is in inhibiting induced conformational polymerisation of proteins such

as tau-tau binding, as reflected by a low K_i.

The stacking of diaminophenothiazines may be less favoured when the molecule is in the oxidised form, since this form is charged, and so can be envisaged to repel other, like molecules. This phenomenon may thus explain the greater efficacy of the reduced form of diaminophenothiazines in the inhibition of tau aggregation (see e.g. Figure 9).

Thus an assessment of the diffusion coefficient (dependent on 'stackability', which is in turn dependent on shape and charge) can be a useful step in the development of effective modulators. One such sterically-relevant parameter is diffusion coefficient which can be diminished by providing diaminophenothiazines in their reduced form.

Thus, the present inventors teach herein that the efficacy of a compound in the blocking, modulation or inhibition of induced conformational polymerisation of a protein (hereinafter referred to as "inhibitory potency" can be tested in an assay method which includes the step of measuring the diffusion coefficient of the compound.

Hence, in its most general form, the present invention provides a method of screening for an agent that blocks, modulates or inhibits induced conformational polymerisation of a protein, which method includes the step of measuring the diffusion coefficient of the agent. The use of the diffusion coefficient value, and in particular the square or third power of its diffusion coefficient, in assessing the inhibitory potency of a phenothiazine (e.g. as described above) for the treatment of a disease as described herein forms a further aspect of the present invention.

The step of measuring the diffusion coefficient of the test agent may be incorporated at any stage of a larger screening programme for identifying or optimising putative or established modulators. The larger method will typically further include assay steps as described herein, or in the prior art (e.g. WO 96/30766). Thus, in

the latter case for instance, when one wishes to screen for agents which block, modulate or inhibit tau-tau aggregation, the method may include the steps of contacting:

(a) a tau protein or a derivative thereof containing the tau core fragment, with;
(b) a substance to be tested for its ability to block, modulate or inhibit tau-tau aggregation; and
(c) a labelled tau protein or a labelled derivative thereof which is capable of binding to the tau protein of step (a) or a tau protein or a derivative thereof which is distinct from the tau protein of step (a) and also capable of binding to the tau protein of step (a).

The diffusion coefficient may be measured by any suitable means, for instance according to the method of Murthy and Reddy (J Chem Soc., Faraday Trans J 1984, 80. 2745-2750). This publication also included some determined values of diffusion coefficients for phenothiazine dyes and its content is specifically incorporated herein by reference.

Thus, the diffusion coefficient may suitably be measured by cyclic voltammetry in an aqueous acidic medium, whereby the magnitude of current flow in a redox cell is tested at the reduction potential of the compound.

The method may include the step of performing further tests on the agent, e.g. to ascertain its specificity as an inhibitor or modulator of induced conformational polymerisation of a particular protein (e.g. tau), or to determine its pharmaceutical acceptability or suitability as an agent for administration to an animal.

The surprising teaching as provided herein, that the efficacy of an agent in blocking, modulating or inhibiting induced conformational polymerisation of a protein is dependent, at least in part, on the diffusion coefficient of the agent, can be utilised in the optimisation of an agent's efficacy. The present inventors have

established that an agent's inhibitory potency towards induced conformational polymerisation of a protein is inversely related to the square or third power of its diffusion coefficient. In other words, the inhibitory potency of an agent can be optimised by providing the agent in a form in which its diffusion coefficient is minimised.

Thus, in a further aspect, the present invention concerns a method of optimising the efficacy of an agent in blocking, modulating or inhibiting induced conformational polymerisation of a protein, which method includes the step of minimising the diffusion coefficient of the agent.

In a further aspect, the present invention provides a pharmaceutical composition for the prophylaxis or treatment of a condition in which induced conformational polymerisation of a protein occurs, the composition comprising a compound which is provided in, or converted into, a form in which its diffusion coefficient is minimised.

This, and further, aspects of the invention will be better understood by reference to the following figures and experimental data, given only by way of example.

Figures

Figure 1 shows a schematic illustration of the structure of a paired helical filament (top) and the immunochemistry of neurofibrillary tangles during progression of Alzheimer's disease (bottom).

Figure 2 shows a conceptual scheme wherein critical nucleating factors provide a 'seed' which initiates tau capture, which then becomes autocatalytic.

Figure 3 shows a putative pathogenic model of Alzheimer's disease. Tau aggregation is a proximal process prior to failure of axonal transport and consequent neuronal death. The tau aggregation

cascade can be triggered either by a seeding/nucleation event arising from upstream changes or from primary mutations in the tau gene.

Figure 4 shows how induction of full-length tau can lead to its conversion into the 12 kD fragment, provided there is some preexisting 12 kD tau in the cell.

Figure 5 shows a table listing proteins which play a role in diseases of protein aggregation. Also listed are the diseases themselves, the aggregating domain and/or mutation believed to be involved, and the putative (maximum) fibril subunit size. One or more literature references for each protein is given.

Figure 6 shows a schematic illustration of the binding sites of various monoclonal antibodies to different forms of N- and C-truncated tau.

Figure 7 shows the nucleotide and predicted amino acid sequences of a human tau protein isoform. The sequence was deduced from cDNA clone htau40.

Figure 8 shows the structures of thionine, tolonium chloride, chlorpromazine and tacrine.

Figure 9 gives cellular assay data for diaminophenothiazines, and a structurally related anthroquinone along with apparent KI values, determined as described herein. In the Figures and Examples herein, a further parameter, B50, has been calculated to express activity in a manner directly related to the conditions of the cell-based assay, and therefore providing an indication of the tissue concentration which would be required to achieve the corresponding activity *in vivo*. The B50 value is the concentration of test compound used in the cell assay at which relative production of the 12 kD band from full-length tau was reduced to 50% of that observed in the absence of the compound. There is a simple linear relationship between apparent KI value and B50 value as follows:

Cellular B50 = 0.0217 x KI

In order to compare the relative usefulness of compounds as therapeutics, it may be desired to calculate an LD50 value. Where inhibitory properties are similar, preferred compounds for clinical use may be those which have the highest LD50 value. A therapeutic index (RxIndx) may be calculated for each of compounds tested in the cell assays as follows:

RxIndx = LD50 / B50

Toxicity of the compounds may be measured by cell numbers after 24 hrs exposure to the compound using a lactate dehydrogenase assay kit TOX-7(Sigma Biosciences) according to the manufacturer's instructions after lysis of remaining cells. Alternatively a kit from Promega UK (CytoTox 96) may be used, again according to the manufacturer's instructions.

Figure 10 shows the results of using reduced thionine in the present invention, based on a data set of 7 experiments. The observed cell data for production of the 12 kD band can be fitted closely (ie observed vs predicted correlation coefficient > 0.9), to a standard function describing inhibition of tau-tau binding in vitro. To obtain this fit, two assumptions need to be made, which are consistent with results from other cell-based and in vitro studies:

1) the intracellular concentration of tau is approximately 500 nM;
2) the tau-tau binding affinity is 22 nM.

using these assumptions, the function for cellular activity predicted via standard inhibition model is:

Activity = [tau] / ([tau] Kd* (1 + [thionine] / KI))

can be solved by standard numerical methods to derive a value for apparent KI. As indicated, the value for the reduced form of

thionine is 100 nM. which is essentially the same as that observed for tau-tau binding in vitro at a tau concentration of 500 nM, where the Kd value for tau-tau binding is known to be 22nM. Therefore, the activity of thionine, where the read-out is production of the 12kD truncation product from full-length tau, can be explained quantitatively on the basis of extent of inhibition of the tau-tau binding occurring through the repeat domain within the cell. This confirms that the extent of tau-tau binding determines production of the proteolytically stable core tau unit of the PHF within the cell.

All subsequent cellular analyses of activities of other compounds are reported in the same standardised format, with the same assumptions regarding intracellular tau concentration (500 nM) and tau-tau binding affinity (22 nM) through the repeat domain.

Figure 11 shows the results for conditions in which the reducing agents have been omitted (i.e. oxidised thionine cf. Figure 10).

Again cellular activity is predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [Ox. Thio.] / KI))

In this case, thionine now has an apparent KI value of 1200 nM. This confirms that the diaminophenothiazines require to be in the reduced form for activity. A similar conclusion was derived from analysis of *in vitro* binding data (results not shown).

Figure 12 shows that by using reducing or partially reducing conditions methylene blue appears much more active in the cell-based assay than predicted from *in vitro* studies in which the time course of the assay (1-2 hours) had not been sufficient to achieve reduction.

Cellular activity is again predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [MB] / KI))

In the cell assay, the apparent KI value for methylene blue is 123 nM, which is within the same range as thionine and tolonium chloride. As indicated in Figure 9, the corresponding brain tissue concentration (i.e. B50 value) required to inhibit tau aggregation would be 2-3 μM.

Figure 13 shows corresponding cell-based activity data for reduced tolonium chloride, indicating again that the predicted KI value derived from in vitro studies can be used to describe production of the 12 kD fragment from full-length tau in cells.

Cellular activity is predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [TC] / KI))

This provides further confirmation of the validity of the mathematical analysis procedure used.

Figure 14 shows that DH12 (anthroquinone) which is structurally related to the diaminophenothiazines is inactive in the conditions of the assay.

Figures 15 & 16 show similar analyses to those given above in Figures 9-14, but for chlorpromazine and tacrine respectively. Using the same assumptions (tau concentration 415 nM, and tau-tau binding Kd 22 nM), and cellular activity predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [cpz] / KI))

the apparent KI values for chlorpromazine and tacrine (2117 nM and 802 nM respectively) are greater than anticipated from the in vitro studies.

Figure 17 shows the extent of reduction of various compounds in the presence of DTT.

Figure 18 shows the percentage reduction of MB plotted against the

ratio of MB:Vitamin C.

Figure 19(a) shows that by assuming a target tissue concentration of 4μM (i.e. 1.5 μg/g) it is possible to determine from the data of DiSanto and Wagner (1972) that tissue concentrations of this order would be achieved at an IV dosage of 0.11 mg/kg.

Figure 19(b) shows a model for the distribution of MB following a single 100 mg dose in a 70 kg subject, assuming instantaneous absorbtion.

Figure 20 summarises the results for the transient expression of tau fragments in 3T3 and COS-7 cells based upon data from both microscopical and biochemical experiments.

Expression of recombinant tau fragments in eukaryotic cells was performed as follows. Eight tau constructs, transiently expressed in 3T3 cells and COS-7 cells were examined by immunocytochemistry and immunoblots. The extent of expression in each cell type was given semi-quantitatively on the basis of both sets of results: -, no detectable expression; ±, very weak immunoreactivity; + to ++++, increasing levels of positive immunoreactivity. In all cases, mAb 7.51 was used with each construct to obtain the results. In addition the specificity was confirmed for each construct by using a panel of antibodies against different domains of tau protein (mAbs 499, T14, Taul, 342, 7.51, 423 and T46). Kozak sequences were absent in the first six contructs, but were present in the cDNA constructs 7 and 8.

Figure 21 illustrates the inducible expression of full-length human tau in 3T6 fibroblasts in two cell lines. T40.22 shows low level background leakage of full length tau in the uninduced state ("U"), and high levels of expression after addition of IPTG (i.e. induced, "I"). T40.37 shows the same, but lower levels of expression without induction.

Figure 22 shows a result of a triple vector system. A vector permitting very low level constitutive expression of the 12 kD

fragment was introduced into cells lines in which inducible expression of full length tau had already been achieved (in fact cell line T40.22 shown in Figure 21 above). Low levels of IPTG are introduced to induce expression of full-length tau. At 0 μM IPTG, there is very low level expression of the 12 kD band, and low "background leakage" expression of full-length tau. As progressively more full-length tau is induced by introducing higher levels of IPTG, more of the full-length tau is converted to the 12 kD species.

Figure 23 shows the inhibitory effects of reduced thionine. In each set of lanes, there is inducible production of the 12 kD band in the presence of increasing concentrations of IPTG inducing higher levels of T40. As the thionine concentration is increased, the production of the 12 kD band from T40 is suppressed.

Figure 24 shows quantitatively the results of Figure 23. In the absence of thionine, induction of T40 at increasing concentrations of IPTG leads to a corresponding increased production of the 12 kD fragment. In the presence of 2 μM thionine, there is still induction of T40, but it is not converted into the 12 kD fragment.

Figure 25 shows comparative in vitro KI values for various compounds, in nM. The KI values relate to the particular assay conditions used (500:1 DTT:compound, 120 minutes - see Figure 17).

Figures 26 and 27 show the inhibitory effect on tau-tau binding of phenothiazines having 0, 2, 3 or 0, 4, 6 methyl groups, respectively.

Figure 28 shows the derivation of two parameters useful for measuring the inhibition of tau-tau association by test compounds. STB is the standardised binding relative to that seen in the absence of compound, taken as the mean observed at 1 and 10 μg/ml. As described in WO 96/30766, an STB value of 1.0 represents binding equivalent to that observed in the absence of compound, whereas a value of 0.2 indicates that the binding was reduced to a mean of 20% at test compound concentrations of 1 and 10 μg/ml. LB50 is log

10 molar ratio of compound:tau producing 50% tau-tau binding compared with that seen in the absence of compound (B50).

Figure 29 shows the relationship between STB and LB50 parameters. STB can be shown to be a linear function of the LB50.

STB is a logarithmic function of the molar ratio of compound:tau at which tau-tau binding is reduced by 50%.

LB50 is the log of the molar ratio of compound with respect to tau at which tau-tau binding is 50% of that observed in the absence of compound

$$LB50 = 0.05 + (2.65 \times STB) \qquad r=0.95$$

The determination of *in vitro* B50 requires that there be some degree of inhibition of tau-tau binding, and a 50% value is obtained by extrapolation. Determination of STB requires no such extrapolation procedure.

Figure 30 shows compounds for which both STB and B50 values have been determined. Assuming that the total tau concentration in cells is approximately 500 nM (i.e. the concentration of tau used in the assay), the B50 values provide an approximation in the *in vitro* assay to the concentration (i.e. [500 x B50] nM) at which the activity might be expected in cell systems.

Figure 31 shows the formal relationship between the *in vitro* LB50 value and the log KI value for the diaminophenothiazine series.

Figure 32 shows the relationship between the number of methyl groups in a diaminophenothiazine (NMETH) and the redox potential (E) and diffusion coefficient (DIF). Italicised figures indicate correlation coefficients (R) and p values after exclusion of MB.

Figure 33 shows the relationship between the percentage of compound that is reduced, as determined experimentally, and the known reduction potential of the compound. The reduction potential

predicts the observed extent of reduction of the diaminophenothiazines.

Figure 34a shows that there is no clear relationship between inhibitory potency and the extent of reduction of compounds. Figure 34b shows that inhibitory potency is not determined simply by reduction potential.

Figure 35 shows that the inhibitory potency can be related directly to the diffusion coefficient (which is a measure of the tendency of the reduced form to stack and aggregate).

Figures 36 and 37 show the predicted relationships between estimated LB50 ("ESTLB50") and STB ("ESTSTB") values, respectively, and reduction potential and diffusion coefficient, in which the diffusion coefficient is given the greater weighting.

Figure 38 shows the crystalline structure of Methylene Blue.

Figure 39 shows tau-tau binding in the presence of 1mM DTT, as measured in the solid phase assay of WO 96/30766. Two different antibodies were used to detect tau-tau binding, namely mAb 342 (top) and 499 (bottom). The vertical axis represents tau-tau binding, the horizontal axis shows the concentration of full-length tau in the aqueous phase, and the key shows varying concentrations of solid-phase tau. As can be seen, tau-tau binding still occurs in the presence of DTT.

Figure 40 shows various species of tau fragments and doublets which are present without induction ("U") and following induction ("I") in a cell line of the present invention. These include species with mobilities equivalent to 12/14 kD, ~25/27 kD, ~30/32 kD, ~36/38 kD and ~42/44 kD (see Example 3).

Figure 41(a) shows how the 12 kD fragment arises via template-induced proteolytic processing of full-length tau molecules at the approximate positions shown by the arrow-heads.

Figure 41(b) shows how the 25/27 kD species arises via template-induced proteolytic processing of full-length tau molecules at the approximate positions shown by the arrow-heads.

Figure 42 shows a plot of the apparent gel mobilities of the species of Figures 40-41 and their lengths in amino-acid residues.

Figure 43 shows the fragments of Figures 40-42 are at intervals of either ~34 residues or ~17 residues which is the equivalent of a single tau repeat, or half of it. All of the fragments may be generated from a basic heptameric aggregate as a simple set of proteolytic cleavages occurring at the positions indicated by the arrowheads.

Figure 44 shows these same fragments in descending order of length and increasing gel mobility.

Figure 45 shows that DMMB is surprisingly potent in the cell model. Its inhibitory activity could be seen both in the absence of IPTG induction and following induction (see Example 4).

Figure 46 shows the activity of DMMB on base-line expression of the 12/14 kD species, using the same set of assumptions regarding intracellular tau concentration and in vitro tau-tau binding affinity used in Figs 10 - 16.

Cellular activity is predicted via standard inhibition model:

activity = [tau] / ([tau] Kd* (1 + [DMMB] / Ki))

DMMB has an apparent KI within the cell of 4.4 nM, and the cellular B50 value is ~100 nM.

Examples

General materials and methods

Production of 3T6H cell lines

3T6 cells were ECACC No: 86120801 Mouse Swiss Albino Embryo Fibroblasts.

For the inducible system, the experiments employed Lac Switch™ from Stratagene using the p3'SS vector to express the Lac repressor protein and pOPRSVICAT to express the full-length tau under the control of the Lac repressor. Expression is induced by the addition of IPTG.

Initially 3T6 cells were transfected, by electroporation, with the p3'SS plasmid and colonies selected by hygromycin resistance. 5 clones that were expressing varying levels of the Lac repressor protein (determined by immunocytochemistry) were picked, and also the non-cloned cells were retained for comparison.

Production of pOPRSVT40 vector

The T40 insert for cloning into the pOPRSVICAT vector was prepared by PCR with Vent polymerase (NEB) using primers (shown below) that included a Not I site and a start or stop codon as appropriate. The PCR product and pOPRSVICAT vector were cut with Not I and purified. The vector was dephosphorylated to prevent re-ligation, and the insert ligated into the vector using standard protocols.

The resulting ligation mix was transfected into competent E. coli cells and the cells plated out on amp plates. Colonies were picked and gridded out on a new amp plate. Colony lifts were taken to Hybond-N 0.45µm nylon membrane (Amersham) and possible positives selected by colony hybridisation using dGA labelled with (α-^{32}P) dCTP (Amersham) (using an oligolabelling kit (Pharmacia Biotech) and purified on a Nap-10 column (Pharmacia Biotech)). Hybridisation was carried out a 65°C overnight in Church buffer followed by 2x20 mins washes in Church wash. Positive colonies, labeled with radioactive probe, were detected by exposing the blots to x-ray film overnight at - 70°C.

Positive colonies were selected and grown, then checked by PCR and restriction digest to confirm the presence of the insert. The use of a single restriction site for the cloning means that T40 can insert into the vector in either orientation. The orientation of the inserts was determined so as to select colonies with the vector containing T40 in the correct orientation for expression.

Primers used

5'-3' T40-Not I

start

5'-gtc gac tct aga ggc ggc cgc ATG GCT GAG CCC CGG CAG GAG-3'

Not I

3'-5' T40- Not I

stop

5'-act ctt aag ggt cgc ggc cgc TCA CAA CAA ACC CTG CTT GGC CAG -3'

Not I

Sequence complementary to T40 sequence is shown in capitals, the start and stop codons are marked. The Not I site to be added is shown underlined. The remaining sequence shown in lower case is a 13 base pair overhang to allow the Not I enzyme to cut efficiently. This was complementary to sequence in the hTau40 plasmid vector to allow efficient binding of the primers.

Determination of Insert Orientation

Orientation was determined using a restriction enzyme that cuts the insert once and the vector at most a few times, and that gives a differing restriction digest pattern for each orientation. Hind III fits these criteria for pOPRSVT40. If the insert is absent two restriction bands are produced. If the insert is present three bands are produced and the size of the bands depends on the orientation of the insert as shown below.

Forward (correct) Orientation	5385 bp	1030 bp	381 bp
Reverse Orientation	6101 bp	381 bp	314 bp

Production of cells expressing T40 under the control of an inducible promoter

The pOPRSVT40 plasmid was produced and purified by CsCl gradient centrifugation. This was transfected (by electroporation) into 3T6H cells (expressing the Lac repressor protein) produced as described above. Positive cells were selected for by resistance to G418 (at 500µg/ml). Resistant colonies were picked and grown on. The level of expression of full-length T40 with and without the addition of IPTG was determined with anti-tau antibodies by both immunocytochemistry and Western blot.

Production of pZeo295-391

The plasmid pZeo295-391 was designed to express protein corresponding to the truncated fragment of tau (residues 295-391; see below). A constitutive system (pcDNA3.1 from InVitrogen, Netherlands) was used - the plasmid imparts resistance to the antibiotic zeocin. The cDNA for this region was amplified by polymerase chain reaction (PCR), using specific oligonucleotide primers (sense and antisense; see below). The sense primer contained an EcoRI site and the antisense, a BamHI site. The fragments were subcloned into pcDNA3.1 (-)zeo (Invitrogen, Netherlands) that had been digested with EcoRI and BamHI. The inserted DNA is downstream from a cytomegalovirus promoter sequence and upstream of a polyadenylation signal. The plasmid contains the DNA sequence for the expression of ampicillin and zeocin resistance for selection in bacteria and eukaryotic cells, respectively. The authenticity of the inserted DNA was confirmed by full-length sequencing of both strands.

Nucleotide and amino acid sequence for truncated tau fragment 295-391

gataatatcaaacacgtcccgggaggcggcagtgtgcaaatagtctacaaaccagttgacctgagca
aggtgacctccaagtgtggctcattaggcaa

catccatcataaaccaggaggtggccaggtggaagtaaaatctgagaagcttgacttcaaggacaga
gtccagtcgaagattgggtccctggacaatat

cacccacgtccctggcggaggaaataaaaagattgaaacccacaagctgaccttccgcgagaacgcc
aaagccaagacagaccacggggcggag

DNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNIT
HVPGGGNKKIETHKLTFRENAKAKTDHGAE

295 sense primer

met asp^{295}
5' - CGG AAT TCC ACC **ATG** GAT AAT ATC AAA CAC GTC CCG - 3'
EcoRI

391 anti-sense primer

stop glu ^{391}
5' - C GCG GGA TCC **TCA** CTC CGC CCC GTG GTC TGT CTT GGC - 3'
BamHI

The start and stop codons are in bold and the EcoRI and BamHI restriction sites to be added are underlined.

Tissue Culture of cells for assay

The medium used was DMEM (with Glutamax I, pyruvate, 4.5g/l glucose) from Life Technologies, Scotland. This was supplemented with 10% FCS (Helena BioSciences),50 U/ml penicillin, 50 µg/ml streptomycin, plsu further antibiotic as appropriate for the selection and maintenance of the relevant plasmid. Antibiotic concentrations were 200 µg/ml hygromycin (p3'SS selection and

maintenance), 500 µg/ml G418(pOPRSVT40 selection and maintenance), 400 or 200 µg/ml zeocin (pZeo295-391 selection or maintenance).

Cells are grown at 37°C, in a humidified atmosphere of 5% CO_2. Cells are maintained in 10cm dishes, and split when they approach confluency. Medium is removed, cells washed with PBS and cells released by trypsinisation with 1 ml of trypsin/EDTA solution / 10cm dish. Cells are resuspended in fresh medium at 1:10 dilution, or optionally in a range of dilutions from 1:5 to 1:20 (approximately 5000 to 20000 cells/cm^2).

For the testing of drugs, cells are plated in 6 well or 24 well plates at an initial density that will allow them to grow to 50-80% confluency within 24 hours. Drugs are added to the well at various concentrations, expression of full-length tau is induced by the addition of IPTG at 0 - 50 µM. Cells are grown for a further 24 hours and then collected for analysis by SDS PAGE/Western blotting.

Preparation of tau protein

Recombinant tau (clone *htau40)* and perchloric acid-soluble tau extracted from rat and human brain were prepared as described previously (Goedert, M. & Jakes, R. (1990) *EMBO* J. 9:4225; Goedert, M. et al (1993) *Proc. Natl. Acad. Sci. USA* 90:5066).

Gel Electrophoresis and Blotting

Cells grown as outlined above are washed once with PBS then lysed in 50 µl (24 well plates) or 100 µl (6 well plates) laemli buffer. Samples are stored at -20°C, boiled for 4 mins prior to running on 15% acrylamide gels using the BioRad miniProtean III mini gel system. Protein is transferred to PVDF membrane by Western blotting using the CAPs buffer system. The membranes are incubated in block buffer (5% non-fat milk powder (Marvel), 0.1% Tween 20 in PBS) for 1 hr to overnight. Tau protein is detected by incubating the membranes with mAb 7.51 diluted 1 :5 with block buffer for 1-3hrs or overnight, washing well with PBS/0.1% Tween20, incubating with

anti-mouse HRP 1:5000 dilution in block buffer for 1 hr, and washing well with PBS/0.1% Tween20. Bound antibody is detected by ECL reaction detected on ECL hyperfilm (Amersham).

Blots are scanned into a computer on a Hewlett Packard Scanjet 6100C flatbed scanner at 600dpi and saved as tiff files. Densitometry of the T40 and dGAE bands is performed with the Scananalysis program on an Apple Power Mac G3.

Drug preparation

Thionine, methylene blue, DMMB, and tolonium chloride are all prepared as a 1 mM stock in ddH2O. Prior to use a 100 μM dilute stock is prepared in HBSS which is added directly to the medium on cells.

For oxidised drug this is prepared simply by diluting the 1mM stock in HBSS and filter sterilising.

For reduced drug the 1 mM is treated with ascorbic acid and DTT to yield 0.5mM drug, 50mM ascorbic acid 50mM DTT, this is allowed to stand for 15mins (turns blue to colourless) before making the dilute stock. This is diluted in HBSS to yield 100 μM drug, 10mM ascorbic acid, 10mM DTT and filter sterilised. Cells are treated with the drug at various concentrations, but for the reduced drug the ascorbic acid and DTT concentrations are maintained at 400 μM throughout by using appropriate quantities of 100 μM reduced stock, 100 μM oxidised stock and 10 mM ascorbic acid/DTT stock.

SDS Gel Electrophoresis and Immunoblotting

Standard electrophoresis and immunoblotting procedures were used as described previously (Wischik, C. M. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4506; Novak, M.,*et al.* (1993) *EMBO J.* 12:365; Jakes, R. et al.(1991) *EMBO J.* 10:2725). Immunoblots were developed with the ABC kit (Vector Laboratories). The monoclonal antibodies (mAbs) 7.51, 21.D10, 499 and 342 were used as undiluted hybridoma

culture supernatant fluids. mAb AT8 (Innogenetics, Belgium) was used at 1/1000 dilution. Anti-tau mAbs 7.51 (which recognises an epitope in the last repeat; see Novak, M. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 5837), 423 (which recognises tau C-terminally truncated at residue Glu-391; see Wischik, C. M. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:4506; Novak, M. et al.(1993) *EMBO J.* 12:365), 499 (which recognises a human-specific tau segment between residues Gly-14 and Gln-26; see Wischik, C. M. et al.(1996) *Proc. Natl. Acad. Sci. USA* 93:11213), and 342 (which recognises a segment between residues Ser-208 and Pro-251). mAb 21.D10 was raised against the A68-tau brain extract (Lee, V. M.-Y. et al.(1991) *Science* 251: 675).

Tau Binding Assay

This was carried out basically as described in Wischik, C. M., et al.(1996) *Proc. Natl. Acad. Sci. USA* 93:11213. Solid phase tau (0-20 µg/ml) was coated on 96-well poly(vinyl chloride) microtitre plates in 50 mM carbonate buffer at 37°C for 1 h. The plate was washed twice with 0.05% Tween 20, then blocked with 2% Marvel in PBST for 1 h at 37°C. After washing again, the plate was incubated for 1 h at 37°C with aqueous phase tau (0 - 300 µg/ml in PBST containing 1% gelatin). In the present application, 1mM DTT was also added.

The plate was washed twice and incubated for 1 h at 37°C with mAb 499 or 342, diluted with an equal volume of 2% Marvel in PBST. After washing, horseradish peroxidase-conjugated goat-anti-mouse antibody (1/1000 in PBST) was incubated for 1 h at 37°C. The plate was washed and incubated with substrate solution containing tetramethylbenzidine and H_2O_2 and the rate of change of absorbance measured using a V_{max} plate reader (Molecular Diagnostics, California) as described previously (Harrington, C. R. et al. (1990) *J. Immunol. Meth.* 134:261). Each experiment was performed in triplicate and included controls in which both solid phase and aqueous phase tau were absent, and also with either one of the two absent.

Data Analysis

This was performed as described in Wischik *et al.* (*supra*) and curves were fitted according to the Langmuir equation with the Kaleidagraph (Synergy, Philadelphia) or Systat (SPSS Inc., Chicago) programs using quasi-Newton approximation. Curve-fitting correlation coefficients are given in the Figures.

Example 1 - constitutive expression of full-length, truncated and mutated tau

Expression of tau in eukaryotic cell lines was sought to generate a cellular model of tau aggregation under physiological conditions which did not suffer from the limitations of lipofectin-based approaches. This involves the expression of full-length tau and truncated tau fragments for both normal tau and tau carrying pathogenic mutations.

Full length tau

When normal full-length tau (T40) was transfected into cells (3T3 and NIE-115) it was expressed and involved in the assembly of the microtubule network within the cells.

Truncated tau

Initially the cDNA for truncated tau fragment from the core of the PHF, corresponding to fragment 297-391, was transfected into non-neuronal 3T3 fibroblasts: this truncated tau was selected since it is: (i) present in the PHF-core; (ii) detected as deposits in AD brain tissue during the early stages of the disease; (iii) capable of supporting the catalytic capture and propagation of tau capture *in vitro*. Subsequently, a series of transfections was performed in which the extent of truncation at either N- or C- termini was varied, based partly on the immunochemical properties of the tau molecule. Six constructs were created with truncation at the N-terminus (186-441 ; 297-441) at the C- and N- termini (186-391;

297-391) and at the C-terminus (1-391). The pattern of immunoreactivity for the six constructs with a limited panel of antibodies was capable of discriminating all of the tau fragments generated in this way.

The constructs were expressed in eukaryotic cells both transiently (using pSG5 as the vector) and stably (using pIF2 and pZeo as vectors). Stable transfectants are selected on the basis of resistance to the antibiotics geneticin and zeocin for pIF2 and pZeo, respectively. Epitope analysis was performed on bacterially expressed proteins using pRK172 as the vector. Figure 20 summarises the results for various fragments in 3T3 and COS-7 cells. Further results showed that the expression of two forms of tau in the same cell can affect the pattern of immunoreactivity. For example, stable expression of 1-391 and 295-391 results in the appearance of abnormal bundles within the cells. However, maintaining such cells in a stable and reproducible state proved elusive.

Mutated tau

Mutagenesis of full-length tau was used to generate known clinical mutations. These were subcloned into pIF2 and stable transfectants generated in 3T3 and NIE cells for a number of mutations including those which affect microtubule assembly properties of tau (G272V, V337M, P301 S, R406W) and S305N, which affects the alternative splicing of the tau gene *in vivo*. In general, cells expressing full-length tau carrying mutations exhibited labelling of the microtubular network and was indistinguishable from cells transfected with wild-type tau. Cell lines expressing certain truncated tau fragments including mutations proved unstable.

Conclusion

In summary, the constitutive expression of truncated tau within eukaryotic cells proved difficult to achieve. Although transient transfection systems permitted the optimisation of expression of tau by manipulating the Kozak consensus surrounding the initiation codon for 297-tau, the expression of e.g. 297-391 was still modest,

suggesting some inherent toxic properties of the fragment. Stable transfections reiterated this conclusion. This latter system demonstrated that truncation at either N- or C-termini resulted in a slightly greater propensity for the tau to assemble in amorphous deposits rather than in a microtubular network. Stable expression of combinations of tau fragments also generated aggregates within the cytoplasm of cells, but this system was not readily reproducible.

Example 2 - inducible expression of truncated tau

In a further attempt to create a stable, reproducible system, without the toxicity associated with constitutive expression, inducible expression of the core-tau fragment of the PHF (i.e. 297-391 - which is 12 kD) was attempted.

Several inducible systems for expression of proteins in eukaryotic cells were tried, although the preferred system was the "lac switch" system. In this system, two vectors are incorporated into cells, typically 3T3 or 3T6 fibroblasts which do not express any endogenous tau protein. The first, the p3'SS vector codes for constitutive expression of the *lac I* gene, and expressors are selected on the basis of hygromycin resistance. The second, pOPRSVICAT incorporates the DNA coding for the tau protein fragment under the control of a strong RSV promoter which contains operator sequences from the *Lac* operon. Cells which incorporate this vector are selected on the basis of neomycin resistance. Cells which have incorporated both vectors have the property that constitutive expression of *lac I* prevents expression of the incorporated protein (i.e. tau) controlled by the *Lac* operon. The addition of the sugar IPTG competes for the binding of *lac I* to the *Lac* operon, and so permits expression of tau protein.

Inducible expression of the 12 kD fragment was carried out in two cell lines. These did not show appreciable levels of tau protein expression until after 3 days treatment with IPTG at which stage high levels of 12 kD suddenly appeared, forming intracellular aggregates which promptly killed the cell. The process of

aggregation was, as expected, non-linear progressing from low level expression to sudden accumulation of toxic aggregates without any clear gradation, making the aggregation and toxicity impossible to control. This non-linear progression prevented a proper control of the system.

Example 3 - expression of tau in stable cell lines according to invention

In view of the results above, a further system was used as follows. Tissue culture cell line DH 19.4.1.4 and clones thereof were based on 3T6 cells (ECACC No: 86120801 Mouse Swiss Albino Embryo Fibroblasts) expressing full-length, four repeat human tau under the control of an inducible promoter and truncated human tau (295-391) under the control of a constitutive promoter.

Cells expressing T40 under the control of an inducible promoter, T40.22.10, were transfected (by lipofection) with the pZeo295-391 plasmid. Positive cells were selected for by resistance to zeocin at 400µg/ml. Expression of truncated tau on a background of inducible expression of full-length tau was confirmed by Western blot analysis with Mab 7.51.

Figure 21 illustrates the inducible expression of full-length human tau only in 3T6 fibroblasts in two cell lines. T40.22 shows low level background leakage of full length tau in the uninduced state ("U"), and high levels of expression after addition of IPTG (i.e. induced, "I"). T40.37 shows the same, but lower levels of expression without induction. Figure 22 shows the results of a triple vector system. A vector permitting very low level constitutive expression of the 12 kD fragment was introduced into cell lines in which inducible expression of full length tau had already been achieved (T40.22 shown in Figure 21). Figure 22 shows what happens when low levels of IPTG are introduced to induce expression of full-length tau. At 0 µM IPTG, there is very low level expression of the 12 kD band, and low "background leakage" expression of full-length tau. As progressively more full-length tau is induced by introducing higher levels of IPTG, more of the

full-length tau is converted to the 12 kD species, and more of the intermediate higher molecular weight fragments (described in more detail in Figs 43 and 44) are produced.

Examination of the original T40-inducible cell line (T40.22.10) which did not contain the vector for constitutive expression of the 12 kD fragment shows that the 12 kD species is not produced as a truncation by-product of full-length tau induction. Enhanced expression of the 12 kD band following induction of T40 was seen only in cells with low level prior expression of the 12 kD fragment (DH19.4.1.4.6). That is, pre-existing 12 kD provides a template for production of more 12 kD following the induction of full-length tau. An additional doublet may also appear with apparent gel mobility of ~25/27 kD when the cells are in the uninduced state (e.g. in the cell line designated DH 19.4.1.4A.B2). Following induction with IPTG, a further series of doublets may appear, with gel mobilities ~30/32 kD, ~36/38 kD and ~42/44 kD.

These species are shown in Figure 40 both without induction ("U") and following induction ("I"). Also shown are the patterns of immunoreactivity of these fragments seen with mAb 342 and a C-terminal polyclonal antibody T46 which recognises epitopes located between residues Ser422 and Leu441.

The derivation of the fragments seen in the uninduced state (i.e. 12/14 kD and 25/27 kD) may be explained by reference to Figure 41.

Figure 41(a) shows how the 12 kD fragment arises via template-induced proteolytic processing of full-length tau molecules at the approximate positions shown by the arrow-heads.

In the case of the 25/27 kD species, these fragments cannot represent dimers of the the 12/14 kD species, as these fragments are immunoreactive with T46. Therefore, a further proteolytic product of the full-length aggregating tau protein must arise via cleavages occurring at the approximate positions shown by the arrowheads in Figure 41(b).

Following induction (Figure 40, I), the further series of doublets is seen. The derivation of these further fragments can be better understood with reference to Figures 42-44.

Figure 42 shows a plot of the apparent gel mobilities of these fragments and their lengths in amino-acid residues, indicating that the apparent gel mobilities can be understood in terms of a characteristic set of fragment lengths.

As illustrated in Figure 43, all of these fragments are at intervals of either ~34 residues or ~17 residues which is the equivalent of a single tau repeat, or half of it. All of the fragments generated can therefore be understood as arising from a simple set of proteolytic cleavages occurring at the positions indicated by the arrowheads in Figure 43 from a basic heptameric aggregate, formed as shown in the figure. In this scheme the fragments arise as the full combinatorial set of the proposed cleavages occurring at the 3 possible approximate positions shown by the arrowheads at either end of the aggregate. The corresponding predicted immunoreactivity patterns seen with mAb 342 and T46 associated with these fragments are also tabulated.

Figure 44 shows these same fragments in descending order of length and increasing gel mobility. Although the heptameric aggregate is shown for convenience as arising entirely from full-length tau molecules, it will be appreciated that the 12/14 kD fragment could be interposed within the proposed aggregate, replacing some of the binding partners, and that the precise pattern of inclusion of these short fragments in the aggregate will determine which precise fragments from the full set predominate in a given instance. Therefore, the production of this family of proteolytic fragments is better understood as a possible repertoire which can be instantiated in various ways within the cell.

