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(54) **URINARY TRYPSIN INHIBITOR TO DIAGNOSE AIDS**

VERWENDUNG VON URINTRYPSININHIBITOR ZUR DIAGNOSE VON AIDS

INHIBITEUR DE TRYPSINE URINAIRE PERMETTANT DE DEPISTER LE SIDA

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(73) Proprietor: **Technologie Integrale Ltd.**
Isle of Man IM8 IRD (GB)

(72) Inventor: **PAPUASHVILI, Marina, N.**
Moscow, 115446 (RU)

(74) Representative: **Becker Kurig Straus**
Patentanwälte
Bavariastrasse 7
80336 München (DE)

(56) References cited:
EP-A- 0 371 706

- **PATENT ABSTRACTS OF JAPAN vol. 18, no. 111 (C-1170), 23 February 1994 (1994-02-23) & JP 05 304983 A (NISSIN FOOD PRODUCTION COMPANY LTD)**

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Description

[0001] The present invention pertains to the use of the urinary trypsin inhibitor for the diagnosis of the onset of AIDS. In particular the present invention relates to the use of antibodies directed against said urinary trypsin inhibitor for the diagnosis of the onset of AIDS.

[0002] In the early 80's patients were observed in hospitals that had developed severe immuno-deficiencies. Some of them developed unusual opportunistic infections while others have been found to suffer from Kaposi's sarcoma, a hitherto rare skin tumor. An immunologic evaluation of these patients showed a marked deficiency of the cellular immune function and a selective decrease of the number of T4 (helper) cells, a subpopulation of T cells that mediate the cellular immune response. This condition with all of its accompanied symptoms was termed "Acquired Immune Deficiency Syndrome" (AIDS). The disease itself was subsequently found to be caused by a pathogenic retrovirus called human immunodeficiency virus (HIV - virus).

[0003] Soon after HIV was found to be the origin of AIDS it was observed that this virus was in particular cytopathic for T4 cells. It was therefore hypothesized that the immunological abnormalities in AIDS resulted at least in part from a progressive depletion of T4 cells due to their infection and destruction by HIV.

[0004] From the clinical point of view the "disease" AIDS is defined as the presence of a reliably diagnosed disease at least moderately indicative of cellular immune deficiency with the absence of known underlying immune deficiency and of any other reduced resistance reported to be associated with the disease, such as e. g. immunosuppressive therapy or lymphoreticular malignancy.

[0005] The disease AIDS, that is the last stage of a slowly but constantly progressing decline of the body's normal constitution, is preceded by a variety of diseases that are generically designated as "predromal AIDS". Such disorders include ARC (Aids Related Complex) which state of disease is considered to be highly predictive of the development of subsequent AIDS. ARC includes the symptoms of e.g. having fever for more than 3 months, exhibiting a loss of weight by more than 10 %, suffering from diarrhoea and showing a reduced number of T4 cells. This state of disease is known to eventually develop into the so called LAIDS (Lesser AIDS) which state is characterized by the development of oral candidasis, herpes zoster, idiopathic thrombocytopenia and other diseases that are not lethal, do, however, indicate immune suppression.

[0006] Another predictive symptom for the prospective development of AIDS is the so called LAS (lymphadenopathy syndrome). which is identified by the presence of at least three or more lymph nodes outside of the regio inguinals having a diameter of more that 1 cm.

[0007] Yet, another indication for the coming development of AIDS is the ADC symptoms (AIDS dementia complex) which is seen in the increasing number of detrimental influences on brain activity.

[0008] With the progress of molecular biology and cell biology techniques the determination of various antigenic determinants of the HIV virus itself has become possible. To this end, antibodies have been prepared with which the presence of the virus in the blood of a patient can be detected. With these tests it is, however, merely possible to determine the presence or absence of an HIV infection while the imminent manifestation of the disease as such can not reliably be predicted thereby. Hence, due to the complex picture of the predromal disease states mentioned above it is often difficult for the physician to determine whether the patient is going to develop AIDS or not within the next time.

[0009] Consequently, there exists a need in the art for a reliable and quick diagnostic tool to reliably predict the onset of the disease.

[0010] The development of an HIV infection in the human body is in general determined by two interacting factors. The unique property of HIV to weaken the infected individual's immune system and the immune response of the host against the invader, which develops along with the infection's progress.

[0011] Prior to the development of the profound immunodeficiency status the immune system still performs its task to a certain extent producing particular molecules that may be detected in the infected individual long before the various conditions described above are perceived. It was therefore considered to use such molecules for the determination of the disease.

[0012] Molecules, which are presently used for this purpose, are e.g. β-microglobulin (WO9507465) or neopterin. The use of these compounds for the determination of the disease suffers, however, from the fact that they are not particularly specific for an HIV infection and that they have to be prepared from blood samples in a complicated manner, which impedes the procedure of diagnosis. Further, the increase in the amount of those molecules in body fluids can only be determined at a stage at which other immune system parameters and virological assays already clearly indicate the development of AIDS.