Example 4 - inhibitory effects of compounds on production of proteolytic fragment

Having achieved a stable cell system in which production of the 12

kD fragment (and others) could be controlled, the model was used to test the inhibitory effects of reduced thionine. This is shown in Figure 23. In each set of lanes, there is inducible production of the 12 kD band in the presence of increasing concentrations of IPTG inducing higher levels of T40. As the thionine concentration is increased, the production of the 12 kD band from T40 is suppressed. This is shown quantitatively in Figure 24. In the absence of thionine, induction of T40 at increasing concentrations of IPTG leads to a corresponding increased production of the 12 kD fragment. In the presence of 2 μM thionine, there is still induction of T40, but it is not converted into to the 12 kD fragment.

As reduced thionine is itself toxic, it is necessary to control for reduction in the levels of T40 induced by corresponding does of IPTG at higher levels of thionine. This can be achieved by determining the ratio of 12 kD : T40, which permits averaging the data across IPTG levels and shows a dose-dependent reduction in the level of the 12 kD relative to full-length tau.

The activities of various compounds in the T40/12 kD assay are shown in Figures 9 to 16.

Results are expressed in terms of the ratio of 12 kD : T40 following induction of full-length tau (T40) by treatment cells with IPTG (0, 10, 25, 50 μM) in the presence of thionine or tolonium chloride introduced at the concentrations shown in the presence of reducing agents (200 μM DTT/ascorbate), or chlorpromazine or tacrine introduced without reducing agents. As can be seen, thionine and tolonium chloride produce essentially identical inhibition, whereas chlorpromazine and tacrine are non-inhibitory in the same concentration range. The effect of the reducing agents alone was tested in control experiments which showed no significant difference was seen in the 12 kD : T40 ratio in the presence of reducing agents alone.

The properties of the cell line producing higher molecular weight

degradation products were also examined with MB and DMMB (dimethyl-methylene blue).

As can be seen in Figure 45, DMMB proved to be surprisingly potent in the cell model. Its inhibitory activity could be seen both in the absence of IPTG induction and following induction. Treatment with 1 μM DMMB effectively abolished **all** degradation products within the cell. Further experience with MB and DMMB has shown that even apparent base-line production of the 12/14 kD species is largely determined by aggregation. That is, the constitutive production of the 295-391 fragment is itself either below the level of detection by immunoblot or else it is stabilised by spontaneous aggregation so as to reach levels within the cell which can be detected by immunoblot. Alternatively, the apparent base-line levels of the 12/14 kD fragment seen without IPTG induction and in the absence of treatment with a tau-aggregation inhibitor may itself be dominated by templated aggregation-dependent production from the leakage levels of T40 produced in absence of induction. Whatever the combination of factors which determines the levels of the 12/14 kD fragment in the base-line condition, its apparent expression can be essentially eliminated, along with higher molecular weight aggregation products, by a potent aggregation inhibitor such as DMMB. These results further confirm that production of the higher molecular weight proteolytic fragments (ie 30/32, 36/38, 42/44 kD) is also dependent on critical tau-tau binding interactions occurring through the repeat domain, as shown in Figures 41, 43 and 44.

Figure 46 shows the activity of DMMB on base-line expression of the 12/14 kD species, using the same set of assumptions regarding intracellular tau concentration and in vitro tau-tau binding affinity used in Figs 10 - 16. In this case DMMB is found to have an apparent KI within the cell of 4.4 nM, and the cellular B50 value is ~100 nM. This indicates that DMMB is highly potent within the cellular milieu.

Example 5 - comparison of inhibitory effects of reduced and oxidised compounds

The mathematical model used for the *in vitro* data was used to analyse the effects of test substances in the T40 : 12kD cell assay. Using the known values for Kd and KI from in vitro data, the expression indicated was used to solve for the intracellular concentration of full-length tau (see e.g. Figure 10).

This was found to be about 500 nM, which is in the range expected from studies of tau in brain and in cell systems. A good fit to the experimental data was obtained implying that for some compounds the inhibition of production of truncated tau within the cell can be predicted from the approximate Kd and KI values determined experimentally in vitro.

Example 6 - examination of inhibitory properties of diaminophenothiazines

In *in vitro* studies, the most active inhibitors of tau-tau binding identified were the reduced forms of diaminophenothiazines having 0, 2 or 3 methyl groups. Figure 25 shows the reduced forms of such compounds. The corresponding tau-tau binding curves are shown as a function of molar ratio with respect to tau in Figures 26 and 27. As shown, compounds of the "desmethyl series" (0, 2 or 3 methyl groups) produce approximately 50% inhibition of tau-tau binding (shown on the vertical axis) at molar ratios of 3:1 - 4:1 of compound:tau 'AMR' shown on log scale on horizontal axis). The mean molar ratio for 50% inhibition of tau-tau binding for this group of compounds is 4:1.

Diaminophenothiazines having 4 or 6 methyl groups (the "methylated group") have a biphasic action, with enhancement of tau-tau binding at lower concentration, and inhibition of tau-tau binding at high concentrations (Figure 27). These compounds thus require much higher molar ratios to effect 50% inhibition of tau-tau binding.

Examination of other features of the diaminophenothiazine compound was also carried out. Substitution of the heterocyclic nitrogen or sulphur atoms was found to severely interfere with inhibitory

potency of the compounds. Likewise, removal of the diamino groups was found to be detrimental to the inhibitory activity. It thus appeared that the diamino and heterocyclic NB and S- structures are important for activity of the molecules in the inhibition of tau-tau binding.

For comparison, two methods were used to determine inhibitory activity in the tau-tau assay: STB is the mean tau-tau binding observed at 1 and 10 µg/ml of compound at standard tau concentrations of 488 nM; LB50 is log10 molar ratio of compound:tau producing 50% inhibition of tau-tau binding (Figure 28). As shown in Figure 29, there is a strong correlation between the STB and LB50 values for a range of compounds, with chlorpromazine and riboflavin being two outliers (see also Figures 30 and 31).

Example 7 - inhibitory activity and diffusion potential

Figure 32 indicates that there is a correlation between the number of methyl groups (NMETH) in a test compound and both the reduction potential (E) and diffusion coefficient (DIF). In all comparisons, the Spearman rank correlation was used. As shown in Figure 32, a strong inverse relationship between the number of methyl groups (NMETH) and the reduction potential can be seen only if methylene blue is excluded (normal type: correlation values including methylene blue; italic type: correlation values excluding methylene blue).

This indicates that methylene blue has a disproportionately high reduction potential relative to number of methyl groups (NMETH) in this series. There is also a strong positive correlation between the number of methyl groups and the diffusion coefficient (DIF, Figure 32).

As well as there being no observed correlation between the number of methyl groups and reduction potential (Figure 33), it was surprisingly found that there was no observed correlation between reduction potential and inhibitory potential (Figure 34b), although the extent of reduction of the diaminophenothiazines in the

conditions of the assay is highly correlated with reduction potential (Figure 33). And indeed, there is no correlation between the extent of reduction of these compounds and inhibitory potency (Figure 34a). On the other hand, there is a strong inverse correlation between the inhibitory potency of a compound and its diffusion coefficient, and it is possible to predict estimated LB50 and STB values as linear functions of reduction coefficient and diffusion coefficient when greater weighting is given to the diffusion coefficient(Figures 35, 36 and 37). Both the LB50 and STB values are found to be uniformly low for NMETH values up to and including 3, but for higher NMETH values (in particular methylene blue, NMETH=4) there is a disproportionately low inhibitory potency relative to the number of methyl groups. This may relate to the symmetric placement of the methyl groups which interferes with the stacking ability of the molecules, as measured by the diffusion coefficient. This can be seen, for example, in the crystalline structure of methylene blue (see Figure 38). The diaminophenothiazine molecule is essentially flat and forms stacking arrays. The presence of charge in the molecule, as in the oxidised form, prevents the formation of such stacking arrays, and it appears to be the propensity of the reduced form of this compound to form such stacking relationships that determines the inhibitory potency of the series.

The experiments carried out by the present inventors examined the binding of full-length tau in the aqueous-phase to the truncated repeat domain fragment of tau in the solid-phase, as described in further detail in WO96/30766. Binding was detected with either mAb 342 or mAb 499. As shown in Figure 39, there is typical tau concentration-dependent tau-tau binding in the presence of a large excess of the standard reducing agent dithiothreitol (DTT, 1 mM). However, the inhibitory activity of phenothiazines is also demonstrated in the presence of DTT (1 mM) in the standard configuration of the assay described above (i.e. the data for STB and LB50). The present inventors thus conclude that the inhibitory activity cannot be attributed to DTT *per se*, but rather to the presence of the phenothiazines in their reduced form, due to an

excess of DTT.

In summary, the present inventors provide herein a potential, significantly improved, system for the treatment and prophylaxis of diseases such as Alzheimer's Disease in which proteins undergo induced conformational polymerisation, e.g. as illustrated in the case of Alzheimer's disease by pathological tau-tau binding. The important teachings of this application, *viz* that the diffusion coefficient of a compound may important in determining its inhibitory potency towards this induced conformational protein polymerisation, are potentially of great benefit in advancing our understanding of, and ability to provide therapy for, diseases such as Alzheimer's Disease. Finally, by combining the findings on the preferality of the reduced form of MB, and demonstration of its activity in the cell-based assay at concentrations substantially below those predicted solely on the basis of *in vitro* data, the inventors have shown that this compound, and others like it, could be used an appropriate reducing formulation for the prophylaxis or treatment of AD and related disorders.

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Claims

1 A method of proteolytically converting a precursor protein to a product fragment in a stable cell line,

which precursor protein is associated with a disease state in which the precursor protein aggregates pathologically,

which method comprises:

(a) providing a stable cell line transfected with nucleic acid encoding:

(i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and

(ii) the precursor protein, which protein is inducibly expressed in the cell in response to a stimulus,

whereby interaction of the template fragment with the precursor protein causes a conformational change in the precursor protein such as to cause aggregation and proteolytic processing of the precursor protein to the product fragment.

2 A method as claimed in claim 1 wherein pathological aggregation leads to proteolytic processing of the precursor protein in a disease state associated with neurodegeneration and\or clinical dementia.

3 A method as claimed in claim 1 or claim 2 wherein pathological aggregation of the precursor protein in the disease state leads to proteolytic processing to a core domain fragment and the template fragment comprises at least the core fragment of the template protein.

4 A method as claimed in claim 3 wherein the template fragment consists essentially of the core fragment.

5 A method as claimed in any one of the preceding claims wherein the product fragment produced in the cell is toxic.

6 A method as claimed in any one of the preceding claims wherein the product fragment is the same as the template fragment.

7 A method as claimed in any one of claims 1 to 5 wherein a plurality of different product fragments are produced.

8 A method as claimed in any one of the preceding claims comprising the step of subjecting the cell to the stimulus such as to inducibly express the precursor protein in the cell.

9 A method as claimed in any one of the preceding claims wherein the production of at least one product fragment is monitored.

10 A method for identifying a modulator of aggregation and\or proteolytic processing of the precursor protein associated with the disease state

which method comprises:

(a) providing an agent suspected of being capable of modulating the aggregation,

(b) performing a method as claimed in claim 9 in the presence of the agent,

(c) correlating the production of the or each product fragment monitored with the modulatory activity of the agent.

11 A method as claimed in any one of claims 10 wherein step (b) is performed by:

(a) culturing the cells on one or more plates,

(b) incubating the cells with the agent for a period of time sufficient to entry of the agent into the cells.

12 A method as claimed in claim 11 wherein the agent is introduced to the cells to give a final concentration of between 1-50 μM.

13 A method as claimed in any one claims 10 to 12 wherein production of a plurality of different product fragments is monitored.

14 A method as claimed in any one claims 10 to 13 wherein the production of the or each product fragment monitored is compared with a reference value.

15 A method as claimed in claim 14 wherein the reference value is obtained by performing the method in the absence of the agent.

16 A method as claimed in any one of claims 10 to 15 wherein the agent which is provided is selected such as to be capable of crossing the blood-brain barrier.

17 A method as claimed in any one of claims 10 to 16 which comprises the step of selecting the agent to be provided by measuring the diffusion coefficient of the agent and correlating the diffusion coefficient with the agents inhibitory potential.

18 A method as claimed in any one of claims 10 to 17 further comprising the step of calculating a B50 for the agent.

19 A method as claimed in any one of claims 10 to 18 further comprising the step of assessing the effect of the agent on cell viability.

20 A method as claimed in claim 19 further comprising the step of calculating an LD50 for the agent.

21 A method as claimed in claim 18 and 20 comprising the step of calculating a therapeutic index for the agent.

22 A method as claimed in any one of the preceding claims wherein the precursor protein is a tau protein.

23 A method as claimed in claim 22 wherein the template fragment comprises a core fragment of tau.

24 A method as claimed in claim 23 wherein the template fragment comprises a fragment of tau extending from amino acids 186-297 to 390-441 of the full-length tau protein shown in Fig 7.

25 A method as claimed in claim 24 wherein the template fragment consists of a fragment of tau extending from amino acids 295, 296 or 297 to amino acid residues 390 or 391 of the full-length tau protein shown in Fig 7.

26 A method as claimed in any one of claims 22 to 25 wherein the production of an approximately 12, 14, 25, 27, 30, 32, 36, 38, 42 or 44 kDa product fragment of tau is monitored.

27 A method as claimed in claim 26 wherein the production of an approximately 12 kDa product fragment of tau is monitored.

28 A method as claimed in any one of claims 22 to 27 wherein production of the or each toxic product fragment is monitored on SDS PAGE.

29 A method as claimed in any one of claims 22 to 28 wherein production of the or each toxic product fragment is monitored immunologically.

30 A method as claimed in claim 29 wherein the monitoring employs an antibody is selected from a monoclonal antibody which (i) is specific for a human-specific epitope located in the region between Gly-16 and Gln-26 of tau; (ii) is specific for the core tau fragment truncated at Glu-391; (iii) is specific for a generic tau epitope in the repeat domain; or (iv) is specific for a non-species specific generic tau epitope located between Ser-208 and Ser-238.

31 A method as claimed in any one of claims 22 to 30 which comprises the step of selecting the agent to be provided by determining the ability of the agent to modulate the ability of a fragment of tau corresponding to the core repeat domain, which has been adsorbed to a solid phase substrate, to capture soluble full-length tau.

32 A method as claimed in any one of claims 22 to 31 wherein the agent is a phenothiazine.

33 A method as claimed in claim 32 wherein the agent is a reduced phenothiazine.

34 A method for screening for a medicament for use as a therapeutic or prognostic agent for the treatment of a tauopathy which method comprises:
(a) performing a method as claimed in any one of claims 22 to 33
(b) selecting modulators having a therapeutic index of greater than 2.

35 A method as claimed in claim 34 wherein the "disease" is selected from Alzheimer's disease, motor neuron disease, Lewy body disease, Pick's disease or Progressive Supranuclear Palsy.

36 A method for producing a medicament for use as a therapeutic or prognostic modulator for the treatment of a tauopathy, which method comprises
(a) carrying out a method as claimed in any of claim 34 or claim 35 to identify the medicament,
(b) providing the medicament agent in isolated form.

37 A method as claimed in claim 36 further comprising formulating the agent as a medicament composition for use in the treatment of the tauopathy.

38 A method as claimed in claim 37 further comprising using the medicament composition in a method of treatment for the tauopathy.

39 Use of a phenothiazine in the preparation of a medicament composition for use in the treatment or prophylaxis of a tauopathy, wherein the preparation comprises the step of pre-reducing the phenothiazine such that it is present in at least 80, 90, 95, or 99% educed (leuco-) form.

40 Use as claimed in claim 39 wherein the phenothiazine is pre-reduced by addition of an exogenous reducing agent.

41 Use as claimed in claim 40 wherein the reduced form is stabilised in the reduced state by addition of a stabilising agent.

42 Use as claimed in claim 41 wherein the reduced form is lyophilised with the stabilising agent.

43 Use of a pre-reduced phenothiazine in the preparation of a medicament composition for use in the treatment or prophylaxis of a tauopathy, wherein the medicament comprises at least 80, 90, 95, or 99% of the reduced (leuco-) form of the phenothiazine.

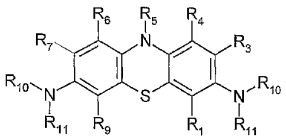
44 Use as claimed in any one of claims 39 to 43 wherein the medicament composition further comprises one or more of the following: a pharmaceutically-acceptable excipients, carriers or buffers.

45 Use as claimed in claim 44 wherein the medicament composition is prepared as a slow release formulation.

46 Use as claimed in any one of claims 39 to 45 wherein the phenothiazine is a diaminophenothiazine.

47 Use as claimed in any one of claims 39 to 46 wherein the pre-reduced (leuco-) phenothiazine has the formula:

(I)



wherein R1, R3, R4, R6, R7 and R9 are independently selected from hydrogen, halogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy; R5 is selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;

and each R10 and R11 are independently selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;

or is a pharmaceutically acceptable salt thereof.

48 Use as claimed in claim 47 wherein R1, R3, R4, R6, R7 and R9 are independently selected from -hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$; each R10 and R11 are independently selected from hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$; and
R5 is hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$.

49 Use as claimed in any one of claims 46 to 48 wherein the phenothiazine is a diaminophenothiazine which has 0, 2, 3 or 4 methyl groups around the diaminophenothiazine nucleus.

50 Use as claimed in any one of claims 46 to 49 wherein the phenothiazine is a diaminophenothiazine which is asymmetrically methylated.

51 Use as claimed in claim 50 wherein the phenothiazine is tolonium chloride, azure A, azure B and thionine.

52 Use as claimed in claim any one of claims 46 to 49 wherein the phenothiazine is selected from Methylene Blue, Toluidine Blue O, or 1,9-Dimethylmethylene Blue.

53 A medicament composition comprising a pre-reduced phenothiazine as described in any one of claims 47 to 52 wherein the phenothiazine is at least 80, 90, 95, or 99% of the reduced (leuco-) form, in combination with a stabilizer.

54 A medicament composition as claimed in claim 53 which is lyophilised with the stabiliser.

55 A medicament composition as claimed in claim 54 or claim 54 wherein the stabiliser is ascorbate.

55 A medicament composition as claimed in any one of claims 53 to 55 for use in the treatment or prophylaxis of a tauopathy.

56 A method of treatment of a tauopathy comprising use of medicament composition as claimed in any one of claims 53 to 55.

57 A method, use, or composition as claimed in any one of claims 34 to 52, claim 55 or claim 56 wherein the treatment or prophylaxis comprises giving a prophylactically effective amount or a therapeutically effective amount of the medicament composition to a patient in need of the same.

58 A method, use, or composition as claimed in any one of claims 39 to 52, claim 55 or claim 56 wherein the treatment or prophylaxis comprises giving a patient in need of same 20 mg tds, 50 mg tds or 100 mg tds, combined with 2x mg ratio of ascorbic acid in such a manner as to achieve more than 90% reduction of the phenothiazine prior to ingestion.

59 A method, use, or composition as claimed in any one of claims 39 to 52, claim 55 or claim 56 wherein the treatment or prophylaxis comprises giving a patient a phenothiazine which is thionine and this is given to the patient in a daily dosage of between 1 and 1000 mg optionally divided into 1 to 8 unit doses.

60 A method, use, or composition as claimed in any one of claims 39 to 52, claim 55 or claim 56 wherein the treatment or prophylaxis comprises giving a patient a phenothiazine which is methylene blue, and the daily dosage is approximately 3.2-3.5 mg/kg.

61 A process for producing a stable cell for use in a method as claimed in claim in any one claims 1 to 38 which process comprises the steps of introducing into a cell nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly expressed in the cell in response to a stimulus.

62 A process as claimed in claim 61 wherein the nucleic acid encoding the precursor protein is operably linked to a lac inducible promoter.

63 A process as claimed in claim 62 wherein expression of the precursor protein is induced by the addition of IPTG at 1 - 50 mM.

64 A process as claimed in any one of claims 61 to 63 wherein the nucleic acid encoding the template fragment is operably linked to a cytomegalovirus promoter sequence.

65 A process as claimed in any one of claims 61 to 64 wherein the nucleic acid encoding the template fragment is introduced as a template vector and the nucleic acid encoding the precursor protein is introduced as a separate precursor protein vector.

66 A process as claimed in claim 65 wherein the precursor protein vector is derived from the pOPRSVICAT vector into which the nucleic acid encoding the precursor protein is cloned.

67 A process as claimed in claim 65 or claim 66 wherein the template fragment vector is derived from the plasmid pZeo295-391 vector into which the nucleic acid encoding the precursor protein is cloned.

68 A process as claimed in any one of claims 61 to 67 wherein the precursor protein is tau.

69 A process as claimed in claim 68 wherein the nucleic acid encoding the template fragment encodes a core fragment of tau.

70 A process as claimed in claim 69 wherein the nucleic acid encoding the template fragment encodes encodes a fragment of tau extending from between amino acids 186-296 to 390-441 of the full-length protein.

71 A process as claimed in claim 70 wherein the nucleic acid encoding the template fragment encodes encodes a fragment of tau extending from between aminoacids 295, 296 or 297 to amino acid residues 390 or 391 of the full-length tau protein shown in Fig 7.

72 A process as claimed in claim 71 wherein the nucleic acid encoding the template fragment encodes encodes a fragment of tau extending between amino acid residues 295 to 391 as shown in Fig 7.

73 A composition of matter comprising nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly expressed in the cell in response to a stimulus, which nucleic acid is described in any one of claims 62 to 72.

74 A mammalian host cell transformed with nucleic acid of claim 73 such as to express (i) a template fragment of the precursor protein such that the template fragment constitutively at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly in response to a stimulus.

75 A cell as claimed in claim 74 which is from a neuronal cell line or a fibroblast cell line.

76 A cell as claimed in claim 75 which is selected from the following cell lines: 3T3; NIE-115; 3T6; N2A; SY5Y; COS-7.

77 A kit comprising a host cell as claimed in any one of claims 74 to 76 plus at least one further component selected from: an agent for stimulating production of the precursor protein or an agent for detecting the interaction of the precursor protein with the template fragment.

78 A kit as claimed in claim 77 wherein the detection agent is an antibody.

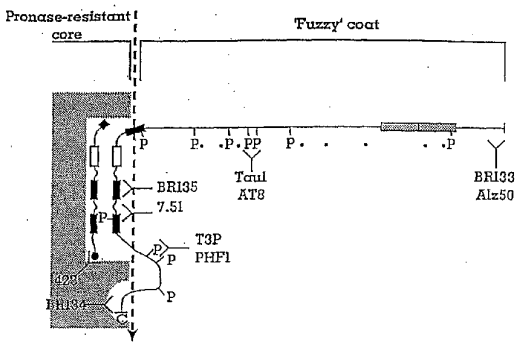
79 A nucleic acid primer selected from:

5'-3' T40-Not I
5'-gtc gac tct aga ggc ggc cgc ATG GCT GAG CCC CGG CAG GAG-3'

3'-5' T40-Not I
5'-act ctt aag ggt cgc ggc cgc TCA CAA CAA ACC CTG CTT GGC CAG -3'

295 sense primer
5' - CGG AAT TCC ACC ATG GAT AAT ATC AAA CAC GTC CCG - 3'

391 anti-sense primer
5' - C GCG GGA TCC TCA CTC CGC CCC GTG GTC TGT CTT GGC - 3'



Antibody	Extracellular Stages 3	Intra-/Extra-Cellular Stages 2	Intracellular Stage 1
BR 133	-	-	+++
mAb 18.8	-	-	+++
BR 134	+	++	+++
mAb 11.57	+/-	++	+++
mAb 25.6E	+/-	++	+++
BR 135	+++	+++	+++
mAb 423	+++	+++	+

Figure 1

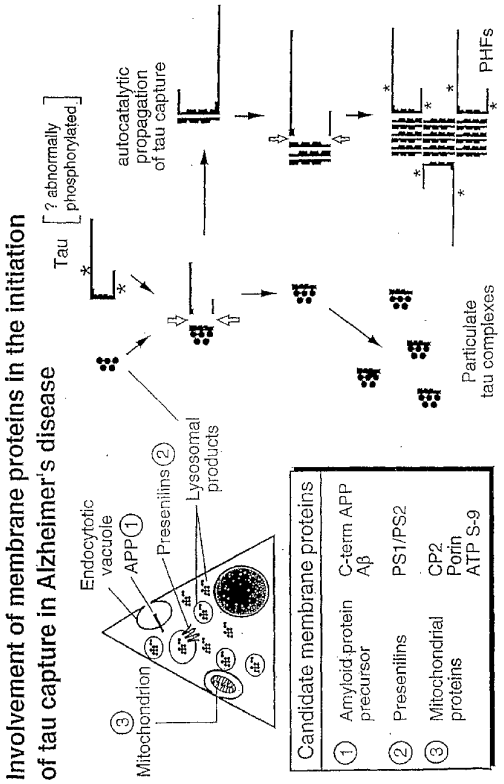

Involvement of membrane proteins in the initiation of tau capture in Alzheimer's disease
Tau
? abnormally phosphorylated
autocatalytic propagation of tau capture
PHFs
Endocytotic vacuole
APP (1)
Presenilins (2)
Lysosomal products
Particulate tau complexes
(3) Mitochondrion
Candidate membrane proteins
(1) Amyloid protein precursor
C-term APP
Aβ
(2) Presenilins
PS1/PS2
(3) Mitochondrial proteins
CP2
Porin
ATP S-9

Figure 2

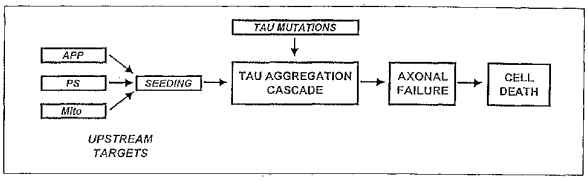

TAU MUTATIONS
APP
PS
Mito
SEEDING
TAU AGGREGATION CASCADE
AXONAL FAILURE
CELL DEATH
UPSTREAM TARGETS

Figure 3

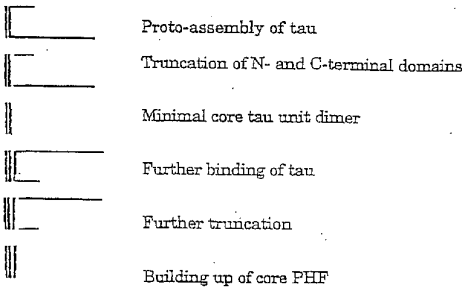

Proto-assembly of tau
Truncation of N- and C-terminal domains
Minimal core tau unit dimer
Further binding of tau
Further truncation
Building up of core PHF

Figure 4

Diseases of protein aggregation

Protein	Disease	Aggregating domain and/or mutations	Rbif subunit size (kDa)	Reference
Neurodegenerative disorders				
Prion protein	Prion diseases CJD, nvCJD, Fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome, Kuru)	*Inherited and sporadic forms* PrP27-30 core fragments Fibrillogenic domains: 113-120, 178-191, 202-218	27	Prusiner (1998) Gasset et al. (1992)
Tau protein	Alzheimer's disease, Down's syndrome FTDP-17, CBD, post-encephalitic parkinsonism, Pick's disease, parkinsonism with dementia complex of Guam	*Inherited and sporadic forms* Truncated tau (tubulin-binding domain) 297-391 Mutations in tau in FTDP-17 Many mutations in presenilin proteins	10-12	Wischik et al. (1988) Hutton et al. (1998) Crowh et al. (2000)
Amyloid β-protein	Alzheimer's disease, Down's syndrome	*Inherited and sporadic forms* Amyloid β-protein; 1-42(3); 11 mutations in APP in rare families	4	Glenner & Wong, (1984) Goate et al., (1991)
Huntingtin Ataxins (1, 2, 3, 7) *Atrophin* Androgen receptor	Huntington's disease Spinocerebellar ataxias (SCA1, 2, 3, 7) Dentatorubropallidoluysian atrophy (DRPLA) Spinal and bulbar muscular atrophy	*N-termini of protein with expanded glutamine repeats* *Proteins with expanded glutamine repeats* *Proteins with expanded glutamine repeats* *Proteins with expanded glutamine repeats*	40	DiFiglia et al. (1997) Paulson et al. (2000) Paulson et al. (2000) Paulson et al. (2000)
Neuroserpin	Familial encephalopathy with neuronal inclusion bodies (FENIB)	Neuroserpin; S49P, S52R	57	Davis et al. (1999)
α-Synuclein	Parkinson's disease, dementia with Lewy bodies, multiple system atrophy	*Inherited and sporadic forms* A53T, A30P In rare autosomal-dominant PD families	19	Spillantini et al. (1998) Polymeropoulos et al. (1997)
Cystatin C	Hereditary cerebral angiopathy (Icelandic)	Cystatin C loss 10 residues; L68Q	12-13	Abrahamson et al. (1992)
Superoxide dismutase 1	Amyotrophic lateral sclerosis	SOD1 mutations		Shibata et al. (1996)
Non-neurodegenerative disorders				
Haemoglobin	Sickle cell anaemia Inclusion body haemolysis	Haemoglobin beta chain (S) Many mutations		Cannell & Ghoptu (1998)
Serpins	α1-Antitrypsin deficiency (emphysema, cirrhosis) Antithrombin deficiency (thromboembolic disease) C1-inhibitor deficiency (angioedema)	Mutations Mutations Mutations		Lomas et al. (1992) Cannell & Ghoptu (1998) Cannell & Ghoptu (1998)
Immunoglobulin light chain	Plasma cell dyscrasias (primary systemic AL amyloidosis)	light chain or fragments	0.5-25	Westermark et al. (1995)
Serum amyloid A	Reactive, secondary systemic AA amyloidosis Chronic inflammatory disease	Variable N-terminal fragments of SAA	4.5-10	Westermark et al. (1986)

(continued.....)

Figure 5a

(.....continued)

Transthyretin	Familial amyloid polyneuropathy (systematic FAP I)	Tetramer dissociated to conformational monomer variant *Many mutations (some not associated with amyloid; several different types of disease)*	10-14	Gustavsson et al. (1991)	
	Senile cardiac amyloidosis	Normal transthyretin	10-14	Gustavsson et al. (1991)	
Gelsolin	Familial amyloidosis - Finnish type (FAP IV)	D187Q leads to truncated 173-225/243 (critical residues 182-192)	9,5	Maury & Baumann (1990)	
β2-Microglobulin	Haemodialysis amyloidosis Prostatic amyloid	β2-Microglobulin	12-25	Gorevic et al. (1985)	
Apolipoprotein AI	Familial amyloid polyneuropathy (systematic FAP III)	N-terminal 83-93 residues; G26R, W50R, L60R	9	Booth et al. (1996)	
Lysozyme	Familial visceral amyloidosis	Lysozyme or fragments (with or without I56T, D67H)	14	Pepys et al., (1993)	
Amylin (islet amyloid polypeptide)	Type II diabetes (NIDDM)	Fragments (critical core of 20-29); no mutations	3,9	Westermark (1990)	
Fibrinogen α-chain	Hereditary renal amyloidosis	Fibrinogen fragments	7-10	Uemichi et al. (1994)	
Procalcitonin	Medullary carcinoma of thyroid	Calcitonin fragments	3,4	Sletten et al. (1976)	
Atrial natriuretic factor	Cardiac amyloidosis	ANF, no mutants	3,5	Johnenson et al. (1987)	
Insulin	Injection localised amyloidosis	Insulin		Dische et al. (1988)	
Other proteins forming amyloid	*(In vitro)*	Other proteins		Chiti et al. (1999)	

Figure 5b

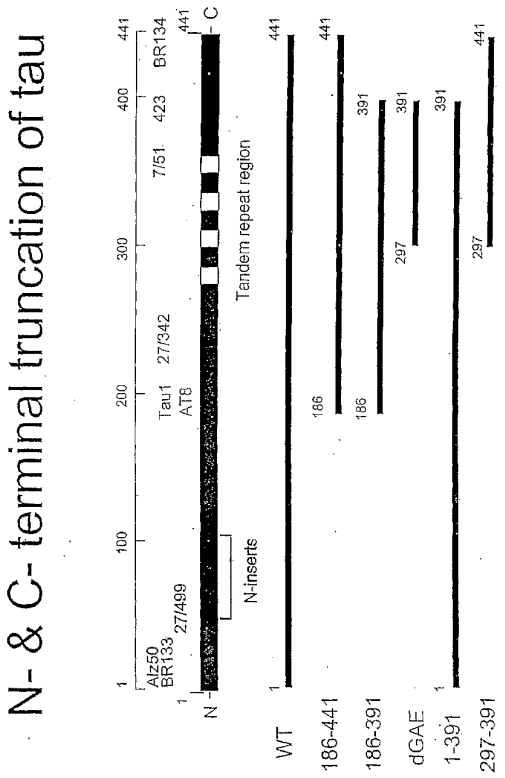

N- & C- terminal truncation of tau
1
100
200
300
400
441
Alz50
BR133
27/499
Tau1
AT8
27/342
7/51
423
BR134
N
C
N-inserts
Tandem repeat region
WT
186-441
186-391
dGAE
1-391
297-391
186
297
391

Figure 6

```
ATG GCT GAG CCC CGC CAG GAG TTC GAA GTG ATG GAA GAT CAC GCT GGG
Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly
  1               5                  10                  15

ACG TAC GGG TTG GGG GAC AGG AAA GAT CAG GGG GGC TAC ACC ATG CAC
Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr Met His
            20                  25                  30

CAA GAC CAA GAG GGT GAC ACG GAC GCT GGC CTG AAA GAA TCT CCC CTG
Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Glu Ser Pro Leu
        35                  40                  45

CAG ACC CCC ACT GAG GAC GGA TCT GAG GAA CCG GGC TCT GAA ACC TCT
Gln Thr Pro Thr Glu Asp Gly Ser Glu Glu Pro Gly Ser Glu Thr Ser
    50                  55                  60

GAT GCT AAG AGC ACT CCA ACA GCG GAA GAT GTG ACA GCA CCC TTA GTG
Asp Ala Lys Ser Thr Pro Thr Ala Glu Asp Val Thr Ala Pro Leu Val
65                  70                  75                  80

GAT GAG GGA GCT CCC GGC AAG CAG GCT GCC GCG CAG CCC CAC ACG GAG
Asp Glu Gly Ala Pro Gly Lys Gln Ala Ala Ala Gln Pro His Thr Glu
                85                  90                  95

ATC CCA GAA GGA ACC ACA GCT GAA GAA GCA GGC ATT GGA GAC ACC CCC
Ile Pro Glu Gly Thr Thr Ala Glu Glu Ala Gly Ile Gly Asp Thr Pro
            100                 105                 110

AGC CTG GAA GAC GAA GCT GCT GGT CAC GTG ACC CAA GCT CGC ATG GTC
Ser Leu Glu Asp Glu Ala Ala Gly His Val Thr Gln Ala Arg Met Val
        115                 120                 125

AGT AAA AGC AAA GAC GGG ACT GGA AGC GAT GAC AAA AAA GCC AAG GGG
Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp Asp Lys Lys Ala Lys Gly
    130                 135                 140

GCT GAT GGT AAA ACG AAG ATC GCC ACA CCG CGG GGA GCA GCC CCT CCA
Ala Asp Gly Lys Thr Lys Ile Ala Thr Pro Arg Gly Ala Ala Pro Pro
145                 150                 155                 160

GGC CAG AAG GGC CAG GCC AAC GCC ACC AGG ATT CCA GCA AAA ACC CCG
Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro
                165                 170                 175

CCC GCT CCA AAG ACA CCA CCC AGC TCT GGT GAA CCT CCA AAA TCA GGG
Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro Lys Ser Gly
            180                 185                 190

GAT CGC AGC GGC TAC AGC AGC CCC GGC TCC CCA GGC ACT CCC GGC AGC
Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser Pro Gly Thr Pro Gly Ser
        195                 200                 205

CGC TCC CGC ACC CCG TCC CTT CCA ACC CCA CCC ACC CGG GAG CCC AAG
Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu Pro Lys
    210                 215                 220
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Figure 7a

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AAG GTG GCA GTG GTC CGT ACT CCA CCC AAG TCG CTG TCT TCC GGC AAG
Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Leu Ser Ser Ala Lys
225                 230                 235                 240

AGC CGC CTG CAG ACA GCC CCC GTG CCC ATG CCA GAC CTG AAG AAT GGC
Ser Arg Leu Gln Thr Ala Pro Val Pro Met Pro Asp Leu Lys Asn Gly
                245                 250                 255

AAG TCC AAG ATC GGC TCC ACT GAG AAC CTG AAG CAC CAG CCG GGA GGC
Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys His Gln Pro Gly Gly
            260                 265                 270

GGG AAG GTG CAG ATA ATT AAT AAG AAG CTG GAT CTT AGC AAC GTC CAG
Gly Lys Val Gln Ile Ile Asn Lys Lys Leu Asp Leu Ser Asn Val Gln
        275                 280                 285

TCC AAG TGT GGC TCA AAG GAT AAT ATC AAA CAG GTC CCG GGA GGC GGC
Ser Lys Cys Gly Ser Lys Asp Asn Ile Lys Gln Val Pro Gly Gly Gly
    290                 295                 300

AGT GTG CAA ATA GTC TAC AAA CCA GTT GAC CTG AGC AAG GTG ACC TCC
Ser Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys Val Thr Ser
305                 310                 315                 320

AAG TGT GGC TCA TTA GGC AAC ATC CAT CAT AAA CCA GGA GGT GGC CAG
Lys Cys Gly Ser Leu Gly Asn Ile His His Lys Pro Gly Gly Gly Gln
                325                 330                 335

GTG GAA GTA AAA TCT GAG AAG CTT GAC TTC AAG GAC AGA GTC CAG TCG
Val Glu Val Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser
            340                 345                 350

AAG ATT GGG TCC CTG GAC AAT ATC ACC CAC GTC CCT GGC GGA GGA AAT
Lys Ile Gly Ser Leu Asp Asn Ile Thr His Val Pro Gly Gly Gly Asn
        355                 360                 365

AAA AAG ATT GAA ACC CAC AAG CTG ACC GTC CGC GAG AAC GCC AAA GCC
Lys Lys Ile Glu Thr His Lys Leu Thr Val Arg Glu Asn Ala Lys Ala
    370                 375                 380

AAG ACA GAC CAC GGG GCG GAG ATC GTG TAC AAG TCG CCA GTG GTG TCT
Lys Thr Asp His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser
385                 390                 395                 400

GGG GAC ACG TCT CCA CGG CAT CTC AGC AAT GTC TCC TCC ACC GGC AGC
Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser
                405                 410                 415

ATT GAC ATG GTA GAC TCG CCC CAG CTC GCC ACG CTA GCT GAC GAG GGG
Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Gly
            420                 425                 430

TCT GCC TCC CTG GCC AAG CAG GGT TTG TGA
Ser Ala Ser Leu Ala Lys Gln Gly Leu ***
        435                 440
```
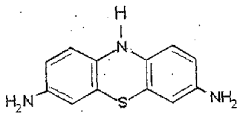
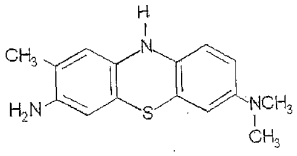