[0013] Therefore, the problem underlying the present invention is to provide a novel diagnostic tool for predicting the onset of AIDS already at an early developmental stage of the disease, preferably already during predromal AIDS conditions.

[0014] The above problem has been solved by using a particular compound to be found in body fluids, the urinary trypsin inhibitor, as a diagnostic tool, and identifying its amount in said body fluids.

[0015] JP05 304 983 describes the use of UTI for the diagnosis of the disease state of a cancer patient and for determining the efficacy of treatments of the patient. This document does not refer to AIDS.

[0016] During the extensive experiments leading to the present invention it has been found that the amount

of urinary trypsin inhibitor (UTI), which may ordinarily be present in a healthy person in the urine in an amount of up to about 3 μg/ml is markedly increased in patients suffering from an HIV infection in whom the disease is just about to develop.

[0017] Thus, according to the present invention there is provided the use of UTI in the diagnosis of the onset of AIDS, wherein the amount of UTI in a body fluid sample of an AIDS patient is determined, which body fluid may be blood or preferably urine. If the amount exceeds a certain level, in the urine 3 μg/ml, this may be taken as a reliable signal that this patient will develop the disease.

[0018] The amount of UTI in a body fluid may be determined according to methods known in the art, such as by means of antibodies, that may be polyclonal or monoclonal antibodies or an antibody containing serum. Other methods of UTI determination include measurement of the antitryptic activity or antichymotryptic activity in the sample.

[0019] In the figures.

[0020] Fig. 1 is a standard curve showing the dependency of the extinction at 492 nm on the UTI concentration

[0021] During the studies leading to the present invention the manifestation of many proteinase inhibitors and the dependency of their levels on tumoral processes in a body suffering from an immunodeficiency condition was examined. One of the blood plasma inhibitors is the inter-α-trypsin inhibitor (ITI), a protein the function of which has not yet been fully elucidated. ITI is a glycoprotein with a molecular weight of about 240 kDa that is synthesized in the liver. Its concentration in blood is about 450 μg/ml.

[0022] Immunologically affixed to ITI there has been found an inhibitor having a lower molecular weight. This specific inhibitor represents a fragment of the ITI light chain containing a large number of bisulfide links which determine its acid-stability. Due to the fact that in certain pathologic conditions this inhibitor is secreted from blood into the urine said molecule is termed urinary trypsin inhibitor (UTI).

[0023] UTI is an acidic glycoprotein with a molecular weight of about 44 kDa. About 30% of its mass is comprised by two major carbohydrate chains. The molecular weight of UTI, without carbohydrates is about 30 kDa. In terms of its tertiary structure, UTI is a two-domain molecule, even though it is comprised of only one polypeptide chain. A structural homology has been noticed between both domains and also to the main pancreatic proteinase inhibitor (MPPI), present in organs of cattle. This essentially also applies to the UTI trypsin-binding domain and to its chymotrypsin-binding domain, in the region of reactive centers.

[0024] The structural homology to MPPI allows to correlate UTI to the "Kunin" inhibitors. Due to the fact that both domains of UTI are homologous to the MPPI, this polypeptide is sometimes called "Bi-Kunin". The UTI's property of inactivating elastase and neutrophilic granulocytes G cathepsin gave rise to the hypothesis that it might exhibit an anti-inflammatory effect.

[0025] On the other hand, already in 1974 a particular protein termed EDC-1 (mol. weight = 27 kDa) was isolated from urine of a patient suffering from acute myeloid leucosis. In most of the oncological patients examined so far the level of EDC-1 in urine was found to correspond to the clinical progress of the disease. It decreases significantly before the appearance of clinical signs of remission and increases prior to a clinical relapse.

[0026] The analysis of the EDC-1 amino acid sequence has shown that its N-terminal sequence generally corresponds to that of UTI while the C-terminal sequences differ. It was sometimes believed that EDC-1 and UTI may emerge by lysis of various peptide links in ITI.

[0027] Meanwhile, a potential mechanism for the increase of EDC-1's content in oncological patients has been proposed. At first stages of tumoral growth the tumor-associated proteolytic activity induces an enhanced formation of α-1-proteinase inhibitor in the host. which is substantially inactivated by the tumor's proteinases. It is only in the second turn that the tumor-associated proteinases decompose the plasma ITI until EDC-1 is formed. As a result, the concentration of EDC-1 in the serum increases while the concentration of ITI decreases.

[0028] In urine and blood serum of patients suffering from different carcinomas, such as e.g. carcinoma of the stomach, the esophagus or of the large intestine, an increased level of UTI has been noticed, which level is in correlation with the progress of the disease. After a successful application of a chemotherapy the level of UTI was found to be decreased.

[0029] Moreover, in a healthy