Figure 7b

Compounds tested in cell-based assays

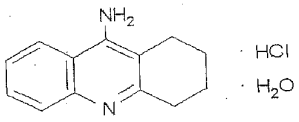
Thionine



Tolonium Chloride



Tacrine



Chlorpromazine

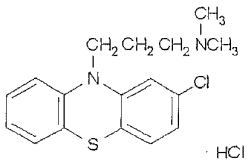


Figure 8

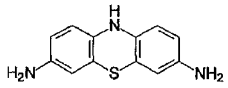
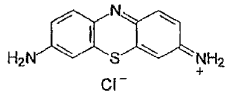
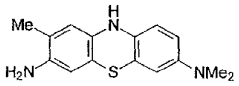
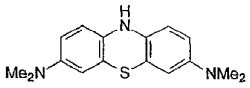
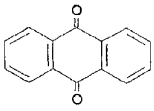
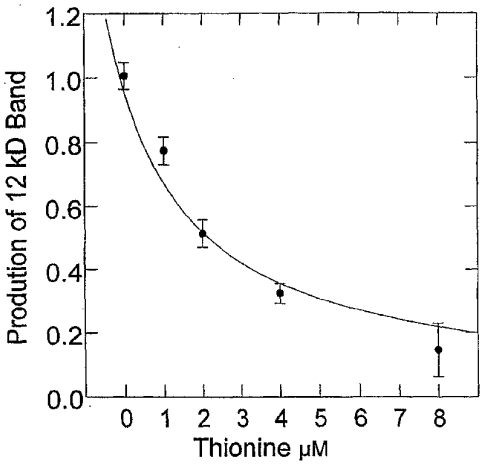
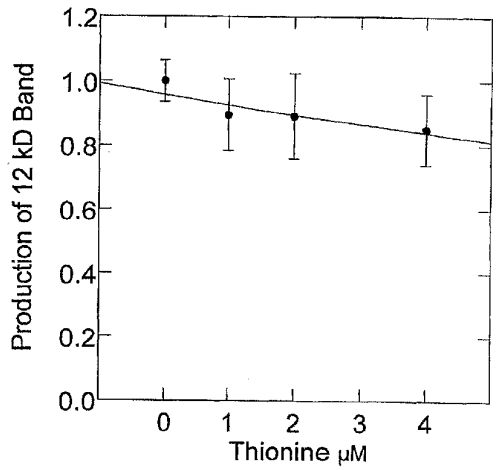
		KI (nM)	B_{50} (μM)
I	 Reduced Thionine	100	2.17
	 Oxidised Thionine	1200	26.07
	 Reduced Tolonium Chloride	105	2.28
	 Reduced Methylene Blue	123	2.67
	 DH12		

Figure 9



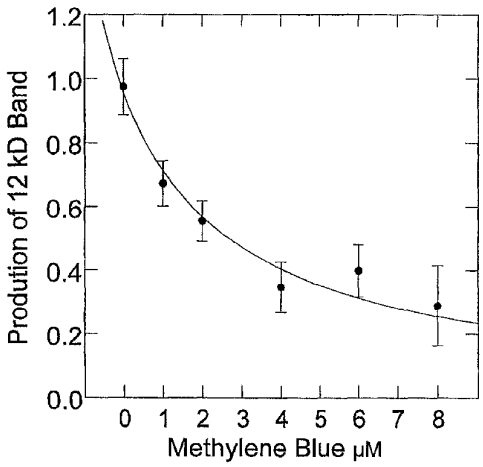
Observed vs predicted activity	r = 0.986
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Thionine KI	100 nM

Figure 10



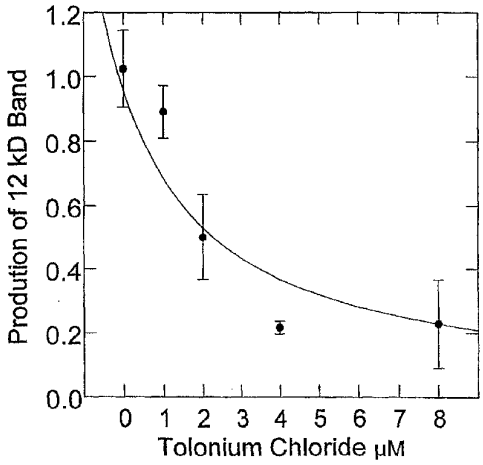
Observed vs predicted activity	r = 0.784
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Oxidised Thionine KI	1200 nm

Figure 11



Observed vs predicted activity	r = 0.962
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Methylene Blue KI	123 nM

Figure 12



Observed vs predicted activity	r = 0.913
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Tolonium Chloride KI	105 nM

Figure 13

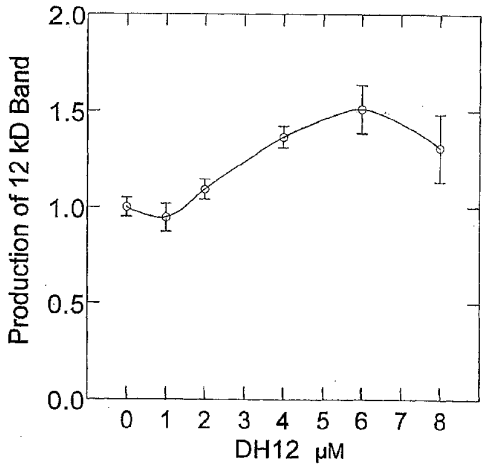
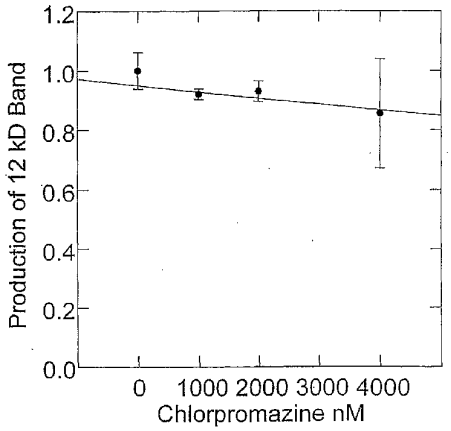
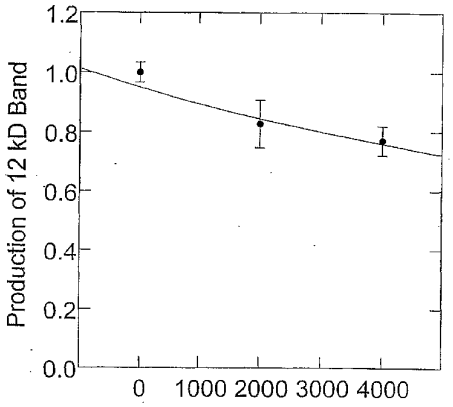


Figure 14



Observed vs predicted activity	r = 0.937
Intracellular tau concentration	415 nM
Tau-tau binding affinity	22 nM
Chlorpromazine KI	2117 nm

Figure 15



Observed vs predicted activity	r = 0.976
Intracellular tau concentration	415 nM
Tau-tau binding affinity	22 nM
Tacrine KI	802 nm

Figure 16

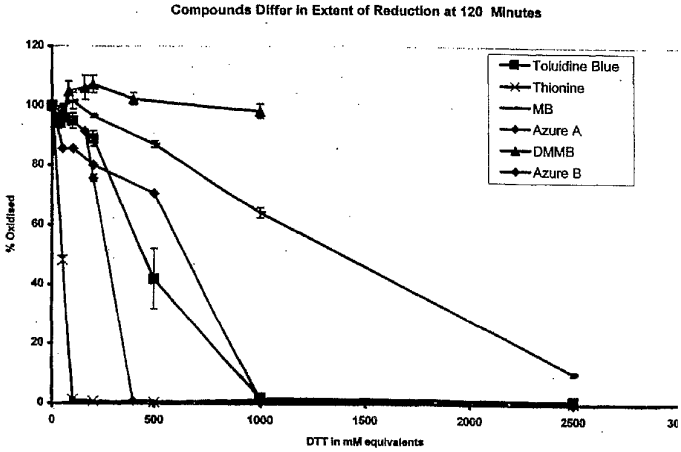

Compounds Differ in Extent of Reduction at 120 Minutes
Toluidine Blue
Thionine
MB
Azure A
DMMB
Azure B
% Oxidised
DTT in mM equivalents
0
20
40
60
80
100
120
0
500
1000
1500
2000
2500

Figure 17

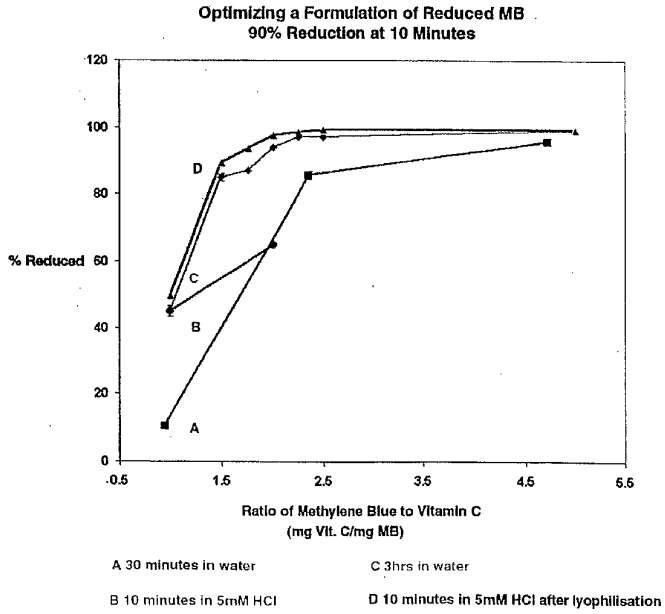

Optimizing a Formulation of Reduced MB
90% Reduction at 10 Minutes
% Reduced
120
100
80
60
40
20
0
-0.5
1.5
2.5
3.5
4.5
5.5
D
C
B
A
Ratio of Methylene Blue to Vitamin C
(mg Vit. C/mg MB)
A 30 minutes in water
B 10 minutes in 5mM HCl
C 3hrs in water
D 10 minutes in 5mM HCl after lyophilisation

Figure 18

Tissue levels vs IV dose of MB
DiSanto and Wagner (1972)

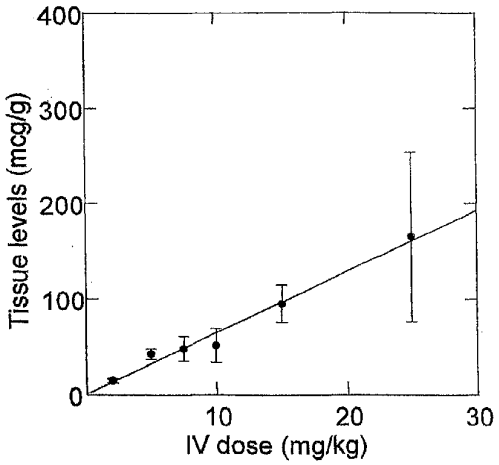


Figure 19a

Blood and Tissue distribution MB (1.43 mg/kg dose)

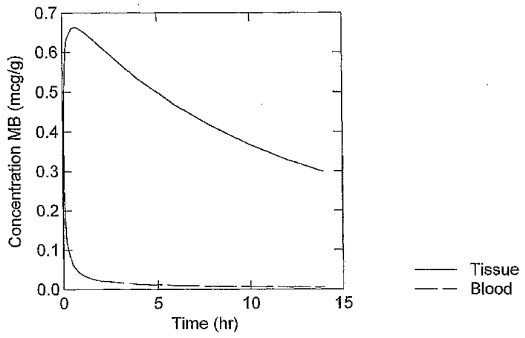


Figure 19b

Length of tau	Apparent Mr (kDa)	Expression in:	
		3T3	COS-7
1) 1-391	55	++	++++
2) m186-391	26	++	++++
3) m297-391	12	+/-	+
4) m186-441	32	++	+++
5) m297-441	18	+	+
6) 1-441	67	++	++++
7) [kozak]m295-391	12	+	+++
8) [kozak]m297-391	12	+/-	++

Figure 20

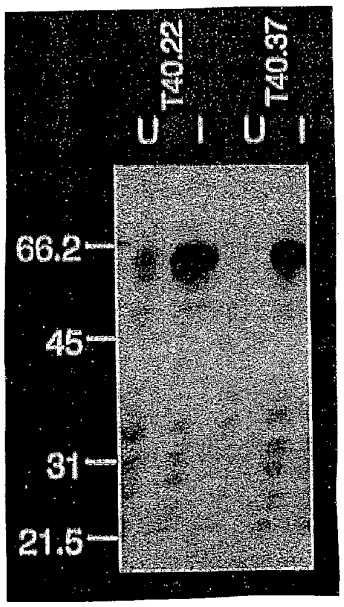


Figure 21

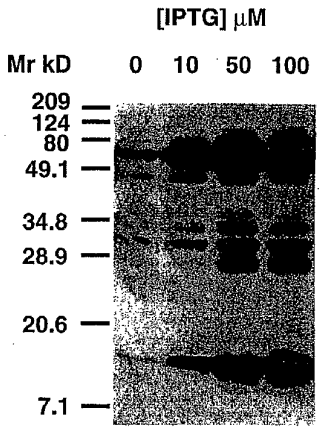


Figure 22

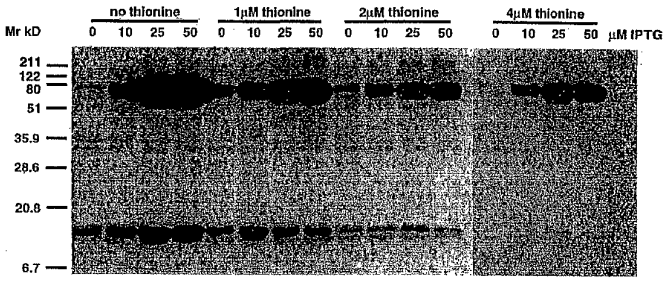

no thionine
1μM thionine
2μM thionine
4μM thionine
Mr kD
0 10 25 50 0 10 25 50 0 10 25 50 0 10 25 50 μM IPTG
211
122
80
51
35.9
28.6
20.8
6.7

Figure 23

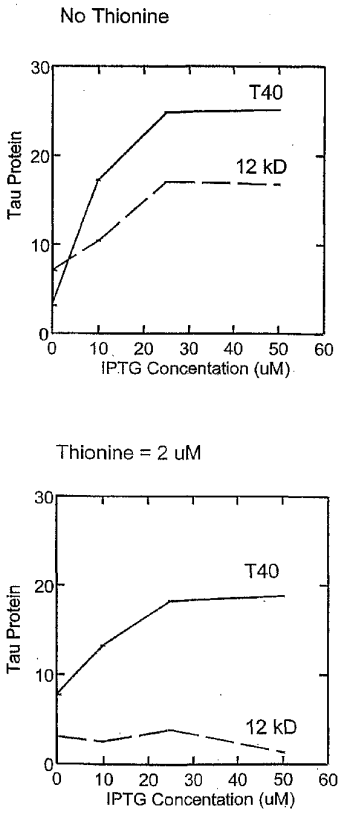

No Thionine
T40
12 kD
Tau Protein
IPTG Concentation (uM)
Thionine = 2 uM
T40
12 kD
Tau Protein
IPTG Concentation (uM)

Figure 24

RANK ORDER OF POTENCY (KI)

REDUCED FORMS

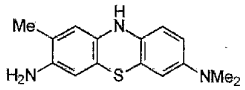
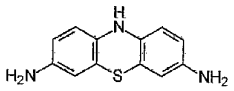
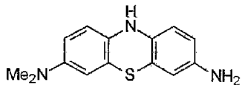
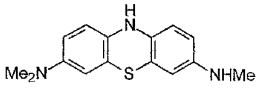
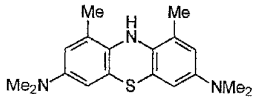
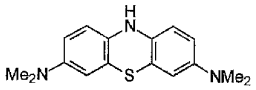
Compound	Structure	KI
Tolonium Chloride		76.05
Thionine		108.34
Azure A		119.01
Azure B		123.91
1,9-Dimethyl-methylene blue		325.41
Methylene Blue		3731.26

Figure 25

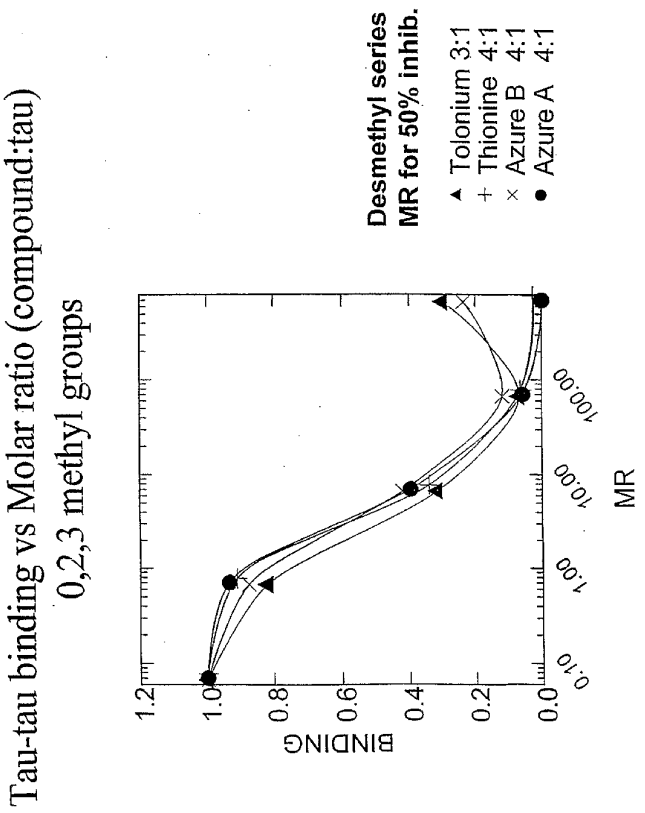

Tau-tau binding vs Molar ratio (compound:tau)
0,2,3 methyl groups
Desmethyl series
MR for 50% inhib.
Tolonium 3:1
Thionine 4:1
Azure B 4:1
Azure A 4:1
BINDING
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.10
1.00
10.00
100.00
MR

Figure 26

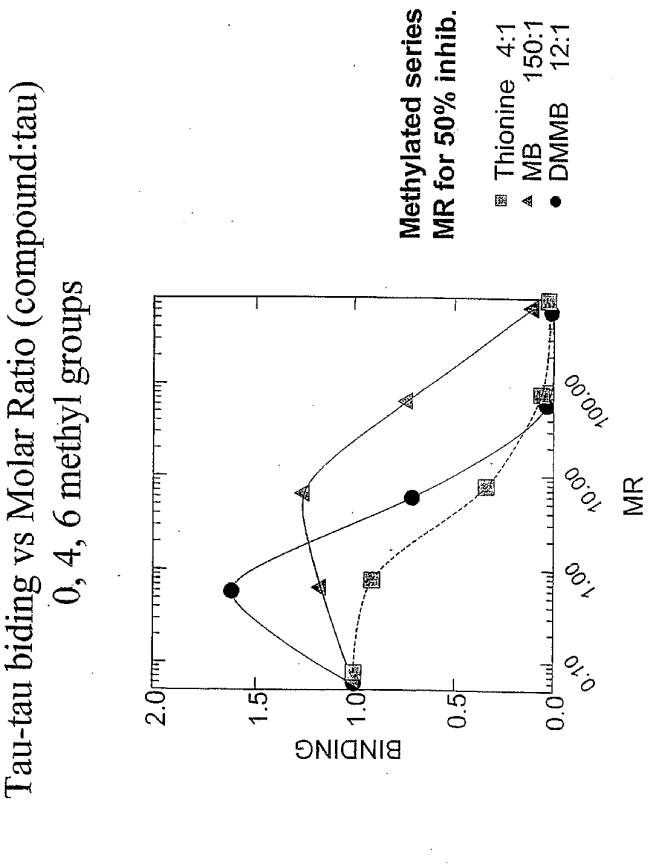

Tau-tau biding vs Molar Ratio (compound:tau)
0, 4, 6 methyl groups
Methylated series
MR for 50% inhib.
Thionine 4:1
MB 150:1
DMMB 12:1
BINDING
2.0
1.5
1.0
0.5
0.0
0.10
1.00
10.00
100.00
MR

Figure 27

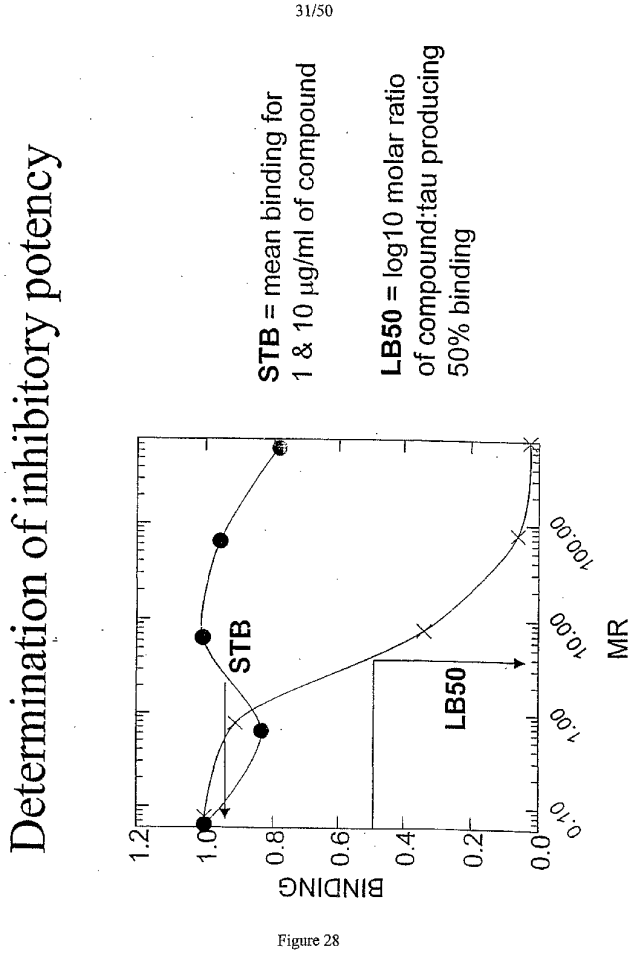

Determination of inhibitory potency
STB = mean binding for 1 & 10 µg/ml of compound
LB50 = log10 molar ratio of compound:tau producing 50% binding
BINDING
1.2
1.0
0.8
0.6
0.4
0.2
0.0
STB
LB50
0.10
1.00
10.00
100.00
MR

Figure 28

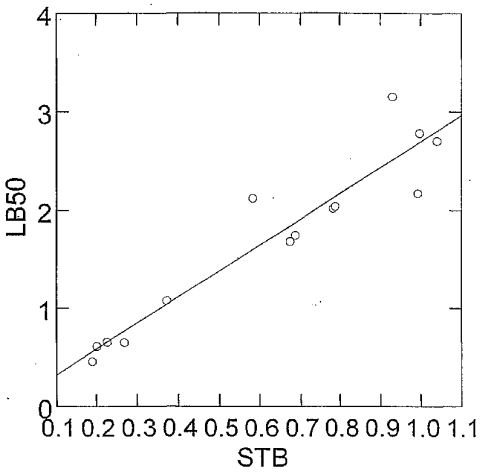


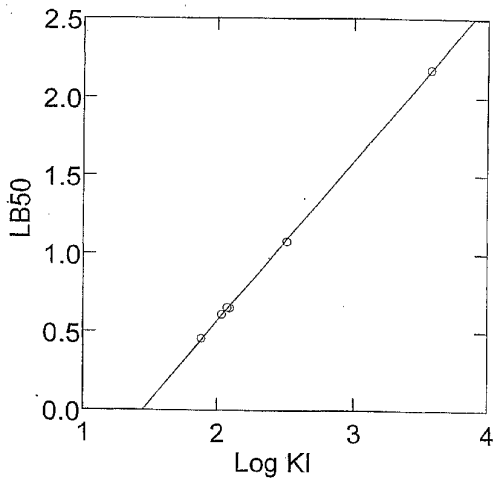
Figure 29

Relationship between STB and B50 values (molar ratio of compound:tau at which tau-tau binding is reduced by 50%)

Compound	STB	B50
Tolonium Chloride	0.190	2.86
Thionine	0.201	4.06
Azure A	0.227	4.49
Azure B	0.269	4.46
Dimethyl MB	0.372	12
Vitamin K	0.674	48
Neutral red	0.787	56
Pyronin Y	0.783	104
Primulin	0.788	109
Acraflavin	0.583	132
Methylene blue (MB)	0.992	150
Phenothiazine	1.040	508
Gallocyanin	0.997	608
Thiazin red	0.929	1419

Figure 30

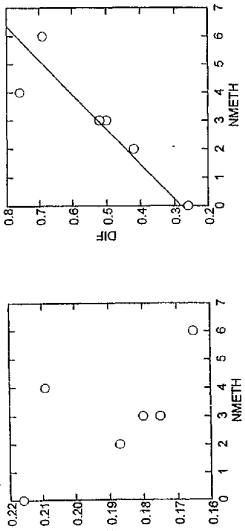
The LB50 value is an alternative representation of the KI value where this can be determined for the diaminophenothiazines



LB50 = (1.019 * Log(KI)) - 1.471

Figure 31

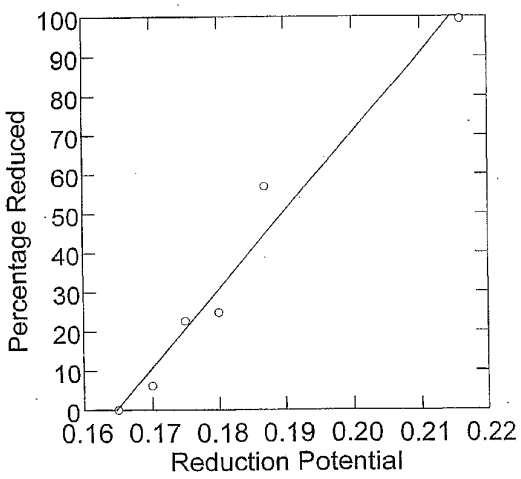
Number of methyl groups vs
Reduction potential and Diffusion coefficient



R=0.928, p=0.004
R=0.975, p=0.002

R=0.601, NS
R=0.975, p=0.002

Figure 32



R = 0.947

Figure 33

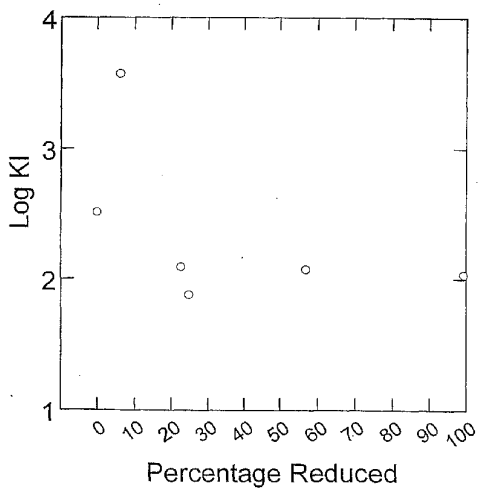
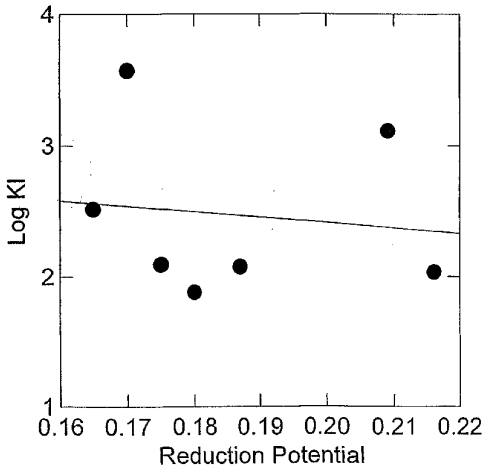
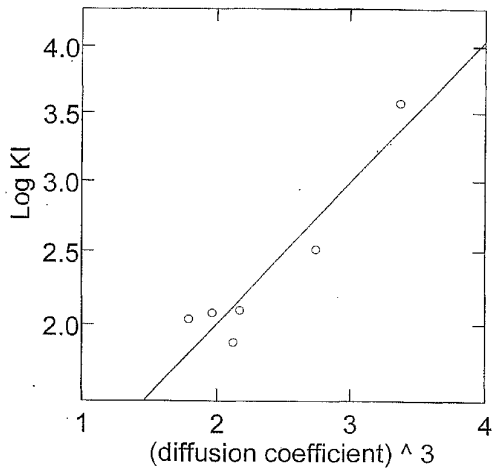


Figure 34a



Inhibitory potency is not determined by Reduction Potential

Figure 34b



Potency appears to be associated with
the aggregation efficiency of the reduced form

Figure 35

Approximation of LB50 values as function of reduction potential and diffusion coefficient

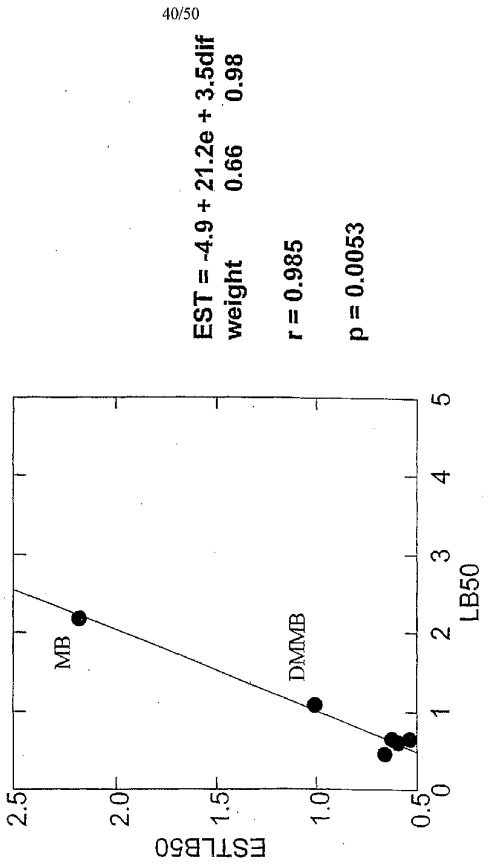


Figure 36

Approximation of STB values as function of reduction potential and diffusion coefficient

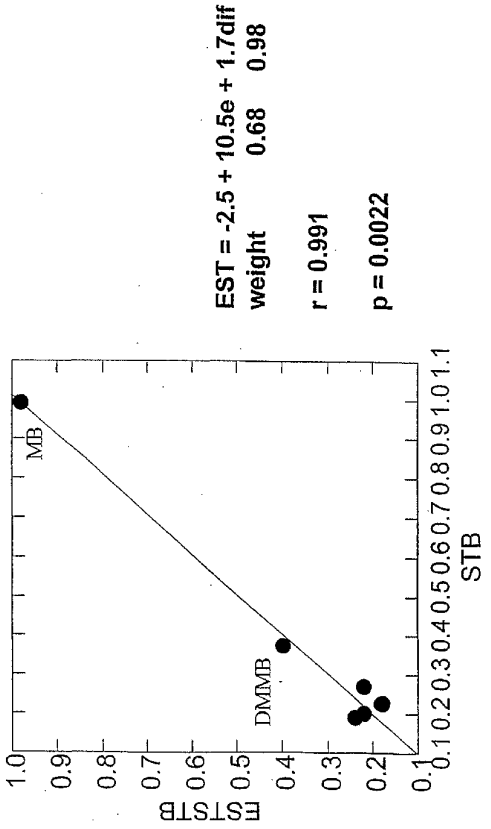


Figure 37

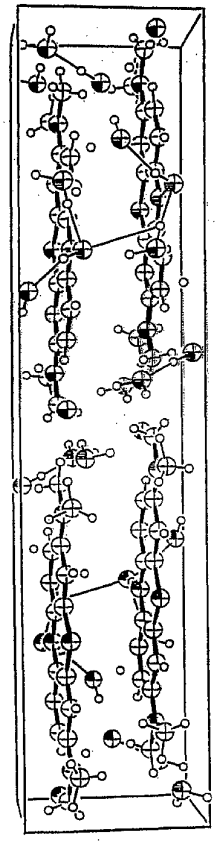


Figure 38

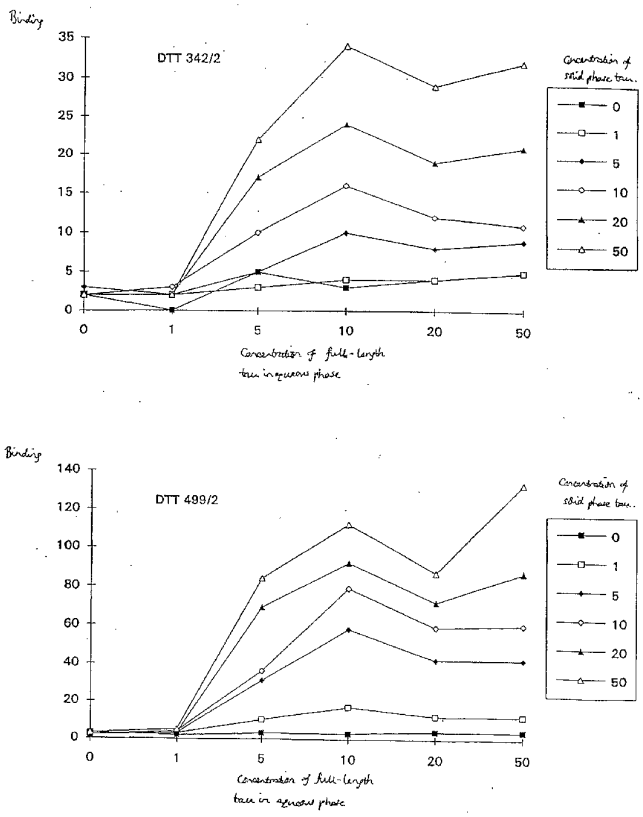

Binding
DTT 342/2
Concentration of solid phase tau
0
1
5
10
20
50
Concentration of full-length tau in aqueous phase
Binding
DTT 499/2
Concentration of solid phase tau
0
1
5
10
20
50
Concentration of full-length tau in aqueous phase

Figure 39

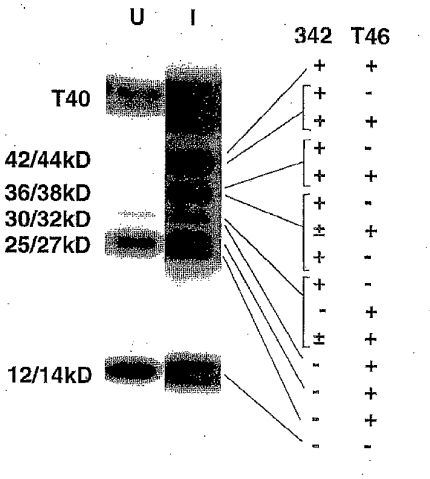


Figure 40

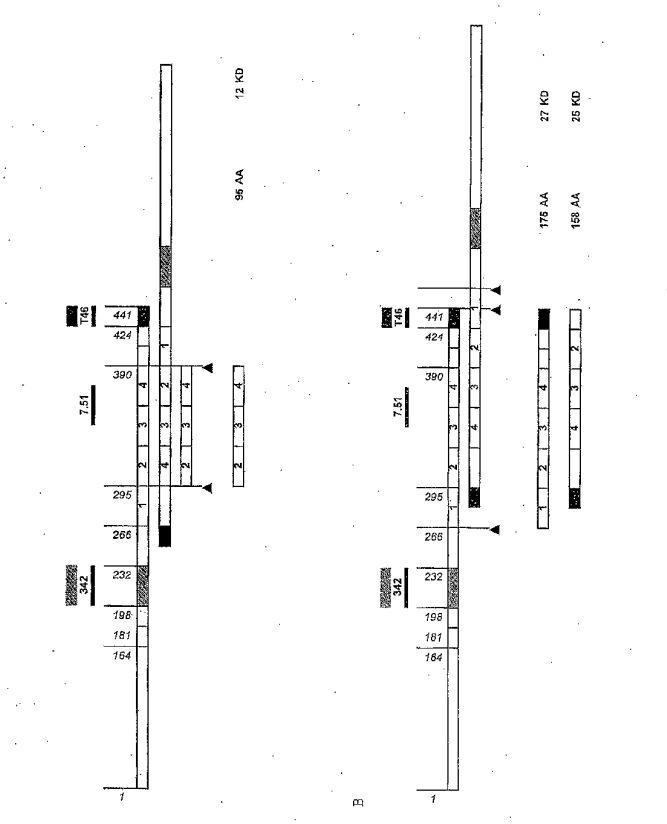

12 KD
96 AA
27 KD
25 KD
176 AA
158 AA
T46
T45
441
424
421
390
7.51
295
266
342
232
198
181
164
1
B

Figure 41

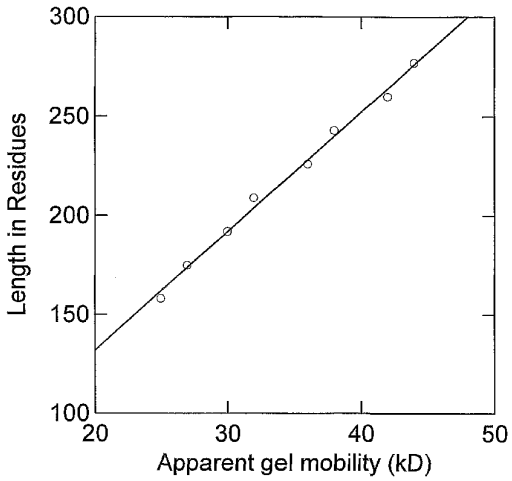

300
250
200
150
100
Length in Residues
20
30
40
50
Apparent gel mobility (kD)

Figure 42

DERIVATION OF OBSERVED FRAGMENTS FROM PROTEOLYTIC PROCESSING OF HEPTAMERIC AGGREGATE

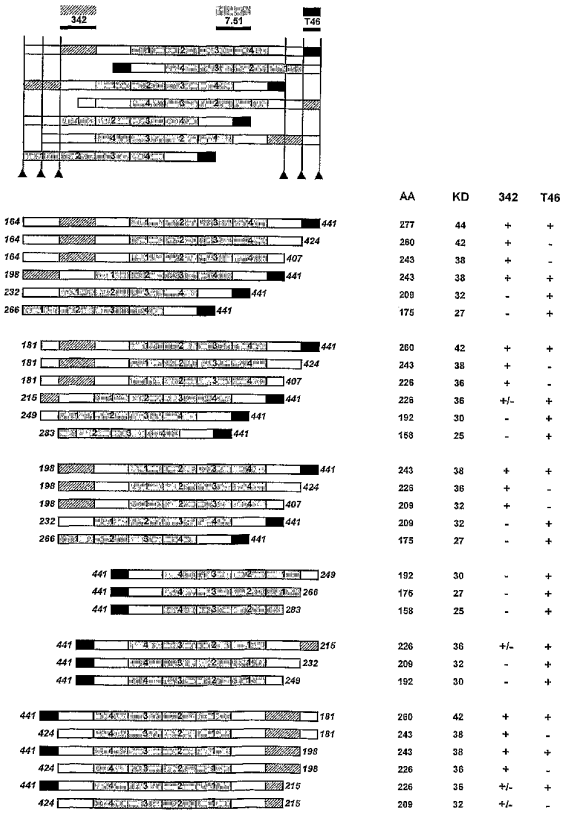
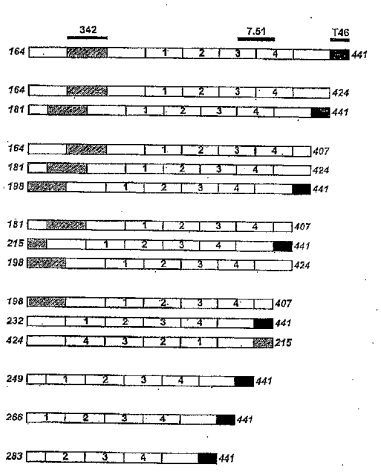


Figure 43

FRAGMENTS DERIVED FROM PROTEOLYTIC PROCESSING OF HEPTAMERIC AGGREGATE



	AA	KD	342	T46
164 1 2 3 4 441	277	44	+	+
164 1 2 3 4 424	260	42	+	-
181 1 2 3 4 441	260	42	+	+
164 1 2 3 4 407	243	38	+	-
181 1 2 3 4 424	243	38	+	-
198 1 2 3 4 441	243	38	+	+
181 1 2 3 4 407	226	36	+	-
215 1 2 3 4 441	226	36	+/-	+
198 1 2 3 4 424	226	36	+	-
198 1 2 3 4 407	209	32	+	-
232 1 2 3 4 441	209	32	-	+
424 4 3 2 1 215	209	32	+/-	-
249 1 2 3 4 441	192	30	-	+
268 1 2 3 4 441	175	27	-	+
283 2 3 4 441	158	25	-	+

Figure 44

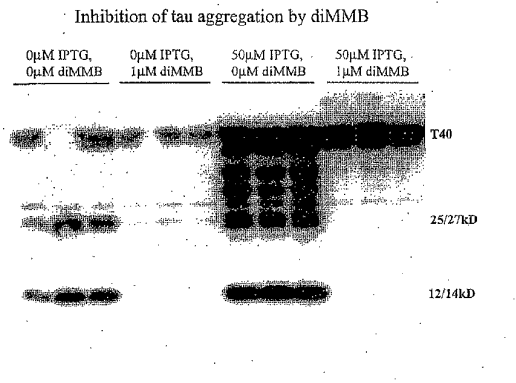
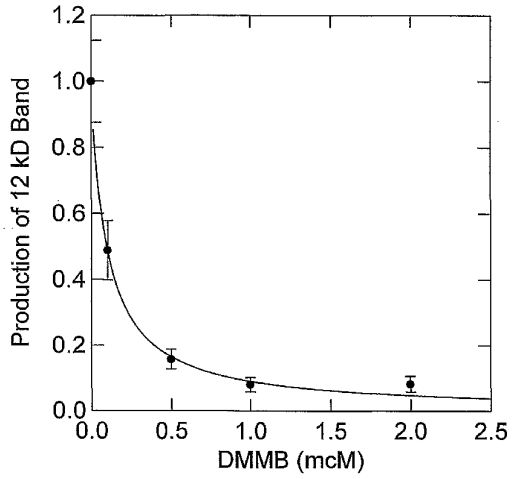

Inhibition of tau aggregation by diMMB
0μM IPTG, 0μM diMMB
0μM IPTG, 1μM diMMB
50μM IPTG, 0μM diMMB
50μM IPTG, 1μM diMMB
T40
25/27kD
12/14kD

Figure 45



Observed vs predicted activity	r = 1.00
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
DMMB KI	4.4 nM
DMMB B50	100 nM

Figure 46

【国際公開パンフレット（コレクトバージョン）】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 July 2002 (18.07.2002)

PCT

(10) International Publication Number
WO 02/055720 A3

(51) International Patent Classification[7]: C12N 15/85, A61P 25/28, G01N 33/68, C07K 14/47, A61K 31/54

(21) International Application Number: PCT/GB02/00153

(22) International Filing Date: 15 January 2002 (15.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0101049.5 15 January 2001 (15.01.2001) GB

(71) Applicant *(for all designated States except US)*: **THE UNIVERSITY COURT OF THE UNIVERSITY OF ABERDEEN** [GB/GB]; Regent Walk, Aberdeen, Grampian AB24 3FX (GB).

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(74) Agents: **KREMER, Simon, M.** et al.; Mewburn Ellis, York House, 23 Kingsway, London, Greater London WC2B 6HP (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
with international search report
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
21 November 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/055720 A3

(54) **Title:** MATERIALS AND METHODS RELATING TO PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASE

(57) **Abstract:** Disclosed are methods of proteolytically converting a precursor protein (e.g. tau) to a product fragment (e.g. a 12 kd fragment) in a stable cell line, wherein the precursor protein is associated with a disease state in which the precursor protein aggregates pathologically (e.g. a tauopathy), and the methods comprise:(a) providing a stable cell line transfected with nucleic acid encoding: (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein, which protein is inducibly expressed in the cell in response to a stimulus, whereby interaction of the template fragment with the precursor protein causes a conformational change in the precursor protein such as to cause aggregation and proteolytic processing of the precursor protein to the product fragment. The method is preferably used to screen for modulators of the aggregation process by monitoring production (or modulation of production) of the product band or bands. Also provided are materials for used in the assays, plus medicaments, and related uses and processes, based on compounds which show high activity in the assay of the invention e.g. reduced diaminophenothiazines.

【国際公開パンフレット（コレクトバージョン）】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 July 2002 (18.07.2002)

PCT

(10) International Publication Number
WO 02/055720 A3

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
21 November 2002

(48) Date of publication of this corrected version:
27 December 2002

(15) Information about Correction:
see PCT Gazette No. 52/2002 of 27 December 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/055720 A3

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MATERIALS AND METHODS RELATING TO PROTEIN AGGREGATION IN NEURODEGENERATIVE DISEASE

Technical field

The present invention concerns cell-based models and other test systems for modelling the aggregation of proteins associated with neurodegenerative disease. It further relates to compounds capable of modulating such aggregation.

Background art

Conditions of dementia such as Alzheimer's disease (AD) are frequently characterised by a progressive accumulation of intracellular and/or extracellular deposits of proteinaceous structures such as β-amyloid plaques and neurofibrillary tangles in the brains of affected patients. The appearance of these lesions largely correlates with pathological neurofibrillary degeneration and brain atrophy, as well as with cognitive impairment (Mukaetova-Ladinska, E.B. *et al.* (2000) Am. J. Pathol. Vol. 157, No. 2, 623-636).

Both neuritic plaques and neurofibrillary tangles contain paired helical filaments (PHFs), of which a major constituent is the microtubule-associated protein tau (Wischik *et al.* (1988) PNAS USA 85, 4506). Plaques also contain extracellular β-amyloid fibrils derived from the abnormal processing of amyloid precursor protein (APP; Kang *et al.* (1987) Nature 325, 733). An article by Wischik *et al.* (in 'Neurobiology of Alzheimer's Disease', 2nd Edition (2000) Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford) discusses in detail the putative role of tau protein in the pathogenesis of neurodegenerative dementias.

Studies of Alzheimer's disease indicate that the loss of the normal form of tau (Mukaetova-Ladinska *et al.* (1993) Am. J. Pathol., 143, 565; Wischik *et al.* (1995a) Neurobiol. Ageing, 16: 409; Lai *et al.*

(1995b) Neurobiol. Ageing, 16: 433), accumulation of pathological PHFs (Mukaetova-Ladinska *et al.* (1993), *loc. cit.*; Harrington *et al.* (1994a) Dementia, 5, 215; Harrington *et al.* (1994b) Am. J. Pathol., 145, 1472; Wischik *et al.*,(1995a), *loc. cit.*) and loss of synapses in the mid-frontal cortex (Terry et al. (1991) Ann. Neurol., 30, 572) correlate with associated cognitive impairment. Furthermore, loss of synapses (Terry *et al.*, *loc. cit.*) and loss of pyramidal cells (Bondareff *et al.* (1993) Arch. Gen. Psychiatry, 50: 350) both correlate with morphometric measures of tau-reactive neurofibrillary pathology, which parallels, at a molecular level, an almost total redistribution of the tau protein pool from a soluble to a polymerised form (PHFs) in Alzheimer's disease (Mukaetova-Ladinska et al. (1993), *loc. cit.*; Lai *et al.* (1995), *loc. cit.*).

Tau exists in alternatively-spliced isoforms, which contain three or four copies of a repeat sequence corresponding to the microtubule-binding domain (Goedert, M., *et al.* (1989) *EMBO J.* 8, 393-399; Goedert, M., *et al.* (1989) *Neuron* 3, 519-526). Tau in PHFs is proteolytically processed to a core domain (Wischik, C.M., *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85, 4884-4888; Wischik *et al.* PNAS USA 1988, 85:4506-4510); Novak, M., *et al.* (1993) *EMBO J.* 12, 365-370) which is composed of a phase-shifted version of the repeat domain; only three repeats are involved in the stable tau-tau interaction (Jakes, R., *et al.* (1991) *EMBO J.* 10, 2725-2729). Once formed, PHF-like tau aggregates act as seeds for the further capture and provide a template for proteolytic processing of full-length tau protein (Wischik *et al.* 1996 Proc Natl Acad Sci USA 93, 11213-11218).

In the course of their formation and accumulation, paired helical filaments (PHFs) first assemble to form amorphous aggregates within the cytoplasm, probably from early tau oligomers which become truncated prior to, or in the course of, PHF assembly (Mena, R., *et al.* (1995) *Acta Neuropathol.* 89, 50-56; Mena, R., *et al.* (1996) *Acta Neuropathol.* 91, 633-641). These filaments then go on to form classical intracellular neurofibrillary tangles. In this state,

the PHFs consist of a core of truncated tau and a fuzzy outer coat containing full-length tau (Wischik., C. M., *et al*, (1996) loc. cit.). The assembly process is exponential, consuming the cellular pool of normal functional tau and inducing new tau synthesis to make up the deficit (Lai, R. Y. K., et al., (1995), *Neurobiology of Ageing*, Vol. 16, No. 3, 433-445). Eventually, functional impairment of the neurone progresses to the point of cell death, leaving behind an extracellular tangle. Cell death is highly correlated with the number of extracellular tangles (Wischik *et al.* 2000, loc.cit). As tangles are extruded into the extracellular space, there is progressive loss of the fuzzy outer coat of the neurone-PHF with corresponding loss of N-terminal tau immunoreactivity, but preservation of tau immunoreactivity associated with the PHF core (Figure 1; also Bondareff, W. *et al.*, (1994) *J. Neuropath. Exper. Neurol.*, Vol. 53, No. 2, 158-164).

The phase shift which is observed in the repeat domain of tau incorporated into PHFs suggests that the repeat domain undergoes an induced conformational change during incorporation into the filament. During the onset of Alzheimer's disease, it is envisaged that this conformational change could be initiated by the binding of tau to a pathological substrate, such as damaged or mutated membrane proteins (see Figure 2 - also Wischik, C.M., *et al.* (1997) in "*Microtubule-associated proteins: modifications in disease*", eds. Avila, J., Brandt, R. and Kosik, K. S. (Harwood Academic Publishers, Amsterdam) pp.185-241).

In the case of Alzheimer's disease, current pharmaceutical therapies are focused on symptomatic treatment of the loss of cholinergic transmission which results from neurodegeneration (Mayeux, R., *et al.* (1999) *New Eng. J. Med.* 341, 1670-1679). However, although the available treatments delay progression of the disease for up to six to twelve months, they do not prevent it. The discovery of drugs that could prevent the aggregation of tau which leads to neurodegeneration would provide a more effective strategy for prophylaxis or for inhibiting the progression of the disease, which would not require an immediate knowledge of the diverse upstream events that initiate the aggregation (see Figure

3).

Models and assays

WO 96/30766 describes an *in vitro* assay for tau aggregation in which a fragment of tau corresponding to the core repeat domain, which has been adsorbed to a solid phase substrate, is able to capture soluble full-length tau and bind tau with high affinity (see Figure 4). This association confers stability against digestion of proteases on the tau molecules on the repeat domains of tau molecules which have aggregated. The process is self-propagating, and can be blocked selectively by prototype pharmaceutical agents ((Wischik *et al.* 1996 Proc Natl Acad Sci USA 93, 11213-11218).

Although the *in vitro* assay described in WO 96/30766 enables the identification of inhibitors or modulators of tau-tau association, the present inventors have also recognized that cell-based models of Alzheimer's disease-like protein aggregation would be useful. Such cellular models could be used both in the primary screening of candidate modulators of tau-tau aggregation, and in the secondary screening of compounds already identified in the *in vitro* assay of WO 96/30766. Furthermore, the demonstration of tau aggregation in cells could also aid in the identification of normal cellular substrates which are involved in the initiation of pathological tau aggregation, which substrates could themselves be targets for pharmaceutical intervention.

However, numerous papers reporting the expression of various tau constructs in tissue culture models have failed to demonstrate aggregation (see e.g. Baum, L. *et al.*, (1995) Mol. Brain Res. 34:1-17). For instance, 3T3 mouse fibroblasts do not possess tau protein and thus present a cellular environment in which recombinant tau can be expressed independent of endogenous mouse tau. Transfection of various cell lines has been reported previously (Kanai *et al.* , 1989; Goedert and Jakes, 1990; Knops *et al*, 1991; Lee and Rook, 1992; Gallo *et al.*, 1992; Lo *et al.*, 1993; Montejo de Garcini *et al.*, 1994; Fasulo *et al.*, 1996). However the

stable long term expression of truncated tau in such cell lines was not achieved. For example, tau constructs for residues 164 or 173 to 338 or 352 did not express protein (Lee and Rook, 1992).

Although Fasulo *et al.* (Alzheimer's Research 1996, 2, 195-200) reported transient expression of truncated tau in COS cells, data for stable long term expression of this tau was not shown. These workers concluded from the use of the transient transfection system that expression of truncated tau by itself was not sufficient to induce tau aggregation in a manner suitable for testing drugs.

Thus far, the aggregation of soluble tau *in vitro* has only been achieved under non-physiological conditions and at high concentrations (reviewed in Wischik (2000), loc. cit).

WO 96/30766 describes two approaches for studying tau aggregation in a cellular environment. In the first approach, full-length tau or fragments of tau were stably expressed in cells. In the second approach, aggregated tau was transiently transfected into cells by use of lipofectin.

Although both of these approaches are useful for the study of tau-tau aggregation, they have some limitations. Transfection of aggregated tau into cells using lipofection is of variable efficiency, as is the production *in vitro* of aggregated tau itself. Moreover, the core tau fragment, which is the most efficient seed for tau aggregation, is found to be toxic when stably expressed in cells, leading to low expression levels. Thus, constitutive expression of the truncated tau fragment of the PHF core in eukaryotic cells is difficult to achieve. Transient expression systems permit the optimization of expression of tau, but the inherent toxicity of the fragments renders even these systems unreliable. Longer fragments of tau are less toxic, but these do not reliably aggregate when expressed in cells.

Thus it would be desirable for an alternative model system to be developed, in which the interaction between e.g. tau molecules and the like could be investigated under physiological conditions, in a

stable and controllable cell line, and which could be used to screen for potential diagnostic, prognostic or therapeutic agents of conditions such as Alzheimer's disease.

Disclosure of the invention

The present inventors have devised a stable cellular test system which can be used to model the template-driven proteolytic processing of a protein, the aggregation of which is associated with neurodegenerative disease. In one test system, exemplified with the tau protein, very low level constitutive expression of a fragment of the tau protein was combined with inducible expression of full-length tau. Induction of the full-length tau lead to its proteolytic conversion to a processed fragment, confirming that "templated proteolytic processing" of the tau was occurring. The system readily permits the demonstration of the effects of tau aggregation inhibitors through their inhibition of production of the processed, 12 kD, fragment from induced full-length tau.

That such a stable system can be achieved notwithstanding the inherent toxic properties of the 12 kD fragment is particularly surprising. For instance, as demonstrated in the Examples below, although partial truncation at either N- or C-termini of full-length tau results in cell lines in which stable expression can be maintained, these longer constructs show only a weak propensity to aggregate, rather than binding to the microtubular network. Stable expression of combinations of tau fragments generates aggregates within the cytoplasm of cells, but this system cannot be maintained reproducibly. Systems based on the inducible expression of the 12 kD fragment lead to toxicity as a result of unpredictable intracellular aggregation of the fragment.

Thus there would appear to be a trade-off in stable expression cell systems between inducing aggregation and hence toxicity on the one hand, which produces cell lines which are either variable or non-viable, and maintaining viable cell lines in which tau has a low propensity to aggregate. Notwithstanding this, the inducible tau expression system of the present invention is both stable, and yet

able to provide controlled aggregation of protein for use in screens and the like.

Additionally, use of the assay has provided evidence that the mechanism of action of certain inhibitors (e.g. phenothiazines) of protein aggregation is primarily steric in nature, rather than essentially redox, as may have been suspected on the basis of the prior art. This discovery has unexpected implications for the choice, assessment, formulation and use of such compounds in the context of the diseases discussed herein. In particular, it shows that assessment of diffusion coefficients can provide a valuable screen for identifying putative inhibitors, or optimising the structure or state of known ones, because the parameters inherently assessed by measuring the diffusion coefficient may be highly relevant to the inhibitors' potency.

The assay further shows that use of phenothiazines in their reduced form can be advantageous for enhancing their inhibitory properties. These observations form the basis of further aspects of the present invention.

In general the present invention provides a method for converting, through proteolytic processing, a precursor protein to a product fragment of the precursor protein, in a stable cell line, which method comprises the steps of: (a) providing a stable cell line transfected with nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein, which protein is inducibly expressed in the cell in response to a stimulus, whereby interaction of the template fragment with the precursor protein causes a conformational change in the precursor protein such as to cause aggregation and proteolytic processing of the precursor protein to the product fragment.

The method may include subjecting the cell to the stimulus such that the precursor protein is expressed in the cell. However in embodiments where an inducible promoter is used which causes low,

but detectable levels of expression even in the absence of the stimulus, then the stimulus step may be omitted.

Generally speaking, the precursor protein will be one which, *in vivo*, is capable of undergoing an induced conformational polymerisation interaction (in a self-propagating manner) leading ultimately to the formation of aggregates comprised of the product fragment, and associated with the disease state. The product fragment obtained in the method provided herein is a measure of the pathological aggregation and proteolysis process which *in vivo* leads to the production of one or more toxic products and the disease state. The product fragment (or one or more of the fragments) of the present method may be toxic, or may simply be used as an indicator of the pathological aggregation process.

The proteins and interactions upon which the method is based are discussed in more detail below.

The present inventors have demonstrated that it is unexpectedly possible to constitutively express the template fragment at a (first) concentration which is not toxic to the cell line i.e. the cell line is viable. Nor does it show cellular abnormalities of the sort shown e.g. in WO 96/30766 at Fig 29.

Nevertheless (e.g. at a time predetermined by addition of the stimulus) it is possible to seed the processing of the precursor protein to a product fragment (which may be the same, broadly equivalent, or quite different to the template fragment) which can thus accumulate to a (second, higher) concentration which is toxic to the cell and which corresponds to the disease state. This in turn provides convenient methods for modeling the disease state associated with the effects of the product fragment, and assessing the effect of modulators on the generation of the product fragment.

In various other, discrete, embodiments the invention provides corresponding methods for any of initiating, seeding, or controlling the proteolytic processing and optionally aggregation of the precursor protein to the product fragment.

In each case the method may involve monitoring (directly or indirectly) the level of proteolytic processing of the precursor protein.

In one embodiment of the present invention fibroblast cells (3T6) express full-length tau ("T40") under the control of an inducible promotor and low constitutive levels of the PHF-core tau fragment (12 kD fragment). When T40 expression is induced in this system, it undergoes aggregation-dependent truncation within the cell, N-terminally at ~a.a.295 and C-terminally at ~a.a.390, thereby producing higher levels of the 12 kD PHF-core domain fragment. Production of the 12 kD fragment can be blocked in a dose-dependent manner by tau-aggregation inhibitors. Indeed the quantitation of inhibitory activity of compounds with respect to proteolytic generation of the 12 kD fragment within cells can be described entirely in terms of the same parameters which describe inhibition of tau-tau binding in vitro. That is, extent of proteolytic generation of the 12 kD fragment within cells is determined entirely by the extent to tau-tau binding through the repeat domain. The availability of the relevant proteases within the cell is non-limiting.

Precursor proteins and diseases (including tauopathies)

As stated above, the invention may be based around the use of any protein which is associated with a disease in which the protein undergoes an induced conformational polymerisation interaction i.e. one in which a conformational change of the protein, or in a fragment thereof, gives rise to templated binding and aggregation of further (precursor) protein molecules in a self-propagating manner.

Once nucleation is initiated, an aggregation cascade may ensue which involves the induced conformational polymerisation of further protein molecules, leading to the formation of toxic product fragments in aggregates which are substantially resistant to further proteolysis. The protein aggregates thus formed are

thought to be a proximal cause of neurodegeneration, clinical dementia, and other pathological symptoms of this group of diseases.

Preferred embodiments of the invention are based on tau protein. Where used herein, the term "tau protein" refers generally to any protein of the tau protein family. Tau proteins are characterised as being one among a larger number of protein families which co-purify with microtubules during repeated cycles of assembly and disassembly (Shelanski *et al.* (1973) Proc. Natl. Acad. Sci. USA, 70., 765-768), and are known as microtubule-associated-proteins (MAPs). Members of the tau family share the common features of having a characteristic N-terminal segment, sequences of approximately 50 amino acids inserted in the N-terminal segment, which are developmentally regulated in the brain, a characteristic tandem repeat region consisting of 3 or 4 tandem repeats of 31-32 amino acids, and a C-terminal tail.

MAP2 is the predominant microtubule-associated protein in the somatodendritic compartment (Matus, A., in "*Microtubules*" [Hyams and Lloyd, eds.] pp 155-166, John Wiley and Sons, NY). MAP2 isoforms are almost identical to tau protein in the tandem repeat region, but differ substantially both in the sequence and extent of the N-terminal domain (Kindler and Garner (1994) Mol. Brain Res. 26, 218-224). Nevertheless, aggregation in the tandem-repeat region is not selective for the tau repeat domain. Thus it will be appreciated that any discussion herein in relation to tau protein or tau-tau aggregation should be taken as relating also to tau-MAP2 aggregation, MAP2-MAP2 aggregation and so on.

Figure 5 shows a Table listing various other disease-associated aggregating proteins which may be used in the present invention. In each case the disease or diseases in which the initiation of aggregation and\or mutation of the protein(s) may play a role is also listed. The domain or mutation responsible for the disease activity is listed, and at least all or part of this minimal portion of the protein would preferably be encompassed by the template fragment used in the present invention.

As can be seen from the table, example diseases which are characterised by pathological protein aggregation include motor neurone disease and Lewy body disease.

Notably it is not only Alzheimer's Disease in which tau protein (and aberrant function or processing thereof) may play a role. The pathogenesis of neurodegenerative disorders such as Pick's disease and Progressive Supranuclear Palsy (PSP) appears to correlate with an accumulation of pathological truncated tau aggregates in the dentate gyrus and stellate pyramidal cells of the neocortex, respectively. Other dementias include fronto-temporal dementia (FTD); parkinsonism linked to chromosome 17 (FTDP-17); disinhibition-dementia-parkinsonism-amyotrophy complex (DDPAC); pallido-ponto-nigral degeneration (PPND); Guam-ALS syndrome; pallido-nigro-luysian degeneration (PNLD); cortico-basal degeneration (CBD) and others (see Wischik *et al.* 2000, loc. cit, for detailed discussion - especially Table 5.1). All of these diseases, which are characterized primarily or partially by abnormal tau aggregation, are referred to herein as "tauopathies".

Thus it will be appreciated, in the light of the above discussion, (and except where context requires otherwise) where the embodiments of the invention are described with respect to tau protein or tau-like proteins (e.g. MAP2) the description should be taken as applying equally to the other proteins discussed above (e.g. β-amyloid, synuclein, prion etc.) or other proteins which may initiate or undergo a similar pathological aggregation by virtue of conformational change in a domain critical for propagation of the aggregation, or which imparts proteolytic stability to the aggregate this formed (article by Wischik *et al.* (in "Neurobiology of Alzheimer's Disease", 2nd Edition (2000) Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford). All such proteins may be referred to herein as "aggregating disease proteins."

Likewise, where mention is made herein of "tau-tau aggregation", or the like, this may also be taken to be applicable to other

"aggregating-protein aggregation", such as β-amyloid aggregation, prion aggregation and synuclein aggregation etc. Likewise "tau proteolytic degradation" and so on.

Template Fragments

In preferred embodiments of the present invention, the template fragment, comprises, consists essentially of, or consists of a "core fragment" of the precursor protein, which term refers to that part of the protein that is able to bind to the precursor protein to initiate or propagate proteolysis and aggregation as described above.

In the case of disease proteins which aggregate, such core fragments are also likely to be those which contribute to the proteolytic stability of the aggregate.

Thus, for example, a "tau core fragment" is a tau fragment comprising a truncated tau protein sequence derived from the tandem repeat region and, which, in the appropriate conditions, is capable of binding to the tandem repeat region of a further tau protein or a MAP2 protein with high affinity. In the case of tau, the preferred fragment is thus exemplified by, but not limited to, the tau fragments present in PHFs (and, ultimately, neurofibrillary tangles) in Alzheimer's disease brains.

A preferred tau fragment may thus be from about (say) between 295-297 extending to about 390-391 (see 'dGAE' in Figure 6) although variants of such fragments may also be used, as discussed below.

In the case of APP (amyloid precursor protein), for instance, expression of a fragment of the APP that encompasses the Aβ domain of 1-40 or 1-42 amino acids as a fusion protein, may be preferred.

Other core fragments may be based e.g. on the domains discussed with reference to Figure 5. Template fragments may include domains from two, or more than two, of these proteins (e.g. as fusions).

The total length of the template fragment may be any which is appropriate to the assay and aggregation disease protein core fragment being used, but will generally be greater than or equal to about 20, 30, 40, 50, 60, 70, 80, 90, or so amino acids in length. However in some embodiments it may be greater than 100, 200 or even 500, if this is desired.

Derivatives

In all instances herein where a named protein (e.g. precursor protein, template or core fragment) or a recited nucleic acid sequence is discussed, a derivative or other variant of the corresponding reference protein (or nucleic acid) may be used as appropriate, provided that it retains appropriate characteristics of the reference sequence. Such derivatives will also share sequence identity with the reference sequence.

For instance the protein used may include an extended N- or C-terminus, which extension may be heterologous to the protein sequence. Equally, the derivative will be one by way of amino acid insertion, deletion, or addition of the reference sequence. For example, a tau protein, or tau core fragment, derivative will comprise at least a partial amino acid sequence resembling the tandem repeat region of the tau proteins, but in which one or more of the amino acids of the natural tau or its fragments have been replaced or deleted, or into which other amino acids have been inserted.

Such changes may be made to enhance or ablate binding activity (the latter case being useful for control experiments). Controls may contain deletions of sequences or domains to see what effect on aggregation these may have.

Preferred derivatives may be those which incorporate mutations corresponding to those known or suspected to be associated with the disease state. These may include changes corresponding to P301S within the tau sequence (see Figure 7). Other mutations include G272V, G389R, P301L, N279K, S305N, V337M, G272V, K280Δ, R406W (see

also Wischik et al, 2000, supra).

Other preferred derivatives may include tandem repeats of the core-fragments discussed above, or binding domains within those fragments.

Yet further derivatives may be based on chimeric products based on multiple, related, disease proteins in which their sequences are mixed or combined. For example restriction enzyme fragments of tau could be ligated together with fragments of MAP2 or even of an unrelated gene to generate recombinant derivatives. An alternative strategy for modifying the core fragments would employ PCR as described by Ho et al., 1989, Gene 77, 51-59 or DNA shuffling (Crameri et al., 1998 Nature *391*).

Use of nucleic acid constructs

Nucleic acids of, or for use in, the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities. Nucleic acids e.g. encoding the template fragment, will be at least partially synthetic in that it will comprise nucleic acid sequences which are not found together in nature (do not run contiguously) but which have been ligated or otherwise combined artificially.

Nucleic acid according to the present invention may be in the form of, or derived from, cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs. Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

As described above, the nucleic acids may encode derivatives or other variants sharing homology with the reference sequences in question. Preferably, the nucleic acid and/or amino acid sequence in question would share about 50%, or 60%, or 70%, or 80% identity,

most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% of the sequence upon which the variant is based. Similarity or homology may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711) using the default parameters. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is: T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex.

Nucleic acid sequences which encode the appropriate proteins or polypeptides can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989, and Ausubel *et al.*, Short Protocols in Molecular Biology, John Wiley and Sons, 1992). These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of the relevant nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparation of cDNA sequences.

DNA encoding e.g. tau core fragments may be generated and used in any suitable way known to those of skilled in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. Modifications to the protein (e.g. tau)-encoding sequences can be made, e.g. using site directed mutagenesis.

Constructs

Thus the invention also relates, in a further aspect, to nucleic acid molecules encoding the appropriate precursor and template fragment proteins. As discussed below, these may be present on the

same or different constructs, and in the latter case, compositions comprising two or more types of construct are also provided.

Nucleic acid sequences which enable a vector to replicate in one or more selected host cells are well known for a variety of bacteria, yeast, and viruses. For Example, various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Expression vectors comprising a nucleic acid as described herein may, for example, be in the form of a plasmid, cosmid, viral particle, phage, or any other suitable vector or construct which can be taken up by a cell and expressed appropriately.

Expression vectors will contain a promoter which is operably linked to the protein-encoding nucleic acid sequence of interest, so as to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional control" of the promoter. Transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems. Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA.

The promoter used for the template fragment will be "constitutive". This promoter may be sufficiently weak that the level of template fragment expressed in the cell is not itself (directly) detectable using conventional techniques, other than (indirectly) by its

affect on precursor protein, leading to aggregation and proteolytic processing thereof (i.e. effectively undetectable when said aggregation is inhibited). Such promoters may be selected by those skilled in the art in the light of the present disclosure without undue burden such as those listed above.

In the case of the precursor protein, the promoter is "inducible" – which is to say, and as is well understood by those skilled in the art, expression is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. In experiments below, a Lac inducible promoter has been used.

Expression vectors of the invention may also contain one or more selection genes. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*. An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the desired protein-encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell, when wild-type DHFR is employed, is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al.*, *Proc. Natl. Acad Sci USA* 77:4216 (1980). A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid Rp7 [Stinchcomb *et al.*, Nature, 282:39 (1979); Kingsman *et al.*, Gene, 7:141 (1979); Tschemper *et al.*, Gene, 10:157 (1980)]. The trp1 gene provides a selection marker for a mutant strain of yeast which lacks the ability to grow in tryptophan, for example, ATCC: No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

Thus a typical vector for use in the present invention may include an origin of replication, one or more protein sequence(s) operably linked to a constitutive or inducible promoter as appropriate, a transcription termination sequence, an enhancer element, a marker gene. Construction of suitable vectors containing various of these components employs standard ligation techniques which are known to the skilled artisan.

Transformation

Also provided by the present invention is a process for producing a stable cell for use in a method as described above, which process comprises the steps of: (a) introducing into a cell nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly expressed in the cell in response to a stimulus.

The introduction, which may be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. The calcium treatment employing calcium chloride, as described in Sambrook *et al., supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens is* used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out

according to the method of Van Solingen *et al.*, J. Bact., 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185:527 537 (1990) and Mansour et al., Nature 336:348-352 (1988).

Host cells

Suitable host cells for use in the invention may include bacteria, eukaryotic cells such as mammalian and yeast cells, and baculovirus systems.

Mammalian cell lines available in the art for expression of a heterologous polypeptide include fibroblast 3T6 cells, HeLa cells, baby hamster kidney cells, COS cells, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651), Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumour cells (MMT 060562, ATCC CCL51); and many others.

Suitable prokaryotic hosts include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *E. coli*. Various E. *coli* strains are publicly available, such as E. *coli* K12 strain MM294 (ATCC 31,446); E. *coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635). Eukaryotic microbes such as filamentous fungi or yeast are also suitable cloning or expression hosts for vectors. *Saccharomyces cerevisiae is* a commonly used lower eukaryotic host microorganism. The selection of the appropriate host cell is deemed to be within the skill in the art.

In a further aspect, the present invention provides a host cell containing heterologous nucleic acid of the invention as described above. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. Alternatively, the nucleic acid may be on an extrachromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

The cell may be produced by a method described above (introduction of nucleic acid construct) or be the ancestor of such a cell. Corresponding cell-lines are also provided. Preferred cell-lines may be based on the fibroblast cell line, e.g. 3T6.

Host cells transfected or transformed with expression or cloning vectors described herein may be cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in "Mammalian Cell Biotechnology: a Practical Approach", M. Butler, ed. JRL Press, (1991) and Sambrook *et al*, *supra*.

Gene expression can be confirmed in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl Acad Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequence of the aggregating disease protein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes.

Gene expression, alternatively, may be measured by immunological methods such as immunohistochemical staining of cells or tissue

sections, and assay of cell culture, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence of the aggregating disease polypeptide.

Thus one aspect of the present invention entails causing or allowing expression from the nucleic acids discussed herein, e.g. by culturing host cells under conditions for expression of the gene (presence of stimulus) so that the product fragment is produced. The present invention also encompasses a method of producing the product fragment, the method including expression from nucleic acid as described above.

Another aspect of the present invention is a kit comprising a transformed cell or cell line as described herein, plus at least one further component e.g. an agent for stimulating production of the precursor protein, or an agent for detecting the interaction of the precursor protein with the template fragment, as described in the following section.

Detection of aggregation and\or proteolytic processing and\or toxic fragment

In various embodiments, the progress of proteolytic processing or aggregation (or modulation thereof - see below) may be detected directly or indirectly by monitoring the concentration or level any one or more of the following species: the precursor protein; the product fragment; any by-product fragments formed during the process; an aggregate of any of these (e.g. based on sedimentation coefficients).

Thus, as exemplified with particular tau proteins and fragments (based on 297-351 fragment and T40), aggregation can be monitored on the basis of increasing levels of a 12kDa processed species, derived primarily from the precursor protein.

Some protein detection methods are discussed in relation to gene expression above. Where antibodies or fragments thereof are used in embodiments of the method of the present invention may be produced by conventional techniques. Polyclonal antibodies may raised e.g. by injecting the corresponding tau antigen into an animal, preferably a rabbit, and recovering the antiserum by immunoaffinity purification, in which the polyclonal antibody is passed over a column to which the antigen is bound and is then eluted in a conventional manner. Preferably the invention will use monoclonal antibodies which are selective to tau epitopes may be prepared by the method of Kohler and Milstein. Suitable monoclonal antibodies to tau epitopes can be modified by known methods to provide Fab fragments or (Fab')2 fragments, chimeric, humanised or single chain antibody embodiments.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Generally speaking, where antibodies are employed for detection, the antibody may carry a reporter molecule. Alternatively, detection of binding may be performed by use of a second antibody capable of binding to a first unlabelled, tau-specific antibody. In this case, the second antibody is linked to a reporter molecule.

Antibodies may be used in any immunoassay system known in the art, including, but not limited to: radioimmunoassays, "sandwich" assays, enzyme-linked immunosorbent assays (ELISA); fluorescent immuno-assays, protein A immunoassays, etc. Typically, an immunoblot method is used. Preferably the immunoassay is performed in the solid phase, as would be well known to the skilled person. For instance, an antibody may be adsorbed to e.g. an assay column, and the cellular sample may then be washed through the column under

conditions suitable for enabling binding to the solid-phase antibody of any aggregate of the protein of interest, e.g. a tau-tau aggregate. Excess reagent is washed away, and the binding of aggregated protein to the column can then be detected by any suitable means, e.g. as exemplified above and below.

Preferred monoclonal antibodies are as follows:

- Those which recognise the N-terminal or C-terminal of the tau epitope permit measuring of binding between truncated and full-length tau species. Especially useful are antibodies recognising human-specific epitopes. One such monoclonal antibody (designated 27/499) recognises a human-specific epitope located in the region between Gly-16 and Gln-26 of tau, and thereby permits measurement of binding between full-length tau species, provided one is derived from a non-human source (Lai (1995); "The role of abnormal phosphorylation of tau protein in the development of neurofibrillary pathology in Alzheimer's disease", PhD Thesis, University of Cambridge).

- Those which recognise the core tau fragment truncated at Glu-391. An example is mAb 423 (Novak et *al.* (1993), loc. cit.). This antibody enables detection of the binding of a truncated core tau fragment terminating at Glu-391 to a similar fragment terminating at Ala-390, which is not recognised by mAb 423. This truncation occurs naturally in the course of PHF assembly in Alzheimer's disease (Mena et *al.* (1995), (1996), *loc.* cit.; Novak et *al.* (1993), *loc.* cit.; Mena et *al.* (1991), *loc. cit.*). Additionally, when tau is bound via the repeat domain *in vitro*, digestion with a protease (e.g. pronase) generates a fragment detectable by mAb 423 (see Wischik et al, 1996, *loc cit*). In the preferred aspects of the present invention, as it relates to tau protein, this antibody may be used to distinguish the generation of proteolytically cleaved product fragment (Glu-391 termination) from constitutive expression of template fragment (Ala-390).

- Those which recognise a generic tau epitope in the repeat domain. A preferred embodiment utilises an antibody (e.g. MAb 7.51). Where

tau-MAP2 or MAP2-MAP2 aggregation is to be detected, an antibody which detects a generic MAP2 epitope could be used. Antibody 7.51 recognises a generic tau epitope located in the antepenultimate repeat of tau (Novak et al. (1991) Proc. Natl. Acad. Sci. USA, 88, 5837-5841), which is occluded when tau is bound in a PHF-like immunochemical configuration but can be exposed after formic acid treatment (Harrington et al. (1990), (1991), *loc. cit.*; Wischik et *al.* (1995a), *loc. cit.*). Normal soluble tau, or tau bound to microtubules, can be detected using mAb 7.51 without formic acid treatment (Harrington *et al.* (1991), *loc.* cit.; Wischik et *al.* (1995a), *loc. cit.*). Binding of full-length tau in the tau-tau binding assay is associated with partial occlusion of the mAb 7.51 epitope.

Antibody 27/342 recognises a non-species specific generic tau epitope located between Ser-208 and Ser-238 which is partially occluded in the course of the tau-tau interaction (Lai, *loc. cit.*).

The binding sites of some monoclonal antibodies are shown in Figure 6.

Screening for modulators and inhibitors

As described above, the invention is preferably concerned with use of a system as provided herein, in a method of modeling, and identifying therapeutic agents for treatment of, the diseases discussed herein.

A typical method for assessing the ability of an agent to modulate the aggregation and\or proteolytic processing of a precursor protein to a product in response to interaction with a template fragment, may comprise:
(a) providing a stable cell or cell line as discussed above,
(b) subjecting the cell to the stimulus such that the precursor protein is expressed in the cell and whereby interaction of the template fragment with the precursor protein causes a conformational change in the protein such as to cause aggregation and proteolytic processing of the precursor protein to a product

fragment,
(c) monitoring the production of the product fragment in the presence of the agent,
(d) optionally comparing the value obtained in step (c) with a reference value.

The reference value may be based on historical observation, or may be based on control experiments carried out in parallel e.g. in which one integer of the assay (template fragment, precursor protein, stimulus, agent) is modified or absent.

The various methods described above may comprise the further step of correlating the result of step (d) with the modulatory activity of the agent(s).

Thus a method of identifying a modulator of aggregation of a protein associated with a disease in which the protein undergoes an induced conformational interaction, may comprise performing a method for inducing aggregation as described above in the presence of one or more agents suspected of being capable of modulating (e.g. inhibiting or reversing) the aggregation. The degree of aggregation (and optionally proteolytic processing) may be observed in the presence or absence of the agent, and the relative values correlated with its activity as a modulator.

For example, a test substance may be added to a cellular system as described above, and the cells incubated for a period of time sufficient to allow binding and to demonstrate inhibition of binding. The bound tau complex can then be detected, e.g. using a suitably-labeled antibody such as MAb 7.51 in an immunoblot of total cell extract, or any other suitable detection method.

Where a screening method is employed for this purpose, i.e. for the identification of modulatory/inhibitory compounds, a non-competitive or competitive assay may be used. For instance, in a competitive assay of the type well known in the art, the effect of a known inhibitor or modulator can be compared in the presence or absence of further test substances or agents, to determine the

ability of the test substance to compete with the known inhibitor/modulator for binding to the protein of interest.

Also provided are methods of producing modulators (e.g. inhibitors) which are as described above, but which further comprise the step of producing the modulator this identified.

Specificity of inhibition

Screening methods according to this aspect of the present invention may be used to screen for compounds which demonstrate the properties of selective competitive inhibition of disease-related protein aggregation (e.g. tau-tau, tau-MAP2, or other protein, binding), without interference with any 'normal' binding in which the precursor protein participates (e.g. tau or MAP2 to tubulin, or by analogy, other precursor proteins with their binding partners insofar as these are known).

Specifically in the case of tau, a method for determining any possible interference of the binding of tau, MAP2 or a derivative thereof to tubulin by potential inhibitors/modulators, comprises contacting a preparation of depolymerised tubulin or taxol-stabilised microtubules with the agent, followed by detection of the tau-tubulin or MAP2-tubulin binding. Tau-tubulin binding could also, for example, be demonstrated by a normal cytoskeletal distribution, as described in e.g. WO 96/30766. Methods for the preparation of tubulin proteins or fragments thereof, possibly in combination with binding partners, are known in the art and are described e.g. by Slobada *et al.* (1976, in: Cell Mobility (R. Goldman, T. Pollard and J. Rosenbaum, eds.), Cold Spring Laboratory, Cold Spring Harbor, New York, pp 1171-1212).

Analogous methods for other proteins having 'disease' and 'normal' functions will occur to those skilled in the art in the light of the present disclosure.

Cell viability

Where desired, methods of the present invention may further include the step of testing the viability of the cells expressing the template protein and optionally precursor protein e.g. by use of a lactate dehydrogenase assay kit (Sigma).

In the case where tau-tau, tau-MAP2 or MAP2-MAP2 aggregation is being investigated (see above, under 'specificity'), this step may also provide an indication of any interference by the test agent of the binding of tau or MAP2 to tubulin, since inhibition or interference of tau-tubulin or MAP2-tubulin binding will correlate to some extent with a decreased ability of the cells to divide, and thus with decreased cell viability.

Cell viability may be used to derive an LD50 value for the agent.

Preferred inhibitors will have a therapeutic index (LD50/B50 - see discussion of Figure 9) of at least 2, 5, 10, or 20.

Choice of test agent

Compounds which are tested may be any which it is desired to assess for the relevant activity.

The methods can serve either as primary screens, in order to identify new inhibitors/modulators, or as secondary screens in order to study known inhibitors/modulators in further detail.

Agents may be natural or synthetic chemical compounds. Antibodies which recognise an Alzheimer's disease-like protein aggregate and/or which modulate Alzheimer's disease-like protein aggregation form one class of putative inhibitory or modulatory compounds with respect to the aggregation process. More usually, relatively small chemical compounds, preferably which are capable of crossing the blood-brain barrier, will be tested. Other qualities which it may be desirable to establish in conjunction with (before, simulataneously with, or after) use of the present invention, include: non-toxic to bone marrow, minimal deleterious cardiovascular activity; minimal liver and renal toxicity; good

oral absorption; non-metabolised to inactive form, and so on. As those skilled in the art are aware, these tests can be performed on a commercial basis by well established methods for compounds which it is desired to test in this way.

For a typical test substance and putative modulator, where possible, the solubility will first be determined e.g. from The Merck Index. Where the substance is soluble in aqueous solution, a concentrated stock solution may be prepared e.g. at 5-20mM in PBS. Immediately prior to use this can be diluted with tissue culture medium to give a working stock solution e.g. at 100μM and introduced to cells to give a final concentration of between 0-10μM for most compounds. Naturally, if it is desired to test compounds at a concentration greater than 10μM, the concentration of the working stock solution may be increased appropriately.

Where the substance is not soluble in aqueous solution, stock solutions may be made in an appropriate solvent (determined from The Merck Index or experimentally) e.g. ethanol at 5-29 nM. This can again be diluted with tissue culture medium immediately prior to use to give a working solution e.g. at 100μM concentration, and added to cells to yield a final concentration of e.g. 0-10μM for most test compounds. As above, if compounds are to be tested at a concentration greater than 10μM the concentration of the working solution will be increased as appropriate.

The skilled person will appreciate that the amount of test substance or compound which is added in a screening assay according to this aspect of the invention, and indeed the manner in which it is introduced, can be determined by those skilled in the art, if necessary by use of a series of trials. Where the administered compound and the cell line have conflicting optimal conditions (e.g. in terms of pH, or ionic strength etc.) a variety of conditions should be tried to find an optimal, compromise, level. Initial concentrations may be selected to be a level which could realistically be used in therapeutic context i.e. would be non-lethal to a patient (see comments on dosages below). In the light of the present disclosure, such an approach will not present any

undue burden to one skilled in the art.

Screening phenothiazines

The present invention extends, in further aspects, to compounds identified by a screening method as provided herein, and to compositions comprising such inhibitors/modulators of induced conformational polymerisation of a protein.

As described in e.g. WO 96/30766, amongst the agents found to be able to inhibit pathological induced conformational polymerisation of proteins such as tau are certain diaminophenothiazines. Examples include such as thionine, methylene blue (MB), tolonium chloride, and dimethyl-methylene blue (DMMB) which are of particular interest as potential therapeutic agents for use in the prevention of tau-tau aggregation in diseases such as Alzheimer's Disease.

Interestingly, as described in more detail in the Examples, the present inventors have used the methods described herein to demonstrate that the mechanism of action of compounds such as MB on induced conformational polymerisation such as tau-tau aggregation is primarily steric in nature. Additionally, it has been shown that the potent steric inhibitory effect, e.g. of the diaminophenothiazines on tau-tau binding, is dependent on the diffusion coefficient of the compound. The various implications of these observations in terms of screening and formulating compounds are discussed in more detail below.

This finding is particularly unexpected when considering the description of the use of the such compounds in the prior art. Thus, for example, such compounds were previously known to be useful in the treatment of methaemoglobinaemia, where their action has been shown to be mediated by the catalytic reduction of oxidised haemoglobin by transfer of electrons from the cell's intrinsic supply of reduced pyridine nucleotides (see, e.g. Hauschild, F. (1936) *Arch. Exp. Pathol. Pharmacol.* 182:118; "*Pharmacological Basis of Therapeutics*", First Edition (1941), Goodman and Gilman; Hrgovic, Z. (1990) *Anästh. Intensivther.*

Notfallmed. 25: 172; and Cudd, L. *et al.* (1996) *Vet Human Toxicol.* 38(5): 329) and in the prophylaxis of manic depressive psychosis (Narsapur, S.L. (1983) *Journal of Affective Disorders* 5:155; Naylor, G.J. (1986) *Biol. Psychiatry* 21:915). Notwithstanding this, MB, thionine and tolonium chloride are actually intrinsically weak oxidising agents and, in the absence of a supply of reduced pyridine nucleotides, they oxidise proteins such as haemoglobin (Morse, E. (1988) *Annals of Clin. Lab. Sci.* 18(1):13). This toxic effect can be used to inactivate viruses, and MB has consequently been exploited therapeutically in a process for removing HIV and hepatitis virus from blood products (Chapman, J. (1994), *Transfusion Today* 20:2; Wagner, S. J. (1995) *Transfusion* 35(5):407). The mechanism of action of this effect is thought to involve intercalation of MB into DNA. The compound is boosted to a higher redox state by photoactivation and, when it drops back down to its ground state, produces singlet oxygen which oxidises the DNA and inactivates it (Ben-Hur, E. *et al.* (1996) *Transfusion Medicine Reviews*, Vol. X, No. 1: 15; Margolis-Nunno, H. et al. (1994), *Transfusion* 34(9): 802). Exploitation of the toxic effect of photoactivated diaminophenothiazines has also been suggested for the treatment of cancer. Within cells, compounds which have been photoactivated to the oxidised form can damage mitochondria (Darzynkiewicz, Z. et al. (1988), *Cancer Research* 48: 1295) and/or microtubules (Stockert, J. et al. (1996) *Cancer Chemother. Pharmacol.* 39: 167).

Thus, on reviewing the prior art, it is apparent that two possible mechanisms have been proposed to account for the action of compounds such as MB and thionine on entities such as DNA or proteins. The first is the catalytic reduction of e.g. oxidised proteins by means of transfer of electrons from reduced pyridine nucleotides in the cell. The second proposed mechanism is the oxidation, and consequent inactivation of e.g. DNA by a photoactivated, oxidised form of compounds such as MB. In the light of these two mechanisms, it could therefore reasonably have been assumed that the inhibitory effect on tau-tau association of compounds such as MB was also attributable to a redox activity.

That is, it might be assumed that such compounds inhibit induced conformational polymerisation such as tau-tau association by acting as weak oxidising agents or as catalytic reducing agents.

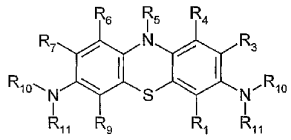
Thus the work of the present inventors, in demonstrating that the mechanism of action is primarily steric in nature, has unexpected implications for the choice, assessment, formulation and use of such compounds in the context of the diseases discussed herein.

In particular, certain compounds have been identified as feasible therapeutics which would have been dismissed based on the result of prior art assays. Specifically, Wischik *et al.* 1996 (*loc cit*) reported on page 1217 that the concentration of MB required for inhibition was higher than could be achieved clinically. However the results herein show that the reduction of MB modifies its stacking ability in such a way as to enhance its inhibitory potential to a level at which it becomes clinically relevant for the treatment of e.g. tau aggregation associated disease. This is discussed in more detail below in relation to the embodiments of the invention concerned with measurement of diffusion coefficients (which are also determined, in part, by the compound's ability to 'stack').

Figure 8 shows the structure of only some of the compounds which have been tested in the cell based assay. Figures 9-16 demonstrate the increased potency of certain compounds in the reduced form, plus some control compounds.

Thus in one aspect of the present invention there is disclosed use, in the treatment of a disease disclosed herein, of a reduced ('leuco') phenothiazine of the formula:

(I)



wherein R_1, R_3, R_4, R_6, R_7 and R_9 are independently selected from hydrogen, halogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;
R_5 is selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy; and each R_{10} and R_{11} are independently selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;
or a pharmaceutically acceptable salt thereof.

Preferably, R_1, R_3, R_4, R_6, R_7 and R_9 are independently selected from -hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$;
each R_{10} and R_{11} are independently selected from hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$; and
R_5 is hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$.

Preferably, the compound is a diaminophenothiazine which has 0, 2, 3 or 4 methyl groups around the diaminophenothiazine nucleus. Preferably, the diaminophenothiazine is asymmetrically methylated (e.g., tolonium chloride, azure A, azure B and thionine).

Preferably the compound is selected from Methylene Blue, Tolonium chloride, Thionine, Azure A, Azure B or 1,9-Dimethylmethylene Blue.

Phenothiazines for use in the present invention may be manufactured by the processes referred to in standard texts (e.g. *Merck Manual*, Houben-Weyl, Beilstein, E. III/IV 27, 1214 ff, *J. Heterocycl. Chem.* 21, 613 (1984)).

Instead of administering these compounds directly, they could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be

treated. For instance, methylene blue may be administered in a precursor form, or it may itself serve as a precursor of the compound Azure A.

Stabilisation of reduced form

Some of these compounds of interest are known to circulate in the body predominantly in the reduced form. For example, for a discussion of the pharmacokinetics of MB, see e.g. DiSanto, A. *et al.* (1972) *Journal Pharm. Sci.* 61(7):1086 and DiSanto, A. *et al.* (1972) *Journal Pharm. Sci* 61(7):1090. Thirdly, only the reduced form of compounds such as MB is found to cross the blood-brain barrier (Chapman, D.M. (1982) *Tissue and Cell* 14(3):475; Müller, T. (1992) *Acta Anat.* 144:39; Müller, T. (1994) *J. Anat.* 184:419; Becker, H. *et al.* (1952) *Zeitschrift für Naturforschung* 7:493; Müller, T. (1995) *It. J. Anat. Embryol.* 100(3):179; Müller, T. (1998) *Histol. Histopathol.* 13:1019).

Such references as these illustrate that the reduced form of compounds such as MB represents a feasible and pharmaceutically-acceptable formulation for administration to subjects. MB has previously been used clinically in an oral preparation. Further toxicological tests are, however, required before its clinical acceptability is achieved. The half live of MB and related compounds (e.g. tolonium chloride) in blood is approximately 100 minutes. It is evident that slow release formulations of compounds with such, relatively short, half lives can substantially improve compound availability and hence therapeutic efficacy.

Figure 17 shows that compounds such as those discussed herein differ greatly in their extent of reduction in the conditions of the assay (approx. 500:1 DTT excess, at 120 minutes). As this figure shows, thionine is completely reduced under these conditions, tolonium chloride is reduced at an intermediate level, and MB and DMMB are relatively little reduced. The amounts of commonly used reductant required to achieve, say, 90% reduction of the oxidized form in 10 minutes, prior to administration\absorption may not be feasible (e.g. 2000:1 ratio of DTT to MB).

As Figure 18 illustrates, the extent of reduction of MB under physiological conditions can be greatly accelerated by allowing reduction over night and then lyophilising the reduced form. The lyophilisate becomes reduced by 90% in 10 minutes, after solubilisation in conditions mimicking gastric acidity (5mM HCl). Capsules containing a form of the diaminophenothiazine pre-reduced with ascorbic acid at a mg ratio of 1.5-2 represent a suitable, if not optimal, formulation for therapeutic use.

The same considerations apply to other compounds, such as thionine and tolonium chloride, which are more readily reduced than MB, but the extent of reduction of which can be accelerated in a manner such as that described above.

Thus in preferred forms the phenothiazine agents of the present invention are provided as pre-reduced compounds e.g. in lyophilised preparations, optionally in the presence of a stabilising agent.

An agent for stabilising the preferred form of the active compound (i.e. a form of the compound having a low diffusion coefficient, e.g. the fully-reduced form of the compound) may be a reducing agent or antioxidant. The agent may serve both to convert one form of the inhibitory compound (e.g. the oxidised form) to the preferred form thereof (e.g. the reduced form), and to stabilise that preferred (e.g. reduced) form. Alternatively, the inhibitory compound may be added to the composition in its preferred (e.g. already-reduced) form, so that the agent merely serves to maintain the compound in this form.

Particularly suitable for use in converting to, and/or stabilising, the reduced form of the active agent (e.g. the diaminophenothiazine) comprised in the formulations of the present invention is the antioxidant ascorbate. Ascorbate has previously been used to minimise oxidative damage of proteins (Parkkinen J. (1996), "Thrombosis and Haemostasis" 75(2): 292). A formulation as provided herein could thus advantageously comprise a diaminophenothiazine, especially MB, tolonium chloride, DMMB or

thionine, in combination with ascorbate, in suitable proportions, concentrations and dosages.

In other embodiments the reduced (leuco) form may be favoured by the addition or selection of appropriate constituent groups.

Thus aspects of the invention further include a method of preparing a medicament for use in the treatment or prophylaxis of a disease as described above, which method comprises the step of reducing the compound (such that it is, say, at least 50, 60, 70, preferably 80, 90, 95, or 99% reduced) and stabilizing it in a lyophilized composition in the reduced form, prior to administration of an appropriate dose to a patient in need of the same.

Dosage of therapeutics

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the disease being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.

CNS penetration of MB following systemic administration has been described by Müller (1992; *Acta Anat.* 144:39). Azure A and B are known to occur as normal metabolic degradation products of MB (Disanto and Wagner (1972a) *J. Pharm. Sci.* 61: 598; Disanto and Wagner (1972b) *J. Pharm. Sci.* 61: 1086). The pharmacokinetics and toxicity of tolonium chloride in sheep is discussed by Cudd et al (1996) Vet Human Toxic 38 (5) 329-332.

For thionine, which is specifically exemplified herein, a daily dosage of between 1 and 1000 mg may be suitable, preferably divided

into 1 to 8 unit doses, which can, for example, be of the same amount. It will, however, be appreciated that these limits given above can be departed from when required, as may be appropriate with the compounds of the invention other than thionine, which have higher or lower activity or bioavailability.

Figure 19 shows the variation of tissue levels of MB vs IV dose.

The pharmacokinetics of methylene blue have been studied in humans, dogs and rats by DiSanto and Wagner, J Pharm Sci 1972, 61:1086-1090 and 1972, 61:1090-1094. Further data on urinary excretion in humans is also available from Moody et al., Biol Psych 1989, 26: 847-858. Combining data on urinary excretion of MB in humans, it is possible to derive an overall model for distribution of MB following single 100 mg dose in a 70 kg subject, assuming instantaneous absorbtion (Fig 19B). Urinary excretion accounts for 54 - 98% of the ingested dose. This variability is most likely due to variability in absorbtion, although variability in metabolism cannot be excluded. From urinary excretion data, it is possible to calculate that whole body clearance is 56 mg/kg/hr. Therefore, the dosage required to achieve an effective target tissue concentration of 4 μM is 1.73 mg/kg/day (0.58 mg/kg tds) if there were complete absorbtion. However, from Moody et al., it is clear that total urinary excretion, and hence effective bioavailability, is itself a function of dose. The oral dose required to deliver 1.73 mg/kg/day is approximately 2x the dosage calculated on the basis of whole-body clearance. Therefore the actual required dosage is on the order of 3.2 mg/kg/day. This is close to the minimum routine oral dosage used clinically in humans, eg in the treatment of chronic urinary tract infection (390 mg/day). The maintenance oral dosage in humans is therefore approximately 225 mg/day, or 75 mg tds. Peak tissue levels are reached at approximately 1 hr and the tissue half-life is about 12 hours.

Methylene blue exists in the charged blue oxidised form, and the uncharged colourless reduced leukomethylene blue form. We have shown experimentally in cells that the target tissue concentration in cells required to prevent tau aggregation by 50% (ie the EC50)

is 4 μM for reduced methylene blue, and that it is the leuko- form which is preferentially active. It is shown by DiSanto and Wagner (1972) that approximately 78% of the methylene blue recovered in urine is in the reduced form, and from anatomical studies following iv administration, the only form which is bound to tissues is the colourless reduced form, which becomes oxidised to the blue colour on exposure to air after post-mortem dissection. The only form of methylene blue which crosses the blood-brain barrier after iv administration is the reduced form (Muller, Acta Anat 1992, 144:39-44 and Becker and Quadbeck, 1952). Therefore, orally absorbed methylene blue is very rapidly reduced in the body, and remains so until excretion, possibly undergoing further chemical modification which stabilises it in a reduced form.

It is highly likely that variability in oral absorbtion is determined largely by the efficiency of initial reduction in the GI tract. One way to achieve more reliable absorbtion is therefore be to pre-reduce methylene blue with ascorbic acid. We have shown from in vitro studies that this conversion is rather slow, so that it takes 3 hours to achieve 90% reduction of methylene blue in water in the presence of 2x mg ratio of ascorbic acid. Therefore, the dosage of methylene blue which is most likely to ensure reliable absorbtion will be 3.5mg/kg/day of methylene blue pre-reduced for at least 3 hours in the presence of 7 mg/kg/day of ascorbic acid.

It is also possible that MB may be active at lower concentrations in man, and that a range of clinically feasible doses would be therefore 20 mg tds, 50 mg tds or 100 mg tds, combined with 2x mg ratio of ascorbic acid in such a manner as to achieve more than 90% reduction prior to ingestion.

Formulation and administration of therapeutics

Suitable compounds, such as those with a formula as shown above or their pharmaceutically-acceptable salts, may be incorporated into compositions of this aspect of the present invention after further testing for toxicity. The compositions may include, in addition to the above constituents, pharmaceutically-acceptable excipients,

carriers, buffers, stabilisers or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration.

Where the composition is formulated into a pharmaceutical composition, the administration thereof can be effected parentally such as orally, in the form of powders, tablets, coated tablets, dragees, hard and soft gelatine capsules, solutions, emulsions or suspensions, nasally (e.g. in the form of nasal sprays) or rectally (e.g. in the form of suppositories). However, the administration can also be effected parentally such as intramuscularly, intravenously, cutaneously, subcutaneously, or intraperitoneally (e.g. in the form of injection solutions).

Where the pharmaceutical composition is in the form of a tablet, it may include a solid carrier such as gelatine or an adjuvant. For the manufacture of tablets, coated tablets, dragees and hard gelatine capsules, the active compounds and their pharmaceutically-acceptable acid addition salts can be processed with pharmaceutically inert, inorganic or organic excipients. Lactose, maize, starch or derivatives thereof, talc, stearic acid or its salts etc. can be used, for example, as such excipients for tablets, dragees and hard gelatine capsules. Suitable excipients for soft gelatine capsules are, for example, vegetable oils, waxes, fats, semi-solid and liquid polyols etc.

Where the composition is in the form of a liquid pharmaceutical formulation, it will generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may also be included. Other suitable excipients for the manufacture of solutions and syrups are, for example, water, polyols, saccharose, invert sugar, glucose, trihalose, etc. Suitable excipients for injection solutions are, for example, water, alcohols, polyols, glycerol, vegetable oils,

etc.

Suitable excipients for suppositories are, for example, natural or hardened oils, waxes, fats, semi-liquid or liquid polyols etc.

Moreover, the pharmaceutical preparations may contain preserving agents, solubilizers, viscosity-increasing substances, stabilising agents, wetting agents, emulsifying agents, sweetening agents, colouring agents, flavouring agents, salts for varying the osmotic pressure, buffers, or coating agents.

For intravenous, cutaneous or subcutaneous injection, or intracatheter infusion into the brain, the active ingredient will be in the form of a parenterally-acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers and/or other additives may be included, as required.

A composition according to the present invention may be administered alone, or in combination with other treatments, either simultaneously or sequentially, dependent upon the condition or disease to be treated.

In accordance with the present invention, the formulations provided herein may be used for the prophylaxis or treatment of Alzheimer's disease, motor neuron disease, Lewy body disease, Pick's disease or Progressive Supranuclear Palsy, or any other condition or disease in which induced conformational polymerisation of a protein is implicated (see Figure 5). In particular, as described in detail below, the formulation may be used for the blocking, modulation and inhibition of pathological tau-tau association.

Examples of the techniques and protocols mentioned above can be found in "*Remington's Pharmaceutical Sciences*", 16th edition, Osol, A. (ed.), 1980.

In a further aspect, the present invention relates to the use of a composition of the preceding aspect, in the diagnosis, prognosis or treatment of a condition in which induced conformational polymerisation of a protein is implicated. The condition may be a disease such as Alzheimer's disease, or any other condition of the type described herein.

Use of diffusion constant as a screen

As stated above, by converting a compound into, and/or stabilising its reduced form, the inhibitory potency of the compound can be optimised.

However, as described in more detail in the examples hereinafter, surprisingly, the redox potential of a compound does not directly determine its inhibitory activity with respect to induced conformational polymerisation of proteins, and that, therefore, neither the oxidation model nor a catalytic reduction model are relevant to an understanding of the activity of compounds as tau-tau aggregation inhibitors.

The inventors have found that there is a strong inverse correlation between the inhibitory potential of a compound towards tau-tau binding and the square or third power of its diffusion coefficient.

The diffusion coefficient is determined by the amount of stacking of discharged molecules at a cathode. Experimentally, this can be evaluated by measuring the current flow in a redox cell at the reduction potential. The diffusion coefficient is inversely correlated with the degree of aggregation of the discharged (i.e. reduced) species within the Helmholtz layer forming at the cathode. These aggregates form by pi-bonded stacking interactions across the phenol ring systems.

In one model, the lower the diffusion coefficient, the higher the tendency to stack, and the more potent the compound is in inhibiting induced conformational polymerisation of proteins such

as tau-tau binding, as reflected by a low K_i.

The stacking of diaminophenothiazines may be less favoured when the molecule is in the oxidised form, since this form is charged, and so can be envisaged to repel other, like molecules. This phenomenon may thus explain the greater efficacy of the reduced form of diaminophenothiazines in the inhibition of tau aggregation (see e.g. Figure 9).

Thus an assessment of the diffusion coefficient (dependent on 'stackability', which is in turn dependent on shape and charge) can be a useful step in the development of effective modulators. One such sterically-relevant parameter is diffusion coefficient which can be diminished by providing diaminophenothiazines in their reduced form.

Thus, the present inventors teach herein that the efficacy of a compound in the blocking, modulation or inhibition of induced conformational polymerisation of a protein (hereinafter referred to as "inhibitory potency" can be tested in an assay method which includes the step of measuring the diffusion coefficient of the compound.

Hence, in its most general form, the present invention provides a method of screening for an agent that blocks, modulates or inhibits induced conformational polymerisation of a protein, which method includes the step of measuring the diffusion coefficient of the agent. The use of the diffusion coefficient value, and in particular the square or third power of its diffusion coefficient, in assessing the inhibitory potency of a phenothiazine (e.g. as described above) for the treatment of a disease as described herein forms a further aspect of the present invention.

The step of measuring the diffusion coefficient of the test agent may be incorporated at any stage of a larger screening programme for identifying or optimising putative or established modulators. The larger method will typically further include assay steps as described herein, or in the prior art (e.g. WO 96/30766). Thus, in

the latter case for instance, when one wishes to screen for agents which block, modulate or inhibit tau-tau aggregation, the method may include the steps of contacting:

(a) a tau protein or a derivative thereof containing the tau core fragment, with;
(b) a substance to be tested for its ability to block, modulate or inhibit tau-tau aggregation; and
(c) a labelled tau protein or a labelled derivative thereof which is capable of binding to the tau protein of step (a) or a tau protein or a derivative thereof which is distinct from the tau protein of step (a) and also capable of binding to the tau protein of step (a).

The diffusion coefficient may be measured by any suitable means, for instance according to the method of Murthy and Reddy (J Chem Soc., Faraday Trans J 1984, 80. 2745-2750). This publication also included some determined values of diffusion coefficients for phenothiazine dyes and its content is specifically incorporated herein by reference.

Thus, the diffusion coefficient may suitably be measured by cyclic voltammetry in an aqueous acidic medium, whereby the magnitude of current flow in a redox cell is tested at the reduction potential of the compound.

The method may include the step of performing further tests on the agent, e.g. to ascertain its specificity as an inhibitor or modulator of induced conformational polymerisation of a particular protein (e.g. tau), or to determine its pharmaceutical acceptability or suitability as an agent for administration to an animal.

The surprising teaching as provided herein, that the efficacy of an agent in blocking, modulating or inhibiting induced conformational polymerisation of a protein is dependent, at least in part, on the diffusion coefficient of the agent, can be utilised in the optimisation of an agent's efficacy. The present inventors have

established that an agent's inhibitory potency towards induced conformational polymerisation of a protein is inversely related to the square or third power of its diffusion coefficient. In other words, the inhibitory potency of an agent can be optimised by providing the agent in a form in which its diffusion coefficient is minimised.

Thus, in a further aspect, the present invention concerns a method of optimising the efficacy of an agent in blocking, modulating or inhibiting induced conformational polymerisation of a protein, which method includes the step of minimising the diffusion coefficient of the agent.

In a further aspect, the present invention provides a pharmaceutical composition for the prophylaxis or treatment of a condition in which induced conformational polymerisation of a protein occurs, the composition comprising a compound which is provided in, or converted into, a form in which its diffusion coefficient is minimised.

This, and further, aspects of the invention will be better understood by reference to the following figures and experimental data, given only by way of example.

Figures

Figure 1 shows a schematic illustration of the structure of a paired helical filament (top) and the immunochemistry of neurofibrillary tangles during progression of Alzheimer's disease (bottom).

Figure 2 shows a conceptual scheme wherein critical nucleating factors provide a 'seed' which initiates tau capture, which then becomes autocatalytic.

Figure 3 shows a putative pathogenic model of Alzheimer's disease. Tau aggregation is a proximal process prior to failure of axonal transport and consequent neuronal death. The tau aggregation

cascade can be triggered either by a seeding/nucleation event arising from upstream changes or from primary mutations in the tau gene.

Figure 4 shows how induction of full-length tau can lead to its conversion into the 12 kD fragment, provided there is some preexisting 12 kD tau in the cell.

Figure 5a-b shows a table listing proteins which play a role in diseases of protein aggregation. Also listed are the diseases themselves, the aggregating domain and/or mutation believed to be involved, and the putative (maximum) fibril subunit size. One or more literature references for each protein is given.

Figure 6 shows a schematic illustration of the binding sites of various monoclonal antibodies to different forms of N- and C-truncated tau.

Figure 7a-b shows the nucleotide and predicted amino acid sequences of a human tau protein isoform. The sequence was deduced from cDNA clone htau40.

Figure 8 shows the structures of thionine, tolonium chloride, chlorpromazine and tacrine.

Figure 9 gives cellular assay data for diaminophenothiazines, and a structurally related anthroquinone along with apparent KI values, determined as described herein. In the Figures and Examples herein, a further parameter, B50, has been calculated to express activity in a manner directly related to the conditions of the cell-based assay, and therefore providing an indication of the tissue concentration which would be required to achieve the corresponding activity *in vivo*. The B50 value is the concentration of test compound used in the cell assay at which relative production of the 12 kD band from full-length tau was reduced to 50% of that observed in the absence of the compound. There is a simple linear relationship between apparent KI value and B50 value as follows:

Cellular B50 = 0.0217 x KI

In order to compare the relative usefulness of compounds as therapeutics, it may be desired to calculate an LD50 value. Where inhibitory properties are similar, preferred compounds for clinical use may be those which have the highest LD50 value. A therapeutic index (RxIndx) may be calculated for each of compounds tested in the cell assays as follows:

RxIndx = LD50 / B50

Toxicity of the compounds may be measured by cell numbers after 24 hrs exposure to the compound using a lactate dehydrogenase assay kit TOX-7(Sigma Biosciences) according to the manufacturer's instructions after lysis of remaining cells. Alternatively a kit from Promega UK (CytoTox 96) may be used, again according to the manufacturer's instructions.

Figure 10 shows the results of using reduced thionine in the present invention, based on a data set of 7 experiments. The observed cell data for production of the 12 kD band can be fitted closely (ie observed vs predicted correlation coefficient > 0.9), to a standard function describing inhibition of tau-tau binding in vitro. To obtain this fit, two assumptions need to be made, which are consistent with results from other cell-based and in vitro studies:

1) the intracellular concentration of tau is approximately 500 nM;
2) the tau-tau binding affinity is 22 nM.

using these assumptions, the function for cellular activity predicted via standard inhibition model is:

Activity = [tau] / ([tau] Kd* (1 + [thionine] / KI))

can be solved by standard numerical methods to derive a value for apparent KI. As indicated, the value for the reduced form of

thionine is 100 nM. which is essentially the same as that observed for tau-tau binding in vitro at a tau concentration of 500 nM, where the Kd value for tau-tau binding is known to be 22nM. Therefore, the activity of thionine, where the read-out is production of the 12kD truncation product from full-length tau, can be explained quantitatively on the basis of extent of inhibition of the tau-tau binding occurring through the repeat domain within the cell. This confirms that the extent of tau-tau binding determines production of the proteolytically stable core tau unit of the PHF within the cell.

All subsequent cellular analyses of activities of other compounds are reported in the same standardised format, with the same assumptions regarding intracellular tau concentration (500 nM) and tau-tau binding affinity (22 nM) through the repeat domain.

Figure 11 shows the results for conditions in which the reducing agents have been omitted (i.e. oxidised thionine cf. Figure 10).

Again cellular activity is predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [Ox. Thio.] / KI))

In this case, thionine now has an apparent KI value of 1200 nM. This confirms that the diaminophenothiazines require to be in the reduced form for activity. A similar conclusion was derived from analysis of *in vitro* binding data (results not shown).

Figure 12 shows that by using reducing or partially reducing conditions methylene blue appears much more active in the cell-based assay than predicted from *in vitro* studies in which the time course of the assay (1-2 hours) had not been sufficient to achieve reduction.

Cellular activity is again predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [MB] / KI))

In the cell assay, the apparent KI value for methylene blue is 123 nM, which is within the same range as thionine and tolonium chloride. As indicated in Figure 9, the corresponding brain tissue concentration (i.e. B50 value) required to inhibit tau aggregation would be 2-3 μM.

Figure 13 shows corresponding cell-based activity data for reduced tolonium chloride, indicating again that the predicted KI value derived from in vitro studies can be used to describe production of the 12 kD fragment from full-length tau in cells.

Cellular activity is predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [TC] / KI))

This provides further confirmation of the validity of the mathematical analysis procedure used.

Figure 14 shows that DH12 (anthroquinone) which is structurally related to the diaminophenothiazines is inactive in the conditions of the assay.

Figures 15 & 16 show similar analyses to those given above in Figures 9-14, but for chlorpromazine and tacrine respectively. Using the same assumptions (tau concentration 415 nM, and tau-tau binding Kd 22 nM), and cellular activity predicted via standard inhibition model:

Activity = [tau] / ([tau] Kd* (1 + [cpz] / KI))

the apparent KI values for chlorpromazine and tacrine (2117 nM and 802 nM respectively) are greater than anticipated from the in vitro studies.

Figure 17 shows the extent of reduction of various compounds in the presence of DTT.

Figure 18 shows the percentage reduction of MB plotted against the

ratio of MB:Vitamin C.

Figure 19(a) shows that by assuming a target tissue concentration of 4μM (i.e. 1.5 μg/g) it is possible to determine from the data of DiSanto and Wagner (1972) that tissue concentrations of this order would be achieved at an IV dosage of 0.11 mg/kg.

Figure 19(b) shows a model for the distribution of MB following a single 100 mg dose in a 70 kg subject, assuming instantaneous absorbtion.

Figure 20 summarises the results for the transient expression of tau fragments in 3T3 and COS-7 cells based upon data from both microscopical and biochemical experiments.

Expression of recombinant tau fragments in eukaryotic cells was performed as follows. Eight tau constructs, transiently expressed in 3T3 cells and COS-7 cells were examined by immunocytochemistry and immunoblots. The extent of expression in each cell type was given semi-quantitatively on the basis of both sets of results: -, no detectable expression; ±, very weak immunoreactivity; + to ++++, increasing levels of positive immunoreactivity. In all cases, mAb 7.51 was used with each construct to obtain the results. In addition the specificity was confirmed for each construct by using a panel of antibodies against different domains of tau protein (mAbs 499, T14, Tau1, 342, 7.51, 423 and T46). Kozak sequences were absent in the first six contructs, but were present in the cDNA constructs 7 and 8.

Figure 21 illustrates the inducible expression of full-length human tau in 3T6 fibroblasts in two cell lines. T40.22 shows low level background leakage of full length tau in the uninduced state ("U"), and high levels of expression after addition of IPTG (i.e. induced, "I"). T40.37 shows the same, but lower levels of expression without induction.

Figure 22 shows a result of a triple vector system. A vector permitting very low level constitutive expression of the 12 kD

fragment was introduced into cells lines in which inducible expression of full length tau had already been achieved (in fact cell line T40.22 shown in Figure 21 above). Low levels of IPTG are introduced to induce expression of full-length tau. At 0 μM IPTG, there is very low level expression of the 12 kD band, and low "background leakage" expression of full-length tau. As progressively more full-length tau is induced by introducing higher levels of IPTG, more of the full-length tau is converted to the 12 kD species.

Figure 23 shows the inhibitory effects of reduced thionine. In each set of lanes, there is inducible production of the 12 kD band in the presence of increasing concentrations of IPTG inducing higher levels of T40. As the thionine concentration is increased, the production of the 12 kD band from T40 is suppressed.

Figure 24 shows quantitatively the results of Figure 23. In the absence of thionine, induction of T40 at increasing concentrations of IPTG leads to a corresponding increased production of the 12 kD fragment. In the presence of 2 μM thionine, there is still induction of T40, but it is not converted into the 12 kD fragment.

Figure 25 shows comparative in vitro KI values for various compounds, in nM. The KI values relate to the particular assay conditions used (500:1 DTT:compound, 120 minutes - see Figure 17).

Figures 26 and 27 show the inhibitory effect on tau-tau binding of phenothiazines having 0, 2, 3 or 0, 4, 6 methyl groups, respectively.

Figure 28 shows the derivation of two parameters useful for measuring the inhibition of tau-tau association by test compounds. STB is the standardised binding relative to that seen in the absence of compound, taken as the mean observed at 1 and 10 μg/ml. As described in WO 96/30766, an STB value of 1.0 represents binding equivalent to that observed in the absence of compound, whereas a value of 0.2 indicates that the binding was reduced to a mean of 20% at test compound concentrations of 1 and 10 μg/ml. LB50 is log

10 molar ratio of compound:tau producing 50% tau-tau binding compared with that seen in the absence of compound (B50).

Figure 29 shows the relationship between STB and LB50 parameters. STB can be shown to be a linear function of the LB50.

STB is a logarithmic function of the molar ratio of compound:tau at which tau-tau binding is reduced by 50%.

LB50 is the log of the molar ratio of compound with respect to tau at which tau-tau binding is 50% of that observed in the absence of compound

LB50 = 0.05 + (2.65 x STB) r=0.95

The determination of *in vitro* B50 requires that there be some degree of inhibition of tau-tau binding, and a 50% value is obtained by extrapolation. Determination of STB requires no such extrapolation procedure.

Figure 30 shows compounds for which both STB and B50 values have been determined. Assuming that the total tau concentration in cells is approximately 500 nM (i.e. the concentration of tau used in the assay), the B50 values provide an approximation in the *in vitro* assay to the concentration (i.e. [500 x B50] nM) at which the activity might be expected in cell systems.

Figure 31 shows the formal relationship between the *in vitro* LB50 value and the log KI value for the diaminophenothiazine series.

Figure 32 shows the relationship between the number of methyl groups in a diaminophenothiazine (NMETH) and the redox potential (E) and diffusion coefficient (DIF). Italicised figures indicate correlation coefficients (R) and p values after exclusion of MB.

Figure 33 shows the relationship between the percentage of compound that is reduced, as determined experimentally, and the known reduction potential of the compound. The reduction potential

predicts the observed extent of reduction of the diaminophenothiazines.

Figure 34a shows that there is no clear relationship between inhibitory potency and the extent of reduction of compounds. Figure 34b shows that inhibitory potency is not determined simply by reduction potential.

Figure 35 shows that the inhibitory potency can be related directly to the diffusion coefficient (which is a measure of the tendency of the reduced form to stack and aggregate).

Figures 36 and 37 show the predicted relationships between estimated LB50 ("ESTLB50") and STB ("ESTSTB") values, respectively, and reduction potential and diffusion coefficient, in which the diffusion coefficient is given the greater weighting.

Figure 38 shows the crystalline structure of Methylene Blue.

Figure 39 shows tau-tau binding in the presence of 1mM DTT, as measured in the solid phase assay of WO 96/30766. Two different antibodies were used to detect tau-tau binding, namely mAb 342 (top) and 499 (bottom). The vertical axis represents tau-tau binding, the horizontal axis shows the concentration of full-length tau in the aqueous phase, and the key shows varying concentrations of solid-phase tau. As can be seen, tau-tau binding still occurs in the presence of DTT.

Figure 40 shows various species of tau fragments and doublets which are present without induction ("U") and following induction ("I") in a cell line of the present invention. These include species with mobilities equivalent to 12/14 kD, ~25/27 kD, ~30/32 kD, ~36/38 kD and ~42/44 kD (see Example 3).

Figure 41(a) shows how the 12 kD fragment arises via template-induced proteolytic processing of full-length tau molecules at the approximate positions shown by the arrow-heads.

Figure 41(b) shows how the 25/27 kD species arises via template-induced proteolytic processing of full-length tau molecules at the approximate positions shown by the arrow-heads.

Figure 42 shows a plot of the apparent gel mobilities of the species of Figures 40-41 and their lengths in amino-acid residues.

Figure 43 shows the fragments of Figures 40-42 are at intervals of either ~34 residues or ~17 residues which is the equivalent of a single tau repeat, or half of it. All of the fragments may be generated from a basic heptameric aggregate as a simple set of proteolytic cleavages occurring at the positions indicated by the arrowheads.

Figure 44 shows these same fragments in descending order of length and increasing gel mobility.

Figure 45 shows that DMMB is surprisingly potent in the cell model. Its inhibitory activity could be seen both in the absence of IPTG induction and following induction (see Example 4).

Figure 46 shows the activity of DMMB on base-line expression of the 12/14 kD species, using the same set of assumptions regarding intracellular tau concentration and in vitro tau-tau binding affinity used in Figs 10 - 16.

Cellular activity is predicted via standard inhibition model:

activity = [tau] / ([tau] Kd* (1 + [DMMB] / Ki))

DMMB has an apparent KI within the cell of 4.4 nM, and the cellular B50 value is ~100 nM.

Examples

General materials and methods

Production of 3T6H cell lines

3T6 cells were ECACC No: 86120801 Mouse Swiss Albino Embryo Fibroblasts.

For the inducible system, the experiments employed Lac Switch™ from Stratagene using the p3'SS vector to express the Lac repressor protein and pOPRSVICAT to express the full-length tau under the control of the Lac repressor. Expression is induced by the addition of IPTG.

Initially 3T6 cells were transfected, by electroporation, with the p3'SS plasmid and colonies selected by hygromycin resistance. 5 clones that were expressing varying levels of the Lac repressor protein (determined by immunocytochemistry) were picked, and also the non-cloned cells were retained for comparison.

Production of pOPRSVT40 vector

The T40 insert for cloning into the pOPRSVICAT vector was prepared by PCR with Vent polymerase (NEB) using primers (shown below) that included a Not I site and a start or stop codon as appropriate. The PCR product and pOPRSVICAT vector were cut with Not I and purified. The vector was dephosphorylated to prevent re-ligation, and the insert ligated into the vector using standard protocols.

The resulting ligation mix was transfected into competent E. coli cells and the cells plated out on amp plates. Colonies were picked and gridded out on a new amp plate. Colony lifts were taken to Hybond-N 0.45µm nylon membrane (Amersham) and possible positives selected by colony hybridisation using dGA labelled with (α-^{32}P) dCTP (Amersham) (using an oligolabelling kit (Pharmacia Biotech) and purified on a Nap-10 column (Pharmacia Biotech)). Hybridisation was carried out a 65°C overnight in Church buffer followed by 2x20 mins washes in Church wash. Positive colonies, labeled with radioactive probe, were detected by exposing the blots to x-ray film overnight at - 70°C.

Positive colonies were selected and grown, then checked by PCR and restriction digest to confirm the presence of the insert. The use of a single restriction site for the cloning means that T40 can insert into the vector in either orientation. The orientation of the inserts was determined so as to select colonies with the vector containing T40 in the correct orientation for expression.

Primers used

5'-3' T40-Not I

start

5'-gtc gac tct aga ggc ggc cgc ATG GCT GAG CCC CGG CAG GAG-3'

Not I

3'-5' T40- Not I

stop

5'-act ctt aag ggt cgc ggc cgc TCA CAA CAA ACC CTG CTT GGC CAG -3'

Not I

Sequence complementary to T40 sequence is shown in capitals, the start and stop codons are marked. The Not I site to be added is shown underlined. The remaining sequence shown in lower case is a 13 base pair overhang to allow the Not I enzyme to cut efficiently. This was complementary to sequence in the hTau40 plasmid vector to allow efficient binding of the primers.

Determination of Insert Orientation

Orientation was determined using a restriction enzyme that cuts the insert once and the vector at most a few times, and that gives a differing restriction digest pattern for each orientation. Hind III fits these criteria for pOPRSVT40. If the insert is absent two restriction bands are produced. If the insert is present three bands are produced and the size of the bands depends on the orientation of the insert as shown below.

Forward (correct) Orientation	5385 bp	1030 bp	381 bp
Reverse Orientation	6101 bp	381 bp	314 bp

Production of cells expressing T40 under the control of an inducible promoter

The pOPRSVT40 plasmid was produced and purified by CsCl gradient centrifugation. This was transfected (by electroporation) into 3T6H cells (expressing the Lac repressor protein) produced as described above. Positive cells were selected for by resistance to G418 (at 500µg/ml). Resistant colonies were picked and grown on. The level of expression of full-length T40 with and without the addition of IPTG was determined with anti-tau antibodies by both immunocytochemistry and Western blot.

Production of pZeo295-391

The plasmid pZeo295-391 was designed to express protein corresponding to the truncated fragment of tau (residues 295-391; see below). A constitutive system (pcDNA3.1 from InVitrogen, Netherlands) was used - the plasmid imparts resistance to the antibiotic zeocin. The cDNA for this region was amplified by polymerase chain reaction (PCR), using specific oligonucleotide primers (sense and antisense; see below). The sense primer contained an EcoRI site and the antisense, a BamHI site. The fragments were subcloned into pcDNA3.1 (-)zeo (Invitrogen, Netherlands) that had been digested with EcoRI and BamHI. The inserted DNA is downstream from a cytomegalovirus promoter sequence and upstream of a polyadenylation signal. The plasmid contains the DNA sequence for the expression of ampicillin and zeocin resistance for selection in bacteria and eukaryotic cells, respectively. The authenticity of the inserted DNA was confirmed by full-length sequencing of both strands.

Nucleotide and amino acid sequence for truncated tau fragment 295-391

gataatatcaaacacgtcccgggaggcggcagtgtgcaaatagtctacaaaccagttgacctgagca
aggtgacctccaagtgtggctcattaggcaa

catccatcataaaccaggaggtggccaggtggaagtaaaatctgagaagcttgacttcaaggacaga
gtccagtcgaagattgggtccctggacaatat

cacccacgtccctggcggaggaaataaaaagattgaaacccacaagctgaccttccgcgagaacgcc
aaagccaagacagaccacggggcggag

DNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNIT
HVPGGGNKKIETHKLTFRENAKAKTDHGAE

295 sense primer

met asp[295]

5' - CGG AAT TCC ACC **ATG** GAT AAT ATC AAA CAC GTC CCG - 3'

EcoRI

391 anti-sense primer

stop glu [391]

5' - C GCG GGA TCC **TCA** CTC CGC CCC GTG GTC TGT CTT GGC - 3'

BamHI

The start and stop codons are in bold and the EcoRI and BamHI restriction sites to be added are underlined.

Tissue Culture of cells for assay

The medium used was DMEM (with Glutamax I, pyruvate, 4.5g/l glucose) from Life Technologies, Scotland. This was supplemented with 10% FCS (Helena BioSciences),50 U/ml penicillin, 50 µg/ml streptomycin, plsu further antibiotic as appropriate for the selection and maintenance of the relevant plasmid. Antibiotic concentrations were 200 µg/ml hygromycin (p3'SS selection and

maintenance), 500 µg/ml G418(pOPRSVT40 selection and maintenance), 400 or 200 µg/ml zeocin (pZeo295-391 selection or maintenance).

Cells are grown at 37°C, in a humidified atmosphere of 5% CO_2. Cells are maintained in 10cm dishes, and split when they approach confluency. Medium is removed, cells washed with PBS and cells released by trypsinisation with 1 ml of trypsin/EDTA solution / 10cm dish. Cells are resuspended in fresh medium at 1:10 dilution, or optionally in a range of dilutions from 1:5 to 1:20 (approximately 5000 to 20000 cells/cm^2).

For the testing of drugs, cells are plated in 6 well or 24 well plates at an initial density that will allow them to grow to 50-80% confluency within 24 hours. Drugs are added to the well at various concentrations, expression of full-length tau is induced by the addition of IPTG at 0 - 50 µM. Cells are grown for a further 24 hours and then collected for analysis by SDS PAGE/Western blotting.

Preparation of tau protein

Recombinant tau (clone *htau40*) and perchloric acid-soluble tau extracted from rat and human brain were prepared as described previously (Goedert, M. & Jakes, R. (1990) *EMBO* J. 9:4225; Goedert, M. et al (1993) *Proc. Natl. Acad. Sci. USA* 90:5066).

Gel Electrophoresis and Blotting

Cells grown as outlined above are washed once with PBS then lysed in 50 µl (24 well plates) or 100 µl (6 well plates) laemli buffer. Samples are stored at -20°C, boiled for 4 mins prior to running on 15% acrylamide gels using the BioRad miniProtean III mini gel system. Protein is transferred to PVDF membrane by Western blotting using the CAPs buffer system. The membranes are incubated in block buffer (5% non-fat milk powder (Marvel), 0.1% Tween 20 in PBS) for 1 hr to overnight. Tau protein is detected by incubating the membranes with mAb 7.51 diluted 1 :5 with block buffer for 1-3hrs or overnight, washing well with PBS/0.1% Tween20, incubating with

anti-mouse HRP 1:5000 dilution in block buffer for 1 hr, and washing well with PBS/0.1% Tween20. Bound antibody is detected by ECL reaction detected on ECL hyperfilm (Amersham).

Blots are scanned into a computer on a Hewlett Packard Scanjet 6100C flatbed scanner at 600dpi and saved as tiff files. Densitometry of the T40 and dGAE bands is performed with the Scananalysis program on an Apple Power Mac G3.

Drug preparation

Thionine, methylene blue, DMMB, and tolonium chloride are all prepared as a 1 mM stock in ddH2O. Prior to use a 100 µM dilute stock is prepared in HBSS which is added directly to the medium on cells.

For oxidised drug this is prepared simply by diluting the 1mM stock in HBSS and filter sterilising.

For reduced drug the 1 mM is treated with ascorbic acid and DTT to yield 0.5mM drug, 50mM ascorbic acid 50mM DTT, this is allowed to stand for 15mins (turns blue to colourless) before making the dilute stock. This is diluted in HBSS to yield 100 µM drug, 10mM ascorbic acid, 10mM DTT and filter sterilised. Cells are treated with the drug at various concentrations, but for the reduced drug the ascorbic acid and DTT concentrations are maintained at 400 µM throughout by using appropriate quantities of 100 µM reduced stock, 100 µM oxidised stock and 10 mM ascorbic acid/DTT stock.

SDS Gel Electrophoresis and Immunoblotting

Standard electrophoresis and immunoblotting procedures were used as described previously (Wischik, C. M. *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4506; Novak, M.,*et al.* (1993) *EMBO J.* 12:365; Jakes, R. et al.(1991) *EMBO J.* 10:2725). Immunoblots were developed with the ABC kit (Vector Laboratories). The monoclonal antibodies (mAbs) 7.51, 21.D10, 499 and 342 were used as undiluted hybridoma

culture supernatant fluids. mAb AT8 (Innogenetics, Belgium) was used at 1/1000 dilution. Anti-tau mAbs 7.51 (which recognises an epitope in the last repeat; see Novak, M. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 5837), 423 (which recognises tau C-terminally truncated at residue Glu-391; see Wischik, C. M. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:4506; Novak, M. et al.(1993) *EMBO* J. 12:365), 499 (which recognises a human-specific tau segment between residues Gly-14 and Gln-26; see Wischik, C. M. et al.(1996) *Proc. Natl. Acad. Sci. USA* 93:11213), and 342 (which recognises a segment between residues Ser-208 and Pro-251). mAb 21.D10 was raised against the A68-tau brain extract (Lee, V. M.-Y. et al.(1991) *Science* 251: 675).

Tau Binding Assay

This was carried out basically as described in Wischik, C. M., et al.(1996) *Proc. Natl. Acad. Sci. USA* 93:11213. Solid phase tau (0-20 µg/ml) was coated on 96-well poly(vinyl chloride) microtitre plates in 50 mM carbonate buffer at 37°C for 1 h. The plate was washed twice with 0.05% Tween 20, then blocked with 2% Marvel in PBST for 1 h at 37°C. After washing again, the plate was incubated for 1 h at 37°C with aqueous phase tau (0 - 300 µg/ml in PBST containing 1% gelatin). In the present application, 1mM DTT was also added.

The plate was washed twice and incubated for 1 h at 37°C with mAb 499 or 342, diluted with an equal volume of 2% Marvel in PBST. After washing, horseradish peroxidase-conjugated goat-anti-mouse antibody (1/1000 in PBST) was incubated for 1 h at 37°C. The plate was washed and incubated with substrate solution containing tetramethylbenzidine and H_2O_2 and the rate of change of absorbance measured using a V_{max} plate reader (Molecular Diagnostics, California) as described previously (Harrington, C. R. et al. (1990) J. *Immunol. Meth.* 134:261). Each experiment was performed in triplicate and included controls in which both solid phase and aqueous phase tau were absent, and also with either one of the two absent.

Data Analysis

This was performed as described in Wischik *et al.* (*supra*) and curves were fitted according to the Langmuir equation with the Kaleidagraph (Synergy, Philadelphia) or Systat (SPSS Inc., Chicago) programs using quasi-Newton approximation. Curve-fitting correlation coefficients are given in the Figures.

Example 1 - constitutive expression of full-length, truncated and mutated tau

Expression of tau in eukaryotic cell lines was sought to generate a cellular model of tau aggregation under physiological conditions which did not suffer from the limitations of lipofectin-based approaches. This involves the expression of full-length tau and truncated tau fragments for both normal tau and tau carrying pathogenic mutations.

Full length tau

When normal full-length tau (T40) was transfected into cells (3T3 and NIE-115) it was expressed and involved in the assembly of the microtubule network within the cells.

Truncated tau

Initially the cDNA for truncated tau fragment from the core of the PHF, corresponding to fragment 297-391, was transfected into non-neuronal 3T3 fibroblasts: this truncated tau was selected since it is: (i) present in the PHF-core; (ii) detected as deposits in AD brain tissue during the early stages of the disease; (iii) capable of supporting the catalytic capture and propagation of tau capture *in vitro*. Subsequently, a series of transfections was performed in which the extent of truncation at either N- or C- termini was varied, based partly on the immunochemical properties of the tau molecule. Six constructs were created with truncation at the N-terminus (186-441 ; 297-441) at the C- and N- termini (186-391;

297-391) and at the C-terminus (1-391). The pattern of immunoreactivity for the six constructs with a limited panel of antibodies was capable of discriminating all of the tau fragments generated in this way.

The constructs were expressed in eukaryotic cells both transiently (using pSG5 as the vector) and stably (using pIF2 and pZeo as vectors). Stable transfectants are selected on the basis of resistance to the antibiotics geneticin and zeocin for pIF2 and pZeo, respectively. Epitope analysis was performed on bacterially expressed proteins using pRK172 as the vector. Figure 20 summarises the results for various fragments in 3T3 and COS-7 cells. Further results showed that the expression of two forms of tau in the same cell can affect the pattern of immunoreactivity. For example, stable expression of 1-391 and 295-391 results in the appearance of abnormal bundles within the cells. However, maintaining such cells in a stable and reproducible state proved elusive.

Mutated tau

Mutagenesis of full-length tau was used to generate known clinical mutations. These were subcloned into pIF2 and stable transfectants generated in 3T3 and NIE cells for a number of mutations including those which affect microtubule assembly properties of tau (G272V, V337M, P301 S, R406W) and S305N, which affects the alternative splicing of the tau gene *in vivo*. In general, cells expressing full-length tau carrying mutations exhibited labelling of the microtubular network and was indistinguishable from cells transfected with wild-type tau. Cell lines expressing certain truncated tau fragments including mutations proved unstable.

Conclusion

In summary, the constitutive expression of truncated tau within eukaryotic cells proved difficult to achieve. Although transient transfection systems permitted the optimisation of expression of tau by manipulating the Kozak consensus surrounding the initiation codon for 297-tau, the expression of e.g. 297-391 was still modest,

suggesting some inherent toxic properties of the fragment. Stable transfections reiterated this conclusion. This latter system demonstrated that truncation at either N- or C-termini resulted in a slightly greater propensity for the tau to assemble in amorphous deposits rather than in a microtubular network. Stable expression of combinations of tau fragments also generated aggregates within the cytoplasm of cells, but this system was not readily reproducible.

Example 2 - inducible expression of truncated tau

In a further attempt to create a stable, reproducible system, without the toxicity associated with constitutive expression, inducible expression of the core-tau fragment of the PHF (i.e. 297-391 - which is 12 kD) was attempted.

Several inducible systems for expression of proteins in eukaryotic cells were tried, although the preferred system was the "lac switch" system. In this system, two vectors are incorporated into cells, typically 3T3 or 3T6 fibroblasts which do not express any endogenous tau protein. The first, the p3'SS vector codes for constitutive expression of the *lac I* gene, and expressors are selected on the basis of hygromycin resistance. The second, pOPRSVICAT incorporates the DNA coding for the tau protein fragment under the control of a strong RSV promoter which contains operator sequences from the *Lac* operon. Cells which incorporate this vector are selected on the basis of neomycin resistance. Cells which have incorporated both vectors have the property that constitutive expression of *lac I* prevents expression of the incorporated protein (i.e. tau) controlled by the *Lac* operon. The addition of the sugar IPTG competes for the binding of *lac I* to the *Lac* operon, and so permits expression of tau protein.

Inducible expression of the 12 kD fragment was carried out in two cell lines. These did not show appreciable levels of tau protein expression until after 3 days treatment with IPTG at which stage high levels of 12 kD suddenly appeared, forming intracellular aggregates which promptly killed the cell. The process of

aggregation was, as expected, non-linear progressing from low level expression to sudden accumulation of toxic aggregates without any clear gradation, making the aggregation and toxicity impossible to control. This non-linear progression prevented a proper control of the system.

Example 3 - expression of tau in stable cell lines according to invention

In view of the results above, a further system was used as follows. Tissue culture cell line DH 19.4.1.4 and clones thereof were based on 3T6 cells (ECACC No: 86120801 Mouse Swiss Albino Embryo Fibroblasts) expressing full-length, four repeat human tau under the control of an inducible promoter and truncated human tau (295-391) under the control of a constitutive promoter.

Cells expressing T40 under the control of an inducible promoter, T40.22.10, were transfected (by lipofection) with the pZeo295-391 plasmid. Positive cells were selected for by resistance to zeocin at 400µg/ml. Expression of truncated tau on a background of inducible expression of full-length tau was confirmed by Western blot analysis with Mab 7.51.

Figure 21 illustrates the inducible expression of full-length human tau only in 3T6 fibroblasts in two cell lines. T40.22 shows low level background leakage of full length tau in the uninduced state ("U"), and high levels of expression after addition of IPTG (i.e. induced, "I"). T40.37 shows the same, but lower levels of expression without induction. Figure 22 shows the results of a triple vector system. A vector permitting very low level constitutive expression of the 12 kD fragment was introduced into cell lines in which inducible expression of full length tau had already been achieved (T40.22 shown in Figure 21). Figure 22 shows what happens when low levels of IPTG are introduced to induce expression of full-length tau. At 0 µM IPTG, there is very low level expression of the 12 kD band, and low "background leakage" expression of full-length tau. As progressively more full-length tau is induced by introducing higher levels of IPTG, more of the

full-length tau is converted to the 12 kD species, and more of the intermediate higher molecular weight fragments (described in more detail in Figs 43 and 44) are produced.

Examination of the original T40-inducible cell line (T40.22.10) which did not contain the vector for constitutive expression of the 12 kD fragment shows that the 12 kD species is not produced as a truncation by-product of full-length tau induction. Enhanced expression of the 12 kD band following induction of T40 was seen only in cells with low level prior expression of the 12 kD fragment (DH19.4.1.4.6). That is, pre-existing 12 kD provides a template for production of more 12 kD following the induction of full-length tau. An additional doublet may also appear with apparent gel mobility of ~25/27 kD when the cells are in the uninduced state (e.g. in the cell line designated DH 19.4.1.4A.B2). Following induction with IPTG, a further series of doublets may appear, with gel mobilities ~30/32 kD, ~36/38 kD and ~42/44 kD.

These species are shown in Figure 40 both without induction ("U") and following induction ("I"). Also shown are the patterns of immunoreactivity of these fragments seen with mAb 342 and a C-terminal polyclonal antibody T46 which recognises epitopes located between residues Ser422 and Leu441.

The derivation of the fragments seen in the uninduced state (i.e. 12/14 kD and 25/27 kD) may be explained by reference to Figure 41.

Figure 41(a) shows how the 12 kD fragment arises via template-induced proteolytic processing of full-length tau molecules at the approximate positions shown by the arrow-heads.

In the case of the 25/27 kD species, these fragments cannot represent dimers of the the 12/14 kD species, as these fragments are immunoreactive with T46. Therefore, a further proteolytic product of the full-length aggregating tau protein must arise via cleavages occurring at the approximate positions shown by the arrowheads in Figure 41(b).

Following induction (Figure 40, I), the further series of doublets is seen. The derivation of these further fragments can be better understood with reference to Figures 42-44.

Figure 42 shows a plot of the apparent gel mobilities of these fragments and their lengths in amino-acid residues, indicating that the apparent gel mobilities can be understood in terms of a characteristic set of fragment lengths.

As illustrated in Figure 43, all of these fragments are at intervals of either ~34 residues or ~17 residues which is the equivalent of a single tau repeat, or half of it. All of the fragments generated can therefore be understood as arising from a simple set of proteolytic cleavages occurring at the positions indicated by the arrowheads in Figure 43 from a basic heptameric aggregate, formed as shown in the figure. In this scheme the fragments arise as the full combinatorial set of the proposed cleavages occurring at the 3 possible approximate positions shown by the arrowheads at either end of the aggregate. The corresponding predicted immunoreactivity patterns seen with mAb 342 and T46 associated with these fragments are also tabulated.

Figure 44 shows these same fragments in descending order of length and increasing gel mobility. Although the heptameric aggregate is shown for convenience as arising entirely from full-length tau molecules, it will be appreciated that the 12/14 kD fragment could be interposed within the proposed aggregate, replacing some of the binding partners, and that the precise pattern of inclusion of these short fragments in the aggregate will determine which precise fragments from the full set predominate in a given instance. Therefore, the production of this family of proteolytic fragments is better understood as a possible repertoire which can be instantiated in various ways within the cell.

Example 4 - inhibitory effects of compounds on production of proteolytic fragment

Having achieved a stable cell system in which production of the 12

kD fragment (and others) could be controlled, the model was used to test the inhibitory effects of reduced thionine. This is shown in Figure 23. In each set of lanes, there is inducible production of the 12 kD band in the presence of increasing concentrations of IPTG inducing higher levels of T40. As the thionine concentration is increased, the production of the 12 kD band from T40 is suppressed. This is shown quantitatively in Figure 24. In the absence of thionine, induction of T40 at increasing concentrations of IPTG leads to a corresponding increased production of the 12 kD fragment. In the presence of 2 μM thionine, there is still induction of T40, but it is not converted into to the 12 kD fragment.

As reduced thionine is itself toxic, it is necessary to control for reduction in the levels of T40 induced by corresponding does of IPTG at higher levels of thionine. This can be achieved by determining the ratio of 12 kD : T40, which permits averaging the data across IPTG levels and shows a dose-dependent reduction in the level of the 12 kD relative to full-length tau.

The activities of various compounds in the T40/12 kD assay are shown in Figures 9 to 16.

Results are expressed in terms of the ratio of 12 kD : T40 following induction of full-length tau (T40) by treatment cells with IPTG (0, 10, 25, 50 μM) in the presence of thionine or tolonium chloride introduced at the concentrations shown in the presence of reducing agents (200 μM DTT/ascorbate), or chlorpromazine or tacrine introduced without reducing agents. As can be seen, thionine and tolonium chloride produce essentially identical inhibition, whereas chlorpromazine and tacrine are non-inhibitory in the same concentration range. The effect of the reducing agents alone was tested in control experiments which showed no significant difference was seen in the 12 kD : T40 ratio in the presence of reducing agents alone.

The properties of the cell line producing higher molecular weight

degradation products were also examined with MB and DMMB (dimethyl-methylene blue).

As can be seen in Figure 45, DMMB proved to be surprisingly potent in the cell model. Its inhibitory activity could be seen both in the absence of IPTG induction and following induction. Treatment with 1 μM DMMB effectively abolished **all** degradation products within the cell. Further experience with MB and DMMB has shown that even apparent base-line production of the 12/14 kD species is largely determined by aggregation. That is, the constitutive production of the 295-391 fragment is itself either below the level of detection by immunoblot or else it is stabilised by spontaneous aggregation so as to reach levels within the cell which can be detected by immunoblot. Alternatively, the apparent base-line levels of the 12/14 kD fragment seen without IPTG induction and in the absence of treatment with a tau-aggregation inhibitor may itself be dominated by templated aggregation-dependent production from the leakage levels of T40 produced in absence of induction. Whatever the combination of factors which determines the levels of the 12/14 kD fragment in the base-line condition, its apparent expression can be essentially eliminated, along with higher molecular weight aggregation products, by a potent aggregation inhibitor such as DMMB. These results further confirm that production of the higher molecular weight proteolytic fragments (ie 30/32, 36/38, 42/44 kD) is also dependent on critical tau-tau binding interactions occurring through the repeat domain, as shown in Figures 41, 43 and 44.

Figure 46 shows the activity of DMMB on base-line expression of the 12/14 kD species, using the same set of assumptions regarding intracellular tau concentration and in vitro tau-tau binding affinity used in Figs 10 - 16. In this case DMMB is found to have an apparent KI within the cell of 4.4 nM, and the cellular B50 value is ~100 nM. This indicates that DMMB is highly potent within the cellular milieu.

Example 5 - comparison of inhibitory effects of reduced and oxidised compounds

The mathematical model used for the *in vitro* data was used to analyse the effects of test substances in the T40 : 12kD cell assay. Using the known values for Kd and KI from in vitro data, the expression indicated was used to solve for the intracellular concentration of full-length tau (see e.g. Figure 10).

This was found to be about 500 nM, which is in the range expected from studies of tau in brain and in cell systems. A good fit to the experimental data was obtained implying that for some compounds the inhibition of production of truncated tau within the cell can be predicted from the approximate Kd and KI values determined experimentally in vitro.

Example 6 - examination of inhibitory properties of diaminophenothiazines

In *in vitro* studies, the most active inhibitors of tau-tau binding identified were the reduced forms of diaminophenothiazines having 0, 2 or 3 methyl groups. Figure 25 shows the reduced forms of such compounds. The corresponding tau-tau binding curves are shown as a function of molar ratio with respect to tau in Figures 26 and 27. As shown, compounds of the "desmethyl series" (0, 2 or 3 methyl groups) produce approximately 50% inhibition of tau-tau binding (shown on the vertical axis) at molar ratios of 3:1 - 4:1 of compound:tau 'AMR' shown on log scale on horizontal axis). The mean molar ratio for 50% inhibition of tau-tau binding for this group of compounds is 4:1.

Diaminophenothiazines having 4 or 6 methyl groups (the "methylated group") have a biphasic action, with enhancement of tau-tau binding at lower concentration, and inhibition of tau-tau binding at high concentrations (Figure 27). These compounds thus require much higher molar ratios to effect 50% inhibition of tau-tau binding.

Examination of other features of the diaminophenothiazine compound was also carried out. Substitution of the heterocyclic nitrogen or sulphur atoms was found to severely interfere with inhibitory

potency of the compounds. Likewise, removal of the diamino groups was found to be detrimental to the inhibitory activity. It thus appeared that the diamino and heterocyclic NB and S- structures are important for activity of the molecules in the inhibition of tau-tau binding.

For comparison, two methods were used to determine inhibitory activity in the tau-tau assay: STB is the mean tau-tau binding observed at 1 and 10 µg/ml of compound at standard tau concentrations of 488 nM; LB50 is log10 molar ratio of compound:tau producing 50% inhibition of tau-tau binding (Figure 28). As shown in Figure 29, there is a strong correlation between the STB and LB50 values for a range of compounds, with chlorpromazine and riboflavin being two outliers (see also Figures 30 and 31).

Example 7 - inhibitory activity and diffusion potential

Figure 32 indicates that there is a correlation between the number of methyl groups (NMETH) in a test compound and both the reduction potential (E) and diffusion coefficient (DIF). In all comparisons, the Spearman rank correlation was used. As shown in Figure 32, a strong inverse relationship between the number of methyl groups (NMETH) and the reduction potential can be seen only if methylene blue is excluded (normal type: correlation values including methylene blue; italic type: correlation values excluding methylene blue).

This indicates that methylene blue has a disproportionately high reduction potential relative to number of methyl groups (NMETH) in this series. There is also a strong positive correlation between the number of methyl groups and the diffusion coefficient (DIF, Figure 32).

As well as there being no observed correlation between the number of methyl groups and reduction potential (Figure 33), it was surprisingly found that there was no observed correlation between reduction potential and inhibitory potential (Figure 34b), although the extent of reduction of the diaminophenothiazines in the

conditions of the assay is highly correlated with reduction potential (Figure 33). And indeed, there is no correlation between the extent of reduction of these compounds and inhibitory potency (Figure 34a). On the other hand, there is a strong inverse correlation between the inhibitory potency of a compound and its diffusion coefficient, and it is possible to predict estimated LB50 and STB values as linear functions of reduction coefficient and diffusion coefficient when greater weighting is given to the diffusion coefficient(Figures 35, 36 and 37). Both the LB50 and STB values are found to be uniformly low for NMETH values up to and including 3, but for higher NMETH values (in particular methylene blue, NMETH=4) there is a disproportionately low inhibitory potency relative to the number of methyl groups. This may relate to the symmetric placement of the methyl groups which interferes with the stacking ability of the molecules, as measured by the diffusion coefficient. This can be seen, for example, in the crystalline structure of methylene blue (see Figure 38). The diaminophenothiazine molecule is essentially flat and forms stacking arrays. The presence of charge in the molecule, as in the oxidised form, prevents the formation of such stacking arrays, and it appears to be the propensity of the reduced form of this compound to form such stacking relationships that determines the inhibitory potency of the series.

The experiments carried out by the present inventors examined the binding of full-length tau in the aqueous-phase to the truncated repeat domain fragment of tau in the solid-phase, as described in further detail in WO96/30766. Binding was detected with either mAb 342 or mAb 499. As shown in Figure 39, there is typical tau concentration-dependent tau-tau binding in the presence of a large excess of the standard reducing agent dithiothreitol (DTT, 1 mM). However, the inhibitory activity of phenothiazines is also demonstrated in the presence of DTT (1 mM) in the standard configuration of the assay described above (i.e. the data for STB and LB50). The present inventors thus conclude that the inhibitory activity cannot be attributed to DTT *per se*, but rather to the presence of the phenothiazines in their reduced form, due to an

excess of DTT.

In summary, the present inventors provide herein a potential, significantly improved, system for the treatment and prophylaxis of diseases such as Alzheimer's Disease in which proteins undergo induced conformational polymerisation, e.g. as illustrated in the case of Alzheimer's disease by pathological tau-tau binding. The important teachings of this application, *viz* that the diffusion coefficient of a compound may important in determining its inhibitory potency towards this induced conformational protein polymerisation, are potentially of great benefit in advancing our understanding of, and ability to provide therapy for, diseases such as Alzheimer's Disease. Finally, by combining the findings on the preferality of the reduced form of MB, and demonstration of its activity in the cell-based assay at concentrations substantially below those predicted solely on the basis of *in vitro* data, the inventors have shown that this compound, and others like it, could be used an appropriate reducing formulation for the prophylaxis or treatment of AD and related disorders.

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Claims

1 A method of proteolytically converting a precursor protein to a product fragment in a stable cell line,

which precursor protein is associated with a disease state in which the precursor protein aggregates pathologically,

which method comprises:

(a) providing a stable cell line transfected with nucleic acid encoding:

(i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and

(ii) the precursor protein, which protein is inducibly expressed in the cell in response to a stimulus,

whereby interaction of the template fragment with the precursor protein causes a conformational change in the precursor protein such as to cause aggregation and proteolytic processing of the precursor protein to the product fragment.

2 A method as claimed in claim 1 wherein pathological aggregation leads to proteolytic processing of the precursor protein in a disease state associated with neurodegeneration and\or clinical dementia.

3 A method as claimed in claim 1 or claim 2 wherein pathological aggregation of the precursor protein in the disease state leads to proteolytic processing to a core domain fragment and the template fragment comprises at least the core fragment of the template protein.

4 A method as claimed in claim 3 wherein the template fragment consists essentially of the core fragment.

5 A method as claimed in any one of the preceding claims wherein the product fragment produced in the cell is toxic.

6 A method as claimed in any one of the preceding claims wherein the product fragment is the same as the template fragment.

7 A method as claimed in any one of claims 1 to 5 wherein a plurality of different product fragments are produced.

8 A method as claimed in any one of the preceding claims comprising the step of subjecting the cell to the stimulus such as to inducibly express the precursor protein in the cell.

9 A method as claimed in any one of the preceding claims wherein the production of at least one product fragment is monitored.

10 A method for identifying a modulator of aggregation and\or proteolytic processing of the precursor protein associated with the disease state

which method comprises:

(a) providing an agent suspected of being capable of modulating the aggregation,

(b) performing a method as claimed in claim 9 in the presence of the agent,

(c) correlating the production of the or each product fragment monitored with the modulatory activity of the agent.

11 A method as claimed in any one of claims 10 wherein step (b) is performed by:

(a) culturing the cells on one or more plates,

(b) incubating the cells with the agent for a period of time sufficient to entry of the agent into the cells.

12 A method as claimed in claim 11 wherein the agent is introduced to the cells to give a final concentration of between 1-50 μM.

13 A method as claimed in any one claims 10 to 12 wherein production of a plurality of different product fragments is monitored.

14 A method as claimed in any one claims 10 to 13 wherein the production of the or each product fragment monitored is compared with a reference value.

15 A method as claimed in claim 14 wherein the reference value is obtained by performing the method in the absence of the agent.

16 A method as claimed in any one of claims 10 to 15 wherein the agent which is provided is selected such as to be capable of crossing the blood-brain barrier.

17 A method as claimed in any one of claims 10 to 16 which comprises the step of selecting the agent to be provided by measuring the diffusion coefficient of the agent and correlating the diffusion coefficient with the agents inhibitory potential.

18 A method as claimed in any one of claims 10 to 17 further comprising the step of calculating a B50 for the agent.

19 A method as claimed in any one of claims 10 to 18 further comprising the step of assessing the effect of the agent on cell viability.

20 A method as claimed in claim 19 further comprising the step of calculating an LD50 for the agent.

21 A method as claimed in claim 18 and 20 comprising the step of calculating a therapeutic index for the agent.

22 A method as claimed in any one of the preceding claims wherein the precursor protein is a tau protein.

23 A method as claimed in claim 22 wherein the template fragment comprises a core fragment of tau.

24 A method as claimed in claim 23 wherein the template fragment comprises a fragment of tau extending from amino acids 186-297 to 390-441 of the full-length tau protein shown in Fig 7.

25 A method as claimed in claim 24 wherein the template fragment consists of a fragment of tau extending from amino acids 295, 296 or 297 to amino acid residues 390 or 391 of the full-length tau protein shown in Fig 7.

26 A method as claimed in any one of claims 22 to 25 wherein the production of an approximately 12, 14, 25, 27, 30, 32, 36, 38, 42 or 44 kDa product fragment of tau is monitored.

27 A method as claimed in claim 26 wherein the production of an approximately 12 kDa product fragment of tau is monitored.

28 A method as claimed in any one of claims 22 to 27 wherein production of the or each toxic product fragment is monitored on SDS PAGE.

29 A method as claimed in any one of claims 22 to 28 wherein production of the or each toxic product fragment is monitored immunologically.

30 A method as claimed in claim 29 wherein the monitoring employs an antibody is selected from a monoclonal antibody which (i) is specific for a human-specific epitope located in the region between Gly-16 and Gln-26 of tau; (ii) is specific for the core tau fragment truncated at Glu-391; (iii) is specific for a generic tau epitope in the repeat domain; or (iv) is specific for a non-species specific generic tau epitope located between Ser-208 and Ser-238.

31 A method as claimed in any one of claims 22 to 30 which comprises the step of selecting the agent to be provided by determining the ability of the agent to modulate the ability of a fragment of tau corresponding to the core repeat domain, which has been adsorbed to a solid phase substrate, to capture soluble full-length tau.

32 A method as claimed in any one of claims 22 to 31 wherein the agent is a phenothiazine.

33 A method as claimed in claim 32 wherein the agent is a reduced phenothiazine.

34 A method for screening for a medicament for use as a therapeutic or prognostic agent for the treatment of a tauopathy which method comprises:
(a) performing a method as claimed in any one of claims 22 to 33
(b) selecting modulators having a therapeutic index of greater than 2.

35 A method as claimed in claim 34 wherein the "disease" is selected from Alzheimer's disease, motor neuron disease, Lewy body disease, Pick's disease or Progressive Supranuclear Palsy.

36 A method for producing a medicament for use as a therapeutic or prognostic modulator for the treatment of a tauopathy, which method comprises
(a) carrying out a method as claimed in any of claim 34 or claim 35 to identify the medicament,
(b) providing the medicament agent in isolated form.

37 A method as claimed in claim 36 further comprising formulating the agent as a medicament composition for use in the treatment of the tauopathy.

38 A method as claimed in claim 37 further comprising using the medicament composition in a method of treatment for the tauopathy.

39 Use of a phenothiazine in the preparation of a medicament composition for use in the treatment or prophylaxis of a tauopathy, wherein the preparation comprises the step of pre-reducing the phenothiazine such that it is present in at least 80, 90, 95, or 99% educed (leuco-) form.

40 Use as claimed in claim 39 wherein the phenothiazine is pre-reduced by addition of an exogenous reducing agent.

41 Use as claimed in claim 40 wherein the reduced form is stabilised in the reduced state by addition of a stabilising agent.

42 Use as claimed in claim 41 wherein the reduced form is lyophilised with the stabilising agent.

43 Use of a pre-reduced phenothiazine in the preparation of a medicament composition for use in the treatment or prophylaxis of a tauopathy, wherein the medicament comprises at least 80, 90, 95, or 99% of the reduced (leuco-) form of the phenothiazine.

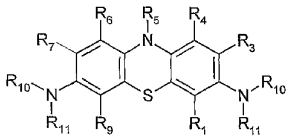
44 Use as claimed in any one of claims 39 to 43 wherein the medicament composition further comprises one or more of the following: a pharmaceutically-acceptable excipients, carriers or buffers.

45 Use as claimed in claim 44 wherein the medicament composition is prepared as a slow release formulation.

46 Use as claimed in any one of claims 39 to 45 wherein the phenothiazine is a diaminophenothiazine.

47 Use as claimed in any one of claims 39 to 46 wherein the pre-reduced (leuco-) phenothiazine has the formula:

(I)



wherein R1, R3, R4, R6, R7 and R9 are independently selected from hydrogen, halogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy; R5 is selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;

and each R10 and R11 are independently selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy;

or is a pharmaceutically acceptable salt thereof.

48 Use as claimed in claim 47 wherein R1, R3, R4, R6, R7 and R9 are independently selected from -hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$; each R10 and R11 are independently selected from hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$; and
R5 is hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$.

49 Use as claimed in any one of claims 46 to 48 wherein the phenothiazine is a diaminophenothiazine which has 0, 2, 3 or 4 methyl groups around the diaminophenothiazine nucleus.

50 Use as claimed in any one of claims 46 to 49 wherein the phenothiazine is a diaminophenothiazine which is asymmetrically methylated.

51 Use as claimed in claim 50 wherein the phenothiazine is tolonium chloride, azure A, azure B and thionine.

52 Use as claimed in claim any one of claims 46 to 49 wherein the phenothiazine is selected from Methylene Blue, Toluidine Blue O, or 1,9-Dimethylmethylene Blue.

53 A medicament composition comprising a pre-reduced phenothiazine as described in any one of claims 47 to 52 wherein the phenothiazine is at least 80, 90, 95, or 99% of the reduced (leuco-) form, in combination with a stabilizer.

54 A medicament composition as claimed in claim 53 which is lyophilised with the stabiliser.

55 A medicament composition as claimed in claim 54 or claim 54 wherein the stabiliser is ascorbate.

56 A medicament composition as claimed in any one of claims 53 to 55 for use in the treatment or prophylaxis of a tauopathy.

57 A method of treatment of a tauopathy comprising use of medicament composition as claimed in any one of claims 53 to 55.

58 A method, use, or composition as claimed in any one of claims 34 to 52, claim 56 or claim 57 wherein the treatment or prophylaxis comprises giving a prophylactically effective amount or a therapeutically effective amount of the medicament composition to a patient in need of the same.

59 A method, use, or composition as claimed in any one of claims 39 to 52, claim 56 or claim 57 wherein the treatment or prophylaxis comprises giving a patient in need of same 20 mg tds, 50 mg tds or 100 mg tds, combined with 2x mg ratio of ascorbic acid in such a manner as to achieve more than 90% reduction of the phenothiazine prior to ingestion.

60 A method, use, or composition as claimed in any one of claims 39 to 52, claim 56 or claim 57 wherein the treatment or prophylaxis comprises giving a patient a phenothiazine which is thionine and this is given to the patient in a daily dosage of between 1 and 1000 mg optionally divided into 1 to 8 unit doses.

61 A method, use, or composition as claimed in any one of claims 39 to 52, claim 56 or claim 57 wherein the treatment or prophylaxis comprises giving a patient a phenothiazine which is methylene blue, and the daily dosage is approximately 3.2-3.5 mg/kg.

62 A process for producing a stable cell for use in a method as claimed in claim in any one claims 1 to 38 which process comprises the steps of introducing into a cell nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly expressed in the cell in response to a stimulus.

63 A process as claimed in claim 62 wherein the nucleic acid encoding the precursor protein is operably linked to a lac inducible promoter.

64 A process as claimed in claim 63 wherein expression of the precursor protein is induced by the addition of IPTG at 1 - 50 mM.

65 A process as claimed in any one of claims 62 to 64 wherein the nucleic acid encoding the template fragment is operably linked to a cytomegalovirus promoter sequence.

66 A process as claimed in any one of claims 62 to 65 wherein the nucleic acid encoding the template fragment is introduced as a template vector and the nucleic acid encoding the precursor protein is introduced as a separate precursor protein vector.

67 A process as claimed in claim 66 wherein the precursor protein vector is derived from the pOPRSVICAT vector into which the nucleic acid encoding the precursor protein is cloned.

68 A process as claimed in claim 66 or claim 67 wherein the template fragment vector is derived from the plasmid pZeo295-391 vector into which the nucleic acid encoding the precursor protein is cloned.

69 A process as claimed in any one of claims 62 to 68 wherein the precursor protein is tau.

70 A process as claimed in claim 69 wherein the nucleic acid encoding the template fragment encodes a core fragment of tau.

71 A process as claimed in claim 70 wherein the nucleic acid encoding the template fragment encodes encodes a fragment of tau extending from between amino acids 186-296 to 390-441 of the full-length protein.

72 A process as claimed in claim 71 wherein the nucleic acid encoding the template fragment encodes encodes a fragment of tau extending from between aminoacids 295, 296 or 297 to amino acid residues 390 or 391 of the full-length tau protein shown in Fig 7.

73 A process as claimed in claim 72 wherein the nucleic acid encoding the template fragment encodes encodes a fragment of tau extending between amino acid residues 295 to 391 as shown in Fig 7.

74 A composition of matter comprising nucleic acid encoding (i) a template fragment of the precursor protein such that the template fragment is constitutively expressed in the cell at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly expressed in the cell in response to a stimulus, which nucleic acid is described in any one of claims 62 to 72.

75 A mammalian host cell transformed with nucleic acid of claim 74 such as to express (i) a template fragment of the precursor protein such that the template fragment constitutively at a level which is not toxic to the cell; and (ii) the precursor protein such that the disease protein is inducibly in response to a stimulus.

76 A cell as claimed in claim 75 which is from a neuronal cell line or a fibroblast cell line.

77 A cell as claimed in claim 76 which is selected from the following cell lines: 3T3; NIE-115; 3T6; N2A; SY5Y; COS-7.

78 A kit comprising a host cell as claimed in any one of claims 75 to 77 plus at least one further component selected from: an agent for stimulating production of the precursor protein or an agent for detecting the interaction of the precursor protein with the template fragment.

79 A kit as claimed in claim 78 wherein the detection agent is an antibody.

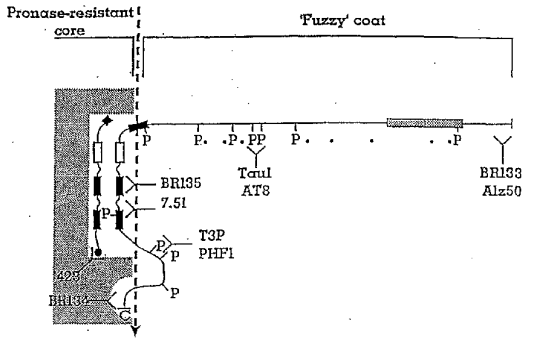
80 A nucleic acid primer selected from:

5'-3' T40-Not I
5'-gtc gac tct aga ggc ggc cgc ATG GCT GAG CCC CGG CAG GAG-3'

3'-5' T40-Not I
5'-act ctt aag ggt cgc ggc cgc TCA CAA CAA ACC CTG CTT GGC CAG -3'

295 sense primer
5' - CGG AAT TCC ACC ATG GAT AAT ATC AAA CAC GTC CCG - 3'

391 anti-sense primer
5' - C GCG GGA TCC TCA CTC CGC CCC GTG GTC TGT CTT GGC - 3'



Antibody	Extracellular Stages 3	Intra-/Extra-Cellular Stages 2	Intracellular Stage 1
BR 133	-	-	+++
mAb 18.8	-	-	+++
BR 134	+	++	+++
mAb 11.57	+/-	++	+++
mAb 25.6E	+/-	++	+++
BR 135	+++	+++	+++
mAb 423	+++	+++	+

Figure 1

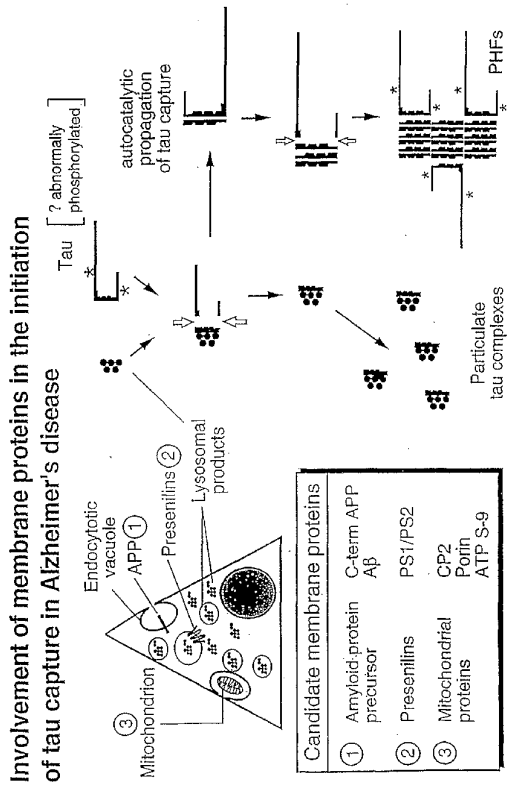

Involvement of membrane proteins in the initiation of tau capture in Alzheimer's disease
Tau
? abnormally phosphorylated
autocatalytic propagation of tau capture
PHFs
Endocytotic vacuole
APP (1)
Presenilins (2)
Lysosomal products
Mitochondrion (3)
Particulate tau complexes
Candidate membrane proteins
(1) Amyloid-protein precursor
C-term APP
Aβ
(2) Presenilins
PS1/PS2
(3) Mitochondrial proteins
CP2
Porin
ATP S-9

Figure 2

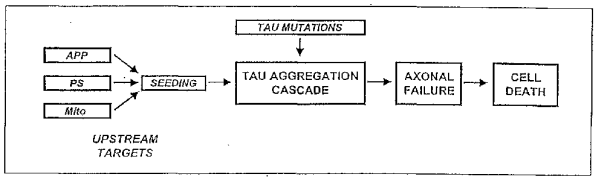

TAU MUTATIONS
APP
PS
Mito
SEEDING
TAU AGGREGATION CASCADE
AXONAL FAILURE
CELL DEATH
UPSTREAM TARGETS

Figure 3

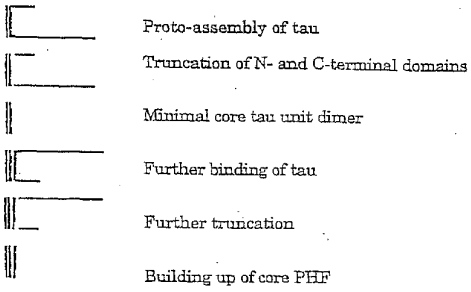

Proto-assembly of tau
Truncation of N- and C-terminal domains
Minimal core tau unit dimer
Further binding of tau
Further truncation
Building up of core PHF

Figure 4

Diseases of protein aggregation

Protein	Disease	Aggregating domain and/or mutations	Fibril subunit size (kDa)	Reference
Neurodegenerative disorders				
Prion protein	Prion diseases (CJD, nvCJD, Fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome, Kuru)	*Inherited and sporadic forms* Pro-227-Asn, many mutations Palindromic domains: 113-120, 178-191, 202-218	27	Prusiner (1998) Gasset et al. (1992)
Tau protein	Alzheimer's disease, Down's syndrome, FTDP-17, Pick's disease, progressive supranuclear palsy, Pick's disease, parkinsonism with dementia complex of Guam	*Inherited and sporadic forms* Three-repeat (3R) and four-repeat (4R) domain) 267-380 Mutations in tau in FTDP-17 Many mutations in presenilin proteins	10-12	Wischik et al. (1988) Hutton et al. (1998) Czech et al. (2000)
Amyloid β-protein	Alzheimer's disease, Down's syndrome	*Inherited and sporadic forms* Amyloid β-protein; 1-40/43; 11 mutations in APP in rare families	4	Glenner & Wong, (1984) Goate et al. (1991)
Huntingtin Ataxins (1, 2, 3, 7) Atrophin Androgen receptor	Huntington's disease Spinocerebellar ataxia (SCA1, 2, 3, 7) Dentatorubropallidoluysian atrophy (DRPLA) Spinal and bulbar muscular atrophy	Mutations in protein with expanded glutamine repeats Proteins with expanded glutamine repeats Proteins with expanded glutamine repeats Proteins with expanded glutamine repeats	40	DiFiglia et al. (1997) Paulson et al. (2000) Paulson et al. (2000) Paulson et al. (2000)
Neuroserpin	Familial encephalopathy with neuronal inclusion bodies (FENIB)	Neuroserpin; S49P, S52R	57	Davis et al. (1999)
α-Synuclein	Parkinson's disease, dementia with Lewy bodies, multiple system atrophy	*Inherited and sporadic forms* A53T, A30P. In rare autosomal-dominant PD families	19	Spillantini et al. (1998) Polymeropoulos et al. (1997)
Cystatin C	Hereditary cerebral angiopathy (Icelandic)	Cystatin C less 10 residues; L68Q	12-13	Abrahamson et al. (1992)
Superoxide dismutase 1	Amyotrophic lateral sclerosis	SOD1 mutations		Shibata et al. (1998)
Non-neurodegenerative disorders				
Haemoglobin	Sickle cell anaemia Indusion body haemolysis	Haemoglobin beta chain (S) Many mutations		Carrell & Gooptu (1998)
Serpins	α1-Antitrypsin deficiency (emphysema, cirrhosis) Antithrombin deficiency (thromboembolic disease) C1-inhibitor deficiency (angioedema)	Mutations Mutations Mutations		Lomas et al. (1992) Carrell & Gooptu (1998) Carrell & Gooptu (1998)
Immunoglobulin light chain	Plasma cell dyscrasias (primary systemic AL amyloidosis)	light chain or fragments	0.5-25	Westermark et al. (1995)
Serum amyloid A	Reactive, secondary systemic AA amyloidosis Chronic inflammatory disease	Variable N-terminal fragments of SAA	4.5-10	Westermark et al. (1995)

(continued.....)

Figure 5a

(.....continued)

Transthyretin	Familial amyloid polyneuropathy (systemic; FAP I)	Tetramer dissociated to conformational monomer variant Many mutations (some not associated with amyloid; several different types of disease)	10-14	Gustavsson et al. (1991)	
	Senile cardiac amyloidosis	Normal transthyretin	10-14	Gustavsson et al. (1991)	
Gelsolin	Familial amyloidosis - Finnish type (FAP IV)	D187G leads to truncated 173-226/243 (critical residues 187-192)	8.5	Maury & Baumann (1990)	
β2-Microglobulin	Haemodialysis amyloidosis Prostatic amyloid	β2-Microglobulin	12-25	Gorevic et al. (1985)	
Apolipoprotein AI	Familial amyloid polyneuropathy (systemic FAP III)	N-terminal 83-93 residues; G26R, W50R, L60R	9	Booth et al. (1996)	
Lysozyme	Familial visceral amyloidosis	Lysozyme or fragments (with or without ISST, D67H)	14	Pepys et al. (1993)	
Amylin (islet amyloid polypeptide)	Type II diabetes (NIDDM)	Fragments (critical core of 20-29); no mutations	3,9	Westermark (1990)	
Fibrinogen α-chain	Hereditary renal amyloidosis	Fibrinogen fragments	7-10	Uemichi et al. (1994)	
Procalcitonin	Medullary carcinoma of thyroid	Calcitonin fragments	3,4	Stefani et al. (1975)	
Atrial natriuretic factor	Cardiac amyloidosis	ANF, no mutants	3,5	Johansson et al. (1987)	
Insulin	Injection localised amyloidosis	Insulin		Dische et al. (1988)	
Other proteins forming amyloid	(*in vitro*)	Other proteins		Chiti et al. (1999)	

Figure 5b

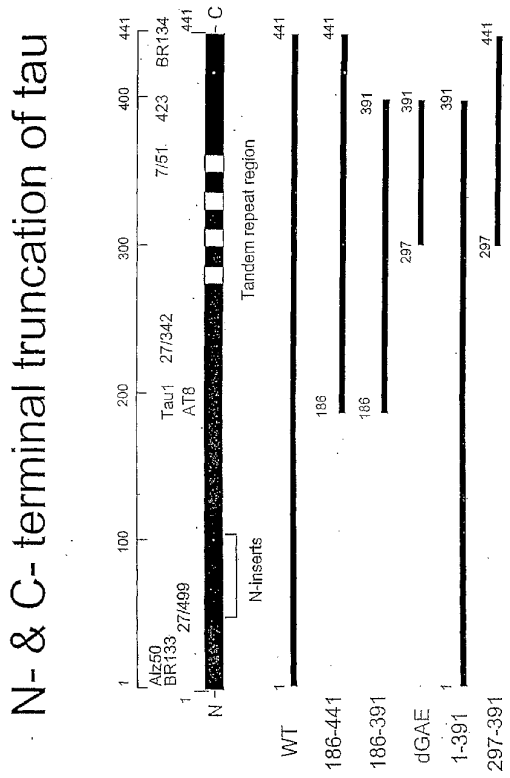

N- & C- terminal truncation of tau
1
100
200
300
400
441
Alz50
BR133
27/499
Tau1
AT8
27/342
7/51
423
BR134
N
C
N-inserts
Tandem repeat region
WT
186-441
186-391
dGAE
1-391
297-391
186
297
391

Figure 6

```
ATG GCT GAG CCC CGC CAG GAG TTC GAA GTG ATG GAA GAT CAC GCT GGG
Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly
  1               5                  10                  15

ACG TAC GGG TTG GGG GAC AGG AAA GAT CAG GGG GGC TAC ACC ATG CAC
Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr Met His
            20                  25                  30

CAA GAC CAA GAG GGT GAC ACG GAC GCT GGC CTG AAA GAA TCT CCC CTG
Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Glu Ser Pro Leu
        35                  40                  45

CAG ACC CCC ACT GAG GAC GGA TCT GAG GAA CCG GGC TCT GAA ACC TCT
Gln Thr Pro Thr Glu Asp Gly Ser Glu Glu Pro Gly Ser Glu Thr Ser
    50                  55                  60

GAT GCT AAG AGC ACT CCA ACA GCG GAA GAT GTG ACA GCA CCC TTA GTG
Asp Ala Lys Ser Thr Pro Thr Ala Glu Asp Val Thr Ala Pro Leu Val
65                  70                  75                  80

GAT GAG GGA GCT CCC GGC AAG CAG GCT GCC GCG CAG CCC CAC ACG GAG
Asp Glu Gly Ala Pro Gly Lys Gln Ala Ala Ala Gln Pro His Thr Glu
                85                  90                  95

ATC CCA GAA GGA ACC ACA GCT GAA GAA GCA GGC ATT GGA GAC ACC CCC
Ile Pro Glu Gly Thr Thr Ala Glu Glu Ala Gly Ile Gly Asp Thr Pro
            100                 105                 110

AGC CTG GAA GAC GAA GCT GCT GGT CAC GTG ACC CAA GCT CGC ATG GTC
Ser Leu Glu Asp Glu Ala Ala Gly His Val Thr Gln Ala Arg Met Val
        115                 120                 125

AGT AAA AGC AAA GAC GGG ACT GGA AGC GAT GAC AAA AAA GCC AAG GGG
Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp Asp Lys Lys Ala Lys Gly
    130                 135                 140

GCT GAT GGT AAA ACG AAG ATC GCC ACA CCG CGG GGA GCA GCC CCT CCA
Ala Asp Gly Lys Thr Lys Ile Ala Thr Pro Arg Gly Ala Ala Pro Pro
145                 150                 155                 160

GGC CAG AAG GGC CAG GCC AAC GCC ACC AGG ATT CCA GCA AAA ACC CCG
Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro
                165                 170                 175

CCC GCT CCA AAG ACA CCA CCC AGC TCT GGT GAA CCT CCA AAA TCA GGG
Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro Lys Ser Gly
            180                 185                 190

GAT CGC AGC GGC TAC AGC AGC CCC GGC TCC CCA GGC ACT CCC GGC AGC
Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser Pro Gly Thr Pro Gly Ser
        195                 200                 205

CGC TCC CGC ACC CCG TCC CTT CCA ACC CCA CCC ACC CGG GAG CCC AAG
Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu Pro Lys
    210                 215                 220
```

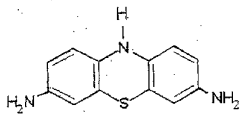
Figure 7a

```
AAG GTG GCA GTG GTC CGT ACT CCA CCC AAG TCG CTG TCT TCC GCC AAG
Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Leu Ser Ser Ala Lys
225                 230                 235                 240

AGC CGC CTG CAG ACA GCC CCC GTG CCC ATG CCA GAC CTG AAG AAT GGC
Ser Arg Leu Gln Thr Ala Pro Val Pro Met Pro Asp Leu Lys Asn Gly
                245                 250                 255

AAG TCC AAG ATC GGC TCC ACT GAG AAC CTG AAG CAC CAG CCG GGA GGC
Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys His Gln Pro Gly Gly
            260                 265                 270

GGG AAG GTG CAG ATA ATT AAT AAG AAG CTG GAT CTT AGC AAC GTC CAG
Gly Lys Val Gln Ile Ile Asn Lys Lys Leu Asp Leu Ser Asn Val Gln
        275                 280                 285

TCC AAG TGT GGC TCA AAG GAT AAT ATC AAA CAG GTC CCG GGA GGC GGC
Ser Lys Cys Gly Ser Lys Asp Asn Ile Lys Gln Val Pro Gly Gly Gly
    290                 295                 300

AGT GTG CAA ATA GTC TAC AAA CCA GTT GAC CTG AGC AAG GTG ACC TCC
Ser Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys Val Thr Ser
305                 310                 315                 320

AAG TGT GGC TCA TTA GGC AAC ATC CAT CAT AAA CCA GGA GGT GGC CAG
Lys Cys Gly Ser Leu Gly Asn Ile His His Lys Pro Gly Gly Gly Gln
                325                 330                 335

GTG GAA GTA AAA TCT GAG AAG CTT GAC TTC AAG GAC AGA GTC CAG TCG
Val Glu Val Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser
            340                 345                 350

AAG ATT GGG TCC CTG GAC AAT ATC ACC CAC GTC CCT GGC GGA GGA AAT
Lys Ile Gly Ser Leu Asp Asn Ile Thr His Val Pro Gly Gly Gly Asn
        355                 360                 365

AAA AAG ATT GAA ACC CAC AAG CTG ACC GTC CGC GAG AAC GCC AAA GCC
Lys Lys Ile Glu Thr His Lys Leu Thr Val Arg Glu Asn Ala Lys Ala
    370                 375                 380

AAG ACA GAC CAC GGG GCG GAG ATC GTG TAC AAG TCG CCA GTG GTG TCT
Lys Thr Asp His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser
385                 390                 395                 400

GGG GAC ACG TCT CCA CGG CAT CTC AGC AAT GTC TCC TCC ACC GGC AGC
Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser
                405                 410                 415

ATT GAC ATG GTA GAC TCG CCC CAG CTC GCC ACG CTA GCT GAC GAG GGG
Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Gly
            420                 425                 430

TCT GCC TCC CTG GCC AAG CAG GGT TTG TGA
Ser Ala Ser Leu Ala Lys Gln Gly Leu ***
        435                 440
```

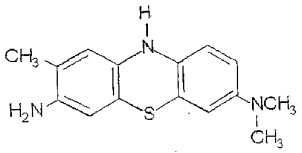
Figure 7b

Compounds tested in cell-based assays

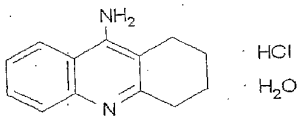
Thionine



Tolonium Chloride



Tacrine



Chlorpromazine

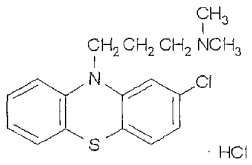


Figure 8

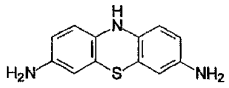
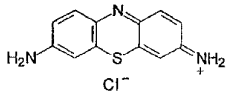
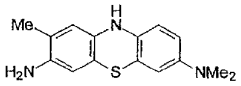
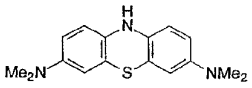
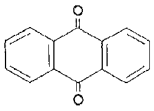
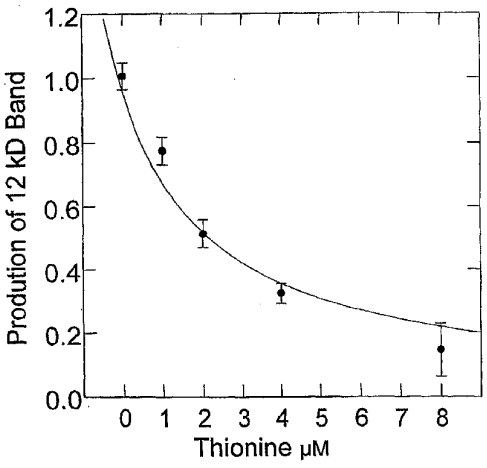
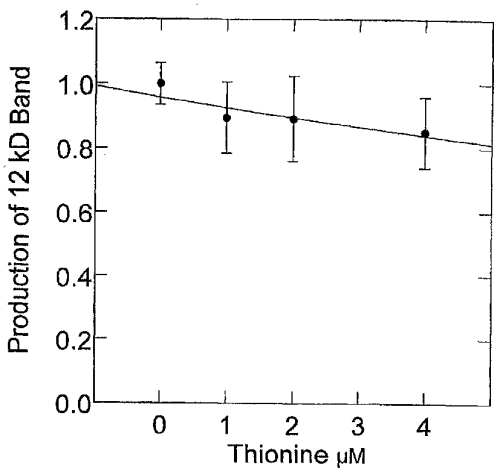
		KI (nM)	B_{50} (μM)
I	 Reduced Thionine	100	2.17
	 Oxidised Thionine	1200	26.07
	 Reduced Tolonium Chloride	105	2.28
	 Reduced Methylene Blue	123	2.67
	 DH12	—	—

Figure 9



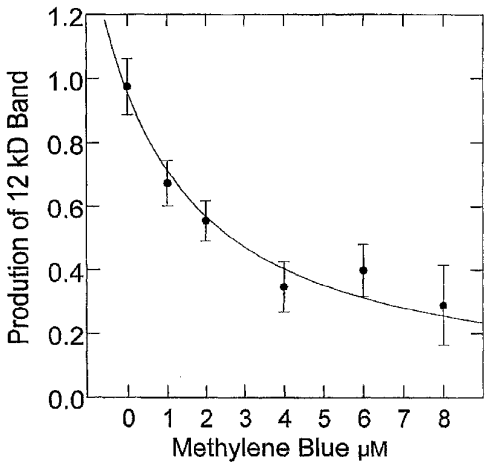
Observed vs predicted activity	r = 0.986
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Thionine KI	100 nM

Figure 10



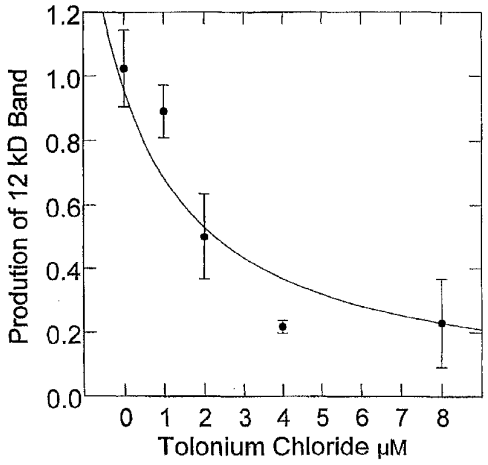
Observed vs predicted activity	r = 0.784
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Oxidised Thionine Ki	1200 nm

Figure 11



Observed vs predicted activity	r = 0.962
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Methylene Blue KI	123 nM

Figure 12



Observed vs predicted activity	r = 0.913
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
Tolonium Chloride Kl	105 nM

Figure 13

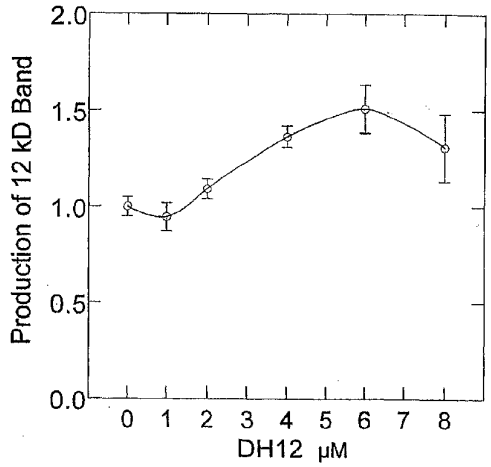
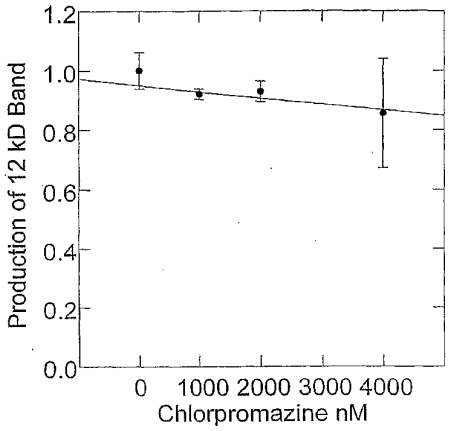
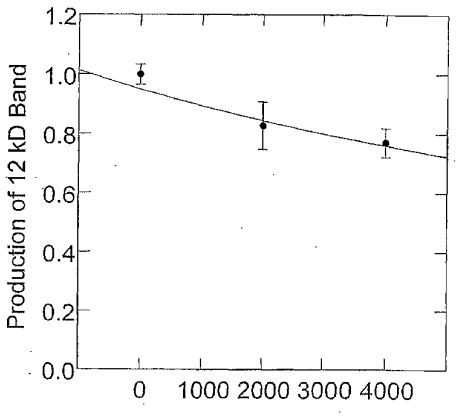


Figure 14



Observed vs predicted activity	r = 0.937
Intracellular tau concentration	415 nM
Tau-tau binding affinity	22 nM
Chlorpromazine KI	2117 nm

Figure 15



Observed vs predicted activity	r = 0.976
Intracellular tau concentration	415 nM
Tau-tau binding affinity	22 nM
Tacrine KI	802 nm

Figure 16

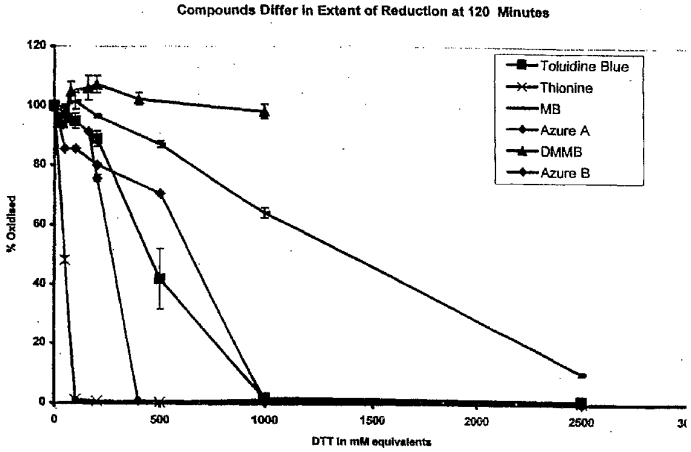

Compounds Differ in Extent of Reduction at 120 Minutes
% Oxidised
DTT in mM equivalents
Toluidine Blue
Thionine
MB
Azure A
DMMB
Azure B
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500

Figure 17

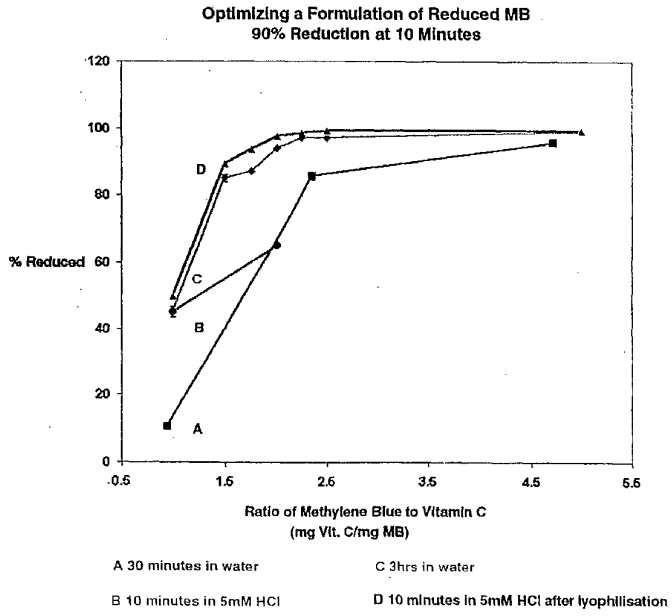

Optimizing a Formulation of Reduced MB
90% Reduction at 10 Minutes
% Reduced
120
100
80
60
40
20
0
.0.5
1.5
2.5
3.5
4.5
5.5
D
C
B
A
Ratio of Methylene Blue to Vitamin C
(mg Vit. C/mg MB)
A 30 minutes in water
B 10 minutes in 5mM HCl
C 3hrs in water
D 10 minutes in 5mM HCl after lyophilisation

Figure 18

Tissue levels vs IV dose of MB
DiSanto and Wagner (1972)

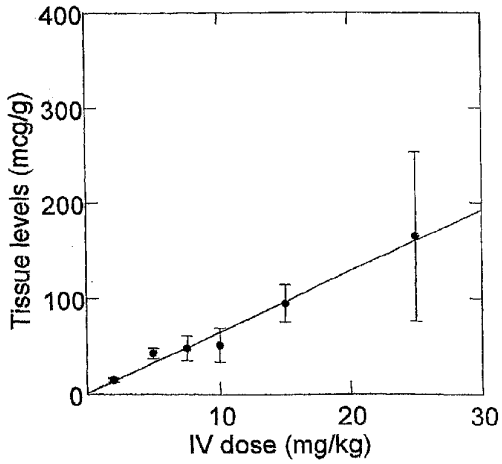


Figure 19a

Blood and Tissue distribution MB (1.43 mg/kg dose)

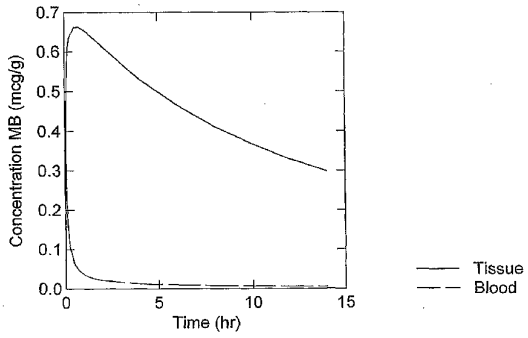


Figure 19b

Length of tau	Apparent Mr (kDa)	Expression in:	
		3T3	COS-7
1) 1-391	55	++	++++
2) m186-391	26	++	++++
3) m297-391	12	+/-	+
4) m186-441	32	++	+++
5) m297-441	18	+	+
6) 1-441	67	++	++++
7) [kozak]m295-391	12	+	+++
8) [kozak]m297-391	12	+/-	++

Figure 20

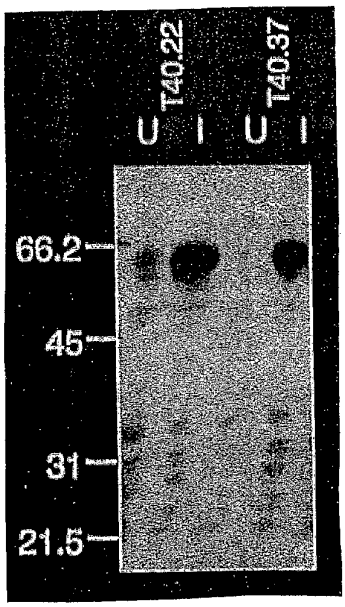


Figure 21

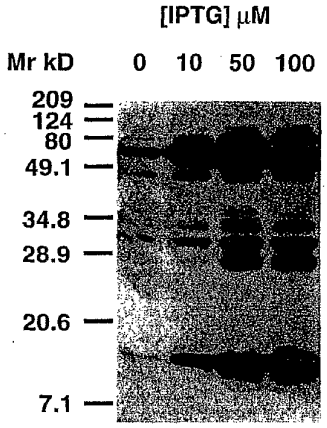


Figure 22

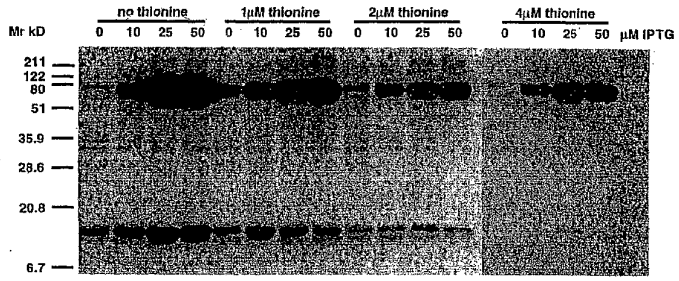

no thionine
1μM thionine
2μM thionine
4μM thionine
Mr kD
0 10 25 50 0 10 25 50 0 10 25 50 0 10 25 50 μM IPTG
211
122
80
51
35.9
28.6
20.8
6.7

Figure 23

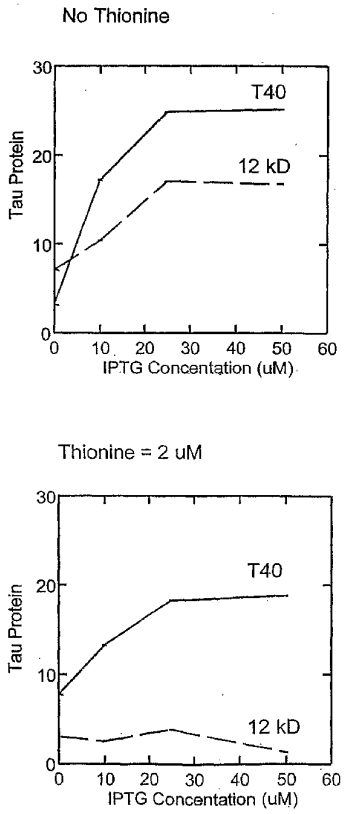

No Thionine
T40
12 kD
Tau Protein
IPTG Concentation (uM)
Thionine = 2 uM
T40
12 kD
Tau Protein
IPTG Concentation (uM)

Figure 24

RANK ORDER OF POTENCY (KI)

REDUCED FORMS

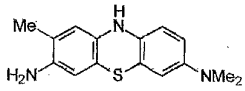
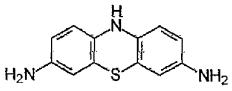
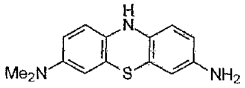
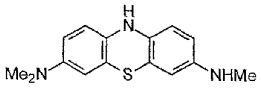
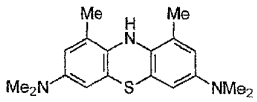
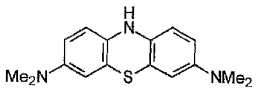
Compound	Structure	KI
Tolonium Chloride		76.05
Thionine		108.34
Azure A		119.01
Azure B		123.91
1,9-Dimethyl-methylene blue		325.41
Methylene Blue		3731.26

Figure 25

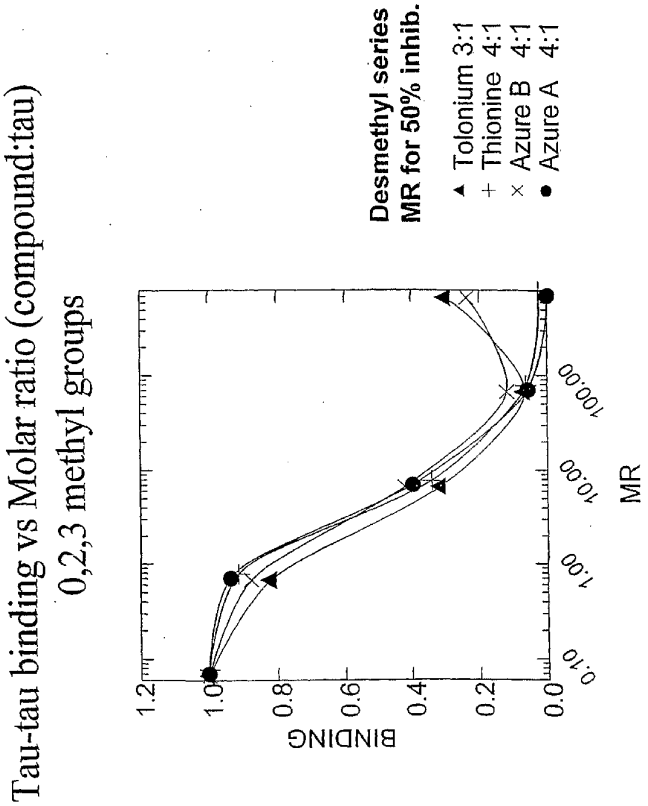

Tau-tau binding vs Molar ratio (compound:tau)
0,2,3 methyl groups
Desmethyl series
MR for 50% inhib.
▲ Tolonium 3:1
+ Thionine 4:1
× Azure B 4:1
● Azure A 4:1
BINDING
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.10
1.00
10.00
100.00
MR

Figure 26

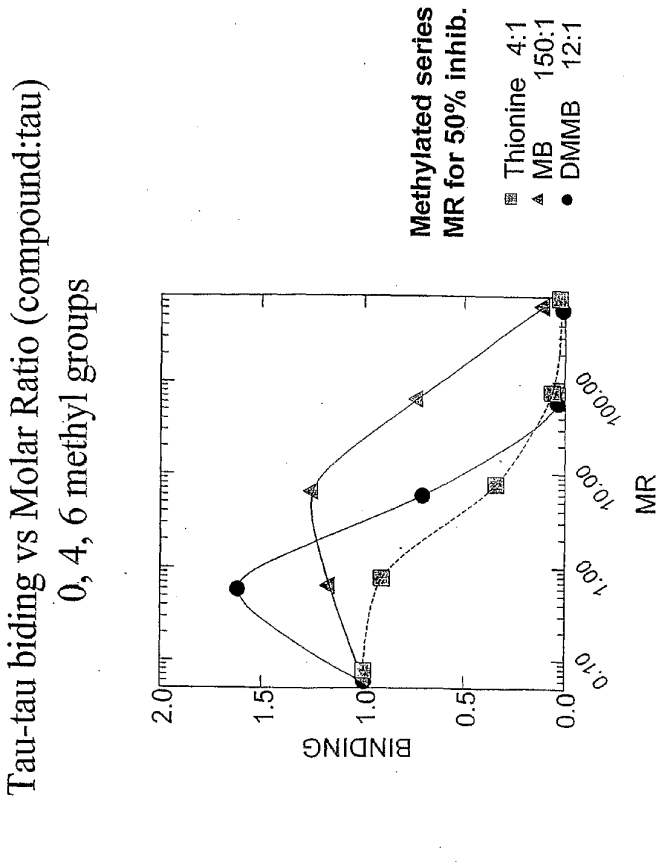

Tau-tau biding vs Molar Ratio (compound:tau)
0, 4, 6 methyl groups
Methylated series
MR for 50% inhib.
Thionine 4:1
MB 150:1
DMMB 12:1
BINDING
2.0
1.5
1.0
0.5
0.0
0:10
1:00
10:00
100:00
MR

Figure 27

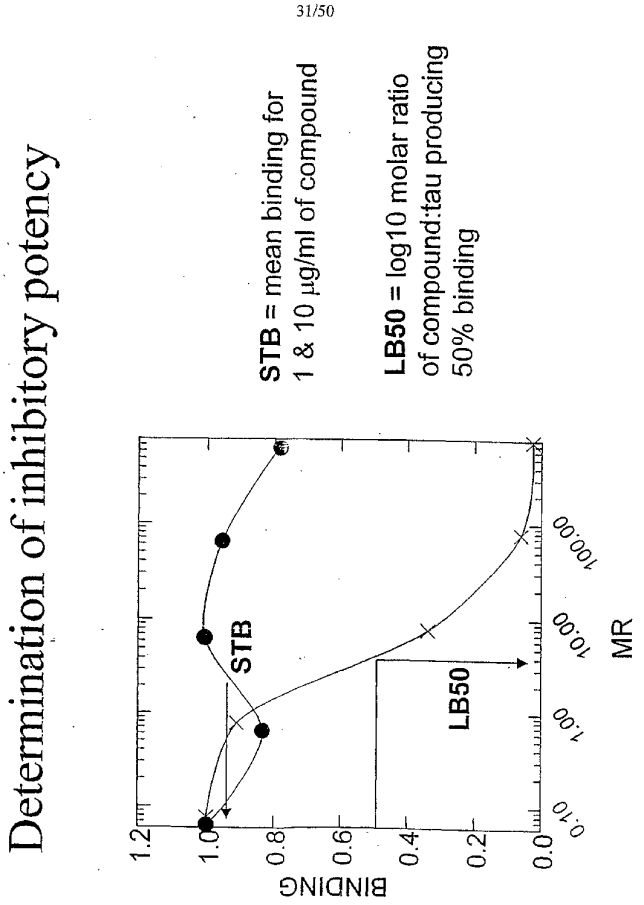

Determination of inhibitory potency
STB = mean binding for 1 & 10 µg/ml of compound
LB50 = log10 molar ratio of compound:tau producing 50% binding
BINDING
1.2
1.0
0.8
0.6
0.4
0.2
0.0
STB
LB50
0.10
1.00
10.00
100.00
MR

Figure 28

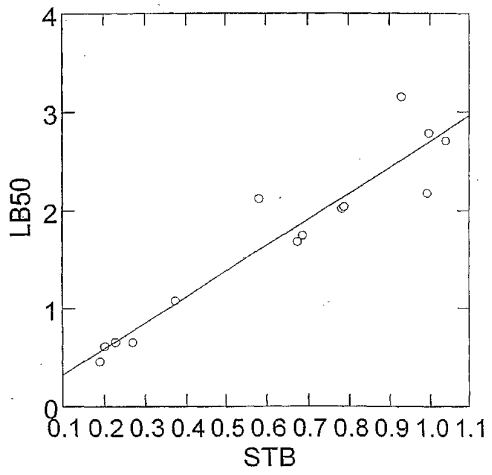


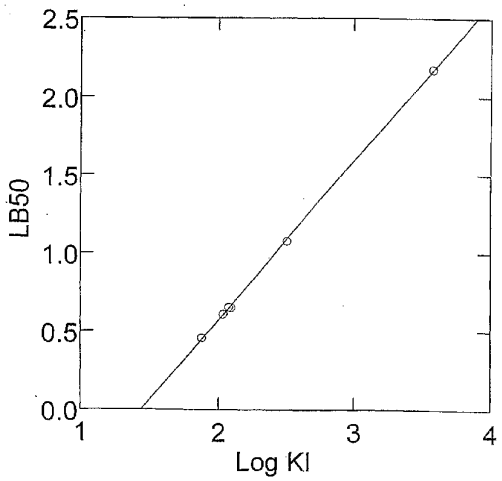
Figure 29

Relationship between STB and B50 values (molar ratio of compound:tau at which tau-tau binding is reduced by 50%)

Compound	STB	B50
Tolonium Chloride	0.190	2.86
Thionine	0.201	4.06
Azure A	0.227	4.49
Azure B	0.269	4.46
Dimethyl MB	0.372	12
Vitamin K	0.674	48
Neutral red	0.787	56
Pyronin Y	0.783	104
Primulin	0.788	109
Acraflavin	0.583	132
Methylene blue (MB)	0.992	150
Phenothiazine	1.040	508
Gallocyanin	0.997	608
Thiazin red	0.929	1419

Figure 30

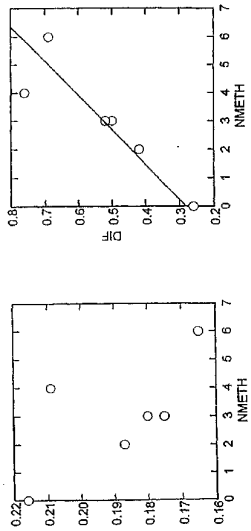
The LB50 value is an alternative representation of the KI value where this can be determined for the diaminophenothiazines



LB50 = (1.019 * Log(KI)) - 1.471

Figure 31

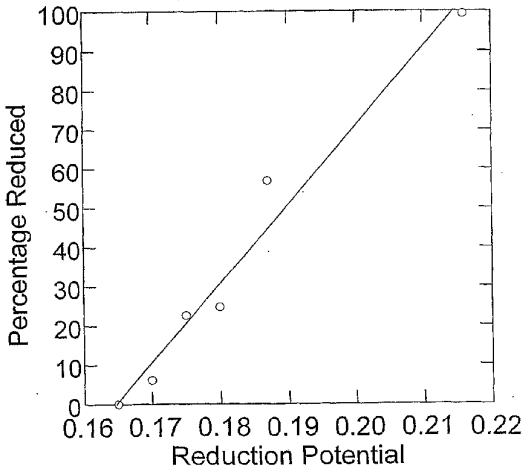
Number of methyl groups vs
Reduction potential and Diffusion coefficient



R=0.928, p=0.004
R=0.975, p=0.002

R=0.601, NS
R=0.975, p=0.002

Figure 32



R = 0.947

Figure 33

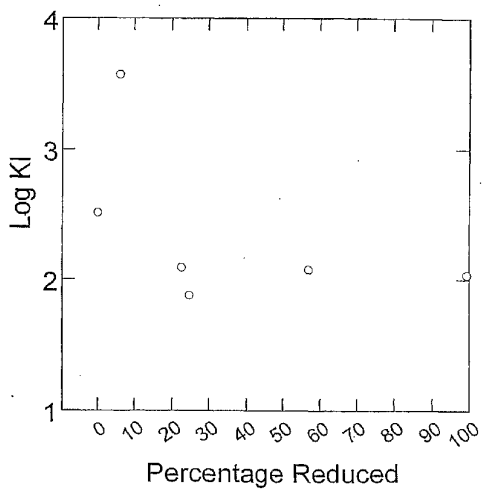
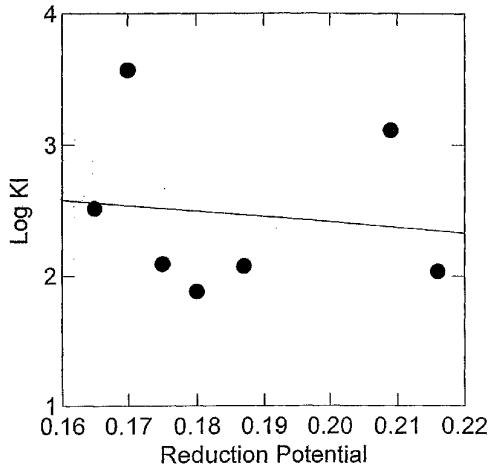
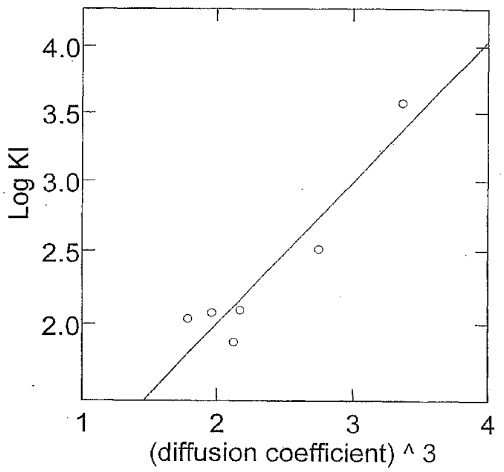


Figure 34a



Inhibitory potency is not determined by Reduction Potential

Figure 34b



Potency appears to be associated with
the aggregation efficiency of the reduced form

Figure 35

Approximation of LB50 values as function of reduction potential and diffusion coefficient

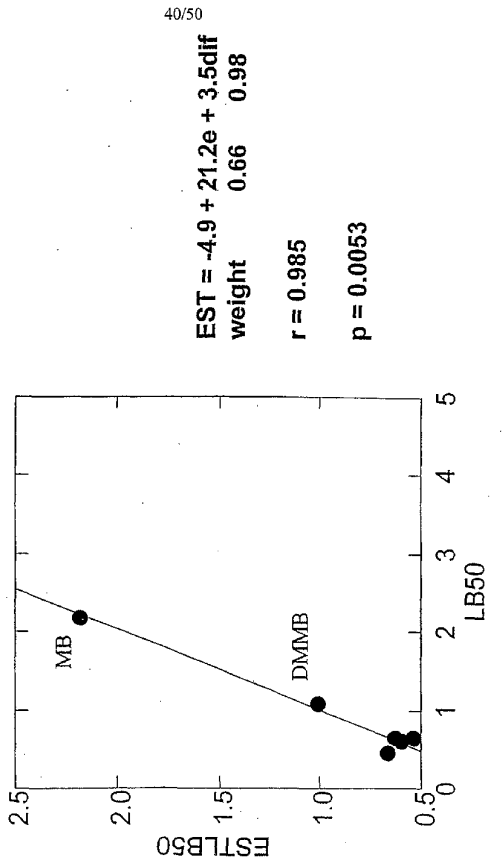


Figure 36

Approximation of STB values as function of reduction potential and diffusion coefficient

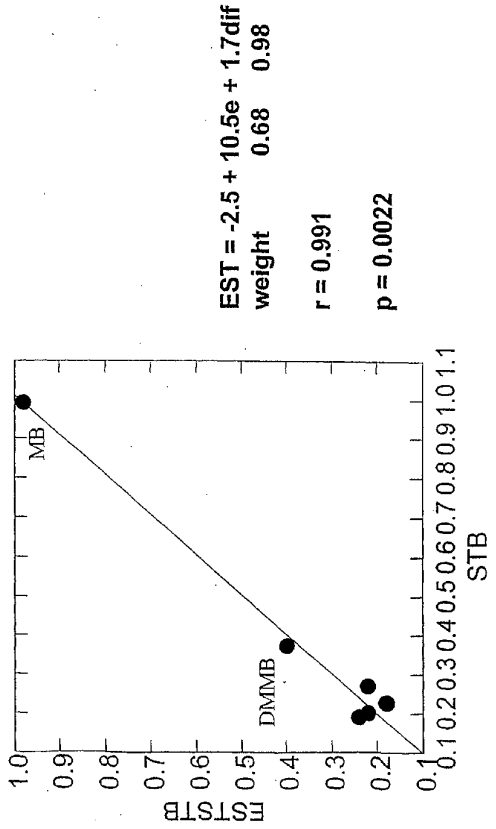


Figure 37

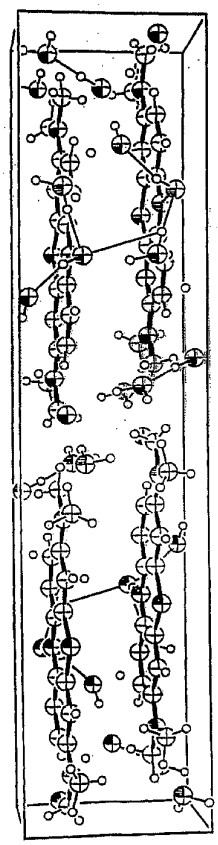


Figure 38

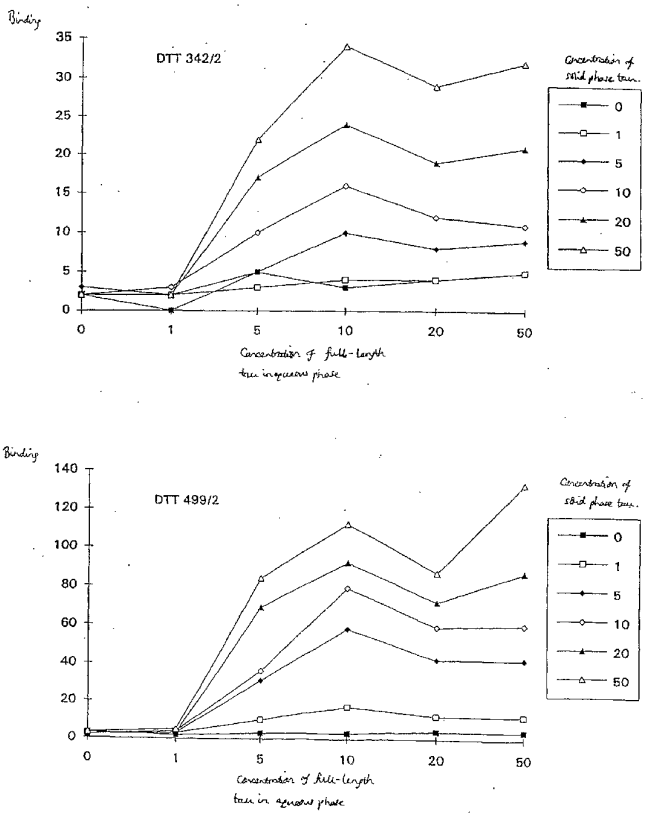

Binding
DTT 342/2
Concentration of solid phase tau
0
1
5
10
20
50
Concentration of full-length tau in aqueous phase
Binding
DTT 499/2
Concentration of solid phase tau
0
1
5
10
20
50
Concentration of full-length tau in aqueous phase

Figure 39

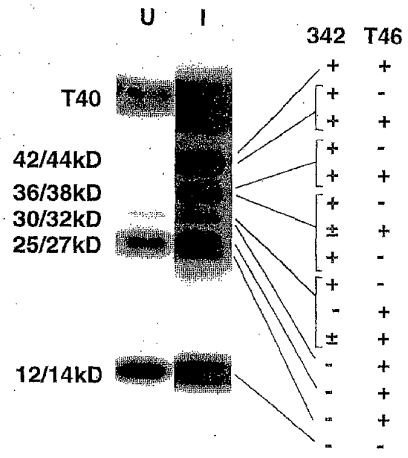


Figure 40

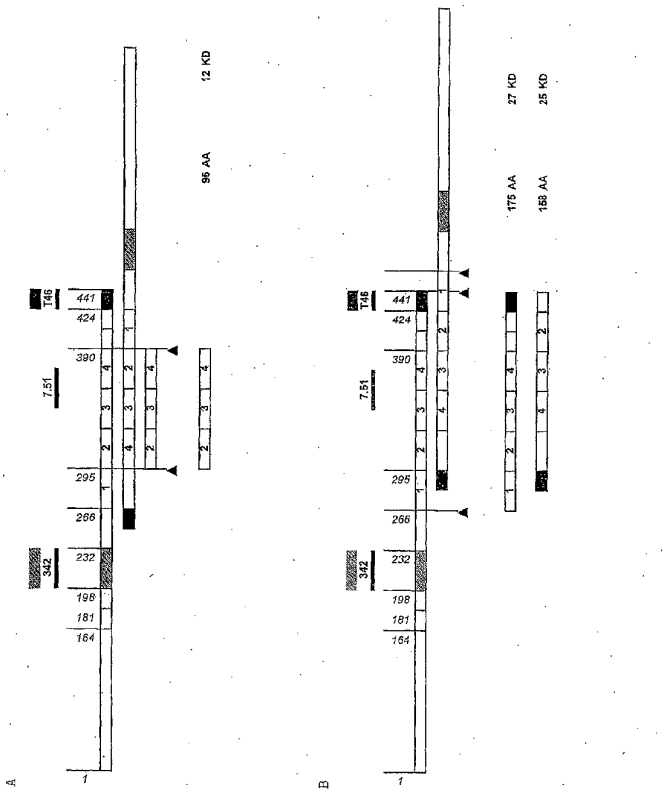

A
B
12 KD
96 AA
27 KD
25 KD
175 AA
168 AA
T46
342
7.51
441
424
390
295
266
232
198
181
164
1

Figure 41

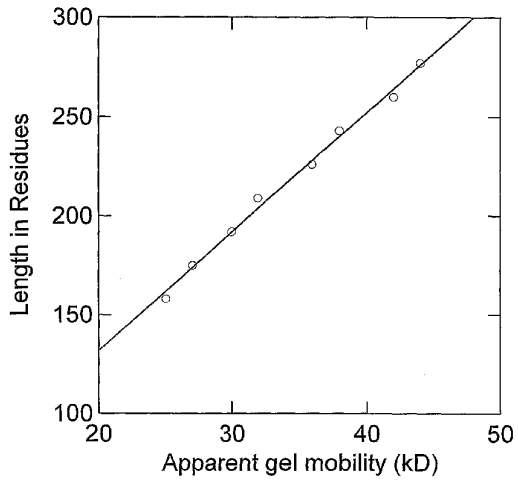
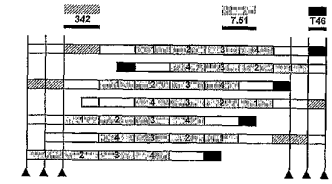

300
250
200
150
100
Length in Residues
20
30
40
50
Apparent gel mobility (kD)

Figure 42

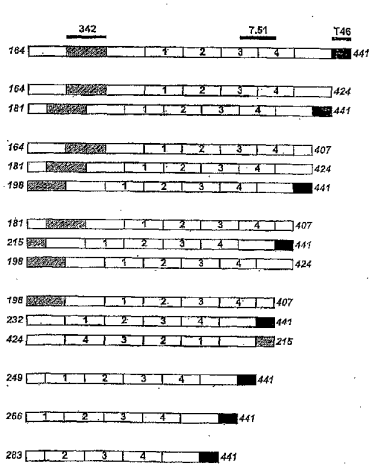
DERIVATION OF OBSERVED FRAGMENTS FROM PROTEOLYTIC PROCESSING OF HEPTAMERIC AGGREGATE



	AA	KD	342	T46
164 — 441	277	44	+	+
164 — 424	260	42	+	-
164 — 407	243	38	+	-
198 — 441	243	38	+	+
232 — 441	209	32	-	+
266 — 441	175	27	-	+
181 — 441	260	42	+	+
181 — 424	243	38	+	-
181 — 407	226	36	+	-
215 — 441	226	36	+/-	+
249 — 441	192	30	-	+
283 — 441	158	25	-	+
198 — 441	243	38	+	+
198 — 424	226	36	+	-
198 — 407	209	32	+	-
232 — 441	209	32	-	+
266 — 441	175	27	-	+
441 — 249	192	30	-	+
441 — 266	175	27	-	+
441 — 283	158	25	-	+
441 — 215	226	36	+/-	+
441 — 232	209	32	-	+
441 — 249	192	30	-	+
441 — 181	260	42	+	+
424 — 181	243	38	+	-
441 — 198	243	38	+	+
424 — 198	226	36	+	-
441 — 215	226	36	+/-	+
424 — 215	209	32	+/-	-

Figure 43

FRAGMENTS DERIVED FROM PROTEOLYTIC PROCESSING OF HEPTAMERIC AGGREGATE



AA	KD	342	T46
277	44	+	+
260	42	+	-
280	42	+	+
243	38	+	-
243	38	+	-
243	38	+	+
226	36	+	-
226	36	+/-	+
226	36	+	-
209	32	+	-
209	32	-	+
209	32	+/-	-
192	30	-	+
175	27	-	+
158	25	-	+

Figure 44

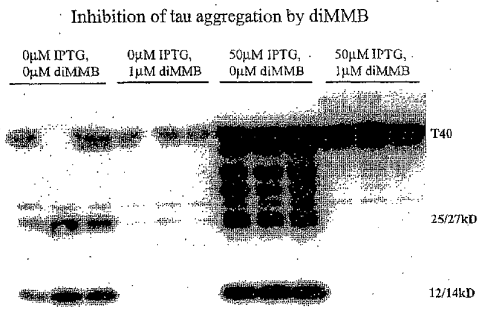
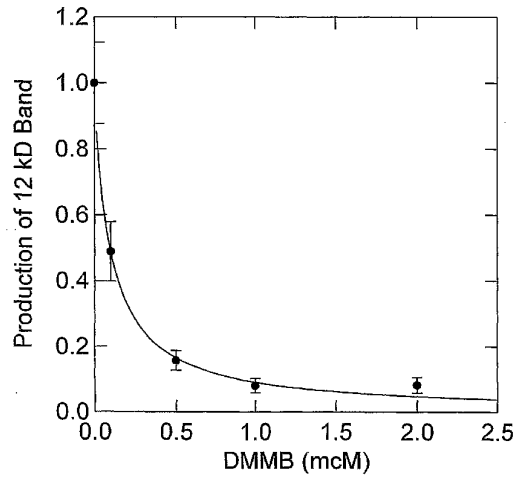

Inhibition of tau aggregation by diMMB
0μM IPTG, 0μM diMMB
0μM IPTG, 1μM diMMB
50μM IPTG, 0μM diMMB
50μM IPTG, 1μM diMMB
T40
25/27kD
12/14kD

Figure 45



Observed vs predicted activity	r = 1.00
Intracellular tau concentration	500 nM
Tau-tau binding affinity	22 nM
DMMB KI	4.4 nM
DMMB B50	100 nM

Figure 46

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/00153

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/85 A61P25/28 G01N33/68 C07K14/47 A61K31/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, MEDLINE, BIOSIS, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WISCHIK C M ET AL: "SELECTIVE INHIBITION OF ALZHEIMER DISEASE-LIKE TAU AGGREGATION BY PHENOTHIAZINES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 93, 1 October 1996 (1996-10-01), pages 11213-11218, XP002067057 ISSN: 0027-8424 cited in the application see whole doc. esp. fig.4 and discussion ---	1-35
X	EP 1 067 386 A (UNIV ABERDEEN) 10 January 2001 (2001-01-10) see whole doc. esp. claims and expl.7, p.19 --- -/--	1-38, 61-79

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2002	13.09.02
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Mueller, F

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 02/00153

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 62548 A (GHETTI BERNARDINO;FARLOW MARTIN R; GOEDERT MICHEL; KLUG AARON; MURRELL) 9 December 1999 (1999-12-09) see whole doc. esp. claims and p.41, 2.par. ---	1-35
X	WO 96 30766 A (HOFFMANN LA ROCHE ;HARRINGTON CHARLES ROBERT (GB); WISCHIK CLAUDE) 3 October 1996 (1996-10-03) cited in the application see whole doc. esp. claims 12ff. and p.46, l.23 ---	1-38, 61-79
A	BERGEN VON M ET AL: "ASSEMBLY OF TAU PROTEIN INTO ALZHEIMER PAIRED HELICAL FILAMENTS DEPENDS ON A LOCAL SEQUENCE MOTIF (306VQIVYK311) FORMING BETA STRUCTURE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 10, 9 May 2000 (2000-05-09), pages 5129-5134, XP000982226 ISSN: 0027-8424 ---	
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A	FR 2 788 436 A (PF MEDICAMENT) 21 July 2000 (2000-07-21) the whole document ---	39-60
E	WO 02 03972 A (WUELFERT ERNEST ;SALOMON ANDREW MARC (GB); AKTINSON ANTHONY (GB);) 17 January 2002 (2002-01-17) see whole doc. esp. p.10,l.11 ff. examples and claims ---	39-60
E	WO 02 04025 A (WUELFERT ERNEST ;SALOMON ANDREW MARC (GB); ATKINSON ANTHONY (GB);) 17 January 2002 (2002-01-17) see whole doc. esp. claims, p.4,l.10-16, p.10, l.11-16, -----	39-60

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/00153

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 38, 56-69 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

International Application No. PCT/GB 02/00153

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-38,61-79 complete

method of proteolytically converting a precursor protein to a fragment product charatcterized by establishing stable cell lines transfected with the related nucleic acid constructs, using this method for identifying modulators of such a system, prepartion of such a system, nucleic acid primers therefore; host cells thereof, kits therefore

2. Claims: 39-60 (complete)

use of phenothiazine for the preparation of a medicament

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/00153

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 1067386	A	10-01-2001	EP 1067386	A2	10-01-2001
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			AU 5334496	A	16-10-1996
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			TR 9701039	T1	21-02-1998
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			AU 7077801	A	21-01-2002
			WO 0203972	A2	17-01-2002
			WO 0204025	A1	17-01-2002

Form PCT/ISA/210 (patent family annex) (July 1998)

フロントページの続き

(51)Int.Cl.[7]	FI		テーマコード（参考）
Ａ６１Ｐ 25/28	Ａ６１Ｐ 25/28		４Ｃ０８４
Ｃ１２Ｎ 5/10	Ｃ１２Ｑ 1/02		４Ｃ０８６
Ｃ１２Ｑ 1/02	Ｇ０１Ｎ 33/15	Ｚ	
Ｇ０１Ｎ 27/447	Ｇ０１Ｎ 33/50	Ｚ	
Ｇ０１Ｎ 33/15	Ｇ０１Ｎ 33/53	Ｄ	
Ｇ０１Ｎ 33/50	Ｇ０１Ｎ 33/577	Ｂ	
Ｇ０１Ｎ 33/53	Ｃ１２Ｎ 5/00	Ｂ	
Ｇ０１Ｎ 33/577	Ｇ０１Ｎ 27/26	３０１Ａ	

(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZM,ZW

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Ｆターム(参考) 2G045 AA35 BB14 CB01 DA36 FA16 FB03 FB05 FB09
4B024 AA01 AA11 BA80 CA04 DA02 EA04 FA04 GA13 GA25 GA27 HA01 HA12 HA15
4B063 QA18 QQ08 QQ13 QQ79 QQ96 QR59 QR69 QR77 QR80 QS24 QS33
4B065 AA91Y AA93Y AB01 AC14 BA05 CA24 CA44 CA46
4C076 CC01 DD59Q FF63
4C084 AA17 MA05 NA14 ZA02 ZA16
4C086 AA01 AA02 BC89 MA01 MA03 MA04 MA05 NA14 ZA02 ZA16

专利名称(译)	<无法获取翻译>		
公开(公告)号	JP2004524831A5	公开(公告)日	2005-12-22
申请号	JP2002556766	申请日	2002-01-15
申请(专利权)人(译)	阿伯丁大学的大学法院		
[标]发明人	ヴィスシッククロードミシェル ホースレイデービッド リッカードジャネットエリザベス ハリントンチャールズロバート		
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IPC分类号	G01N33/50 A61K31/00 A61K31/5415 A61K45/00 A61K47/22 A61P25/14 A61P25/28 C07K14/47 C12N5/10 C12N15/09 C12Q1/02 G01N27/447 G01N33/15 G01N33/53 G01N33/577 G01N33/68		
CPC分类号	A61K31/00 A61K31/5415 A61P25/00 A61P25/14 A61P25/16 A61P25/28 G01N33/6896		
FI分类号	C12N15/00.ZNA.A A61K31/5415 A61K45/00 A61K47/22 A61P25/14 A61P25/28 C12Q1/02 G01N33/15.Z G01N33/50.Z G01N33/53.D G01N33/577.B C12N5/00.B G01N27/26.301.A		
F-TERM分类号	2G045/AA35 2G045/BB14 2G045/CB01 2G045/DA36 2G045/FA16 2G045/FB03 2G045/FB05 2G045/FB09 4B024/AA01 4B024/AA11 4B024/BA80 4B024/CA04 4B024/DA02 4B024/EA04 4B024/FA04 4B024/GA13 4B024/GA25 4B024/GA27 4B024/HA01 4B024/HA12 4B024/HA15 4B063/QA18 4B063/QQ08 4B063/QQ13 4B063/QQ79 4B063/QQ96 4B063/QR59 4B063/QR69 4B063/QR77 4B063/QR80 4B063/QS24 4B063/QS33 4B065/AA91Y 4B065/AA93Y 4B065/AB01 4B065/AC14 4B065/BA05 4B065/CA24 4B065/CA44 4B065/CA46 4C076/CC01 4C076/DD59Q 4C076/FF63 4C084/AA17 4C084/MA05 4C084/NA14 4C084/ZA02 4C084/ZA16 4C086/AA01 4C086/AA02 4C086/BC89 4C086/MA01 4C086/MA03 4C086/MA04 4C086/MA05 4C086/NA14 4C086/ZA02 4C086/ZA16		
代理人(译)	津国 肇 筱田文雄		
优先权	2001001049 2001-01-15 GB		
其他公开文献	JP2004524831A JP4422407B2		

摘要(译)

公开了在稳定细胞系中将前体蛋白（例如tau）蛋白水解为产物片段（例如12kd片段）的方法，其中该前体蛋白与其中该前体蛋白在病理学上聚集的疾病状态相关联（例如 tauopathy），并且该方法包括：（a）提供一种稳定的细胞系，该细胞系已被编码以下核酸的转染： 到牢房 （ii）前体蛋白，该蛋白响应刺激而在细胞中诱导表达，从而模板片段与前体蛋白的相互作用引起前体蛋白的构象变化，从而引起前体蛋白的聚集和蛋白水解加工。 产品片段的前体蛋白。 该方法优选地用于通过监测一个或多个产物带的生产（或生产的调制）来筛选聚集过程的调节剂。 还提供了基于在本发明的测定法中显示出高活性的化合物，例如，在本发明的测定法中使用的化合物，以及用于相关测定法和药物的测定法的材料。 还原二氨基吩噻嗪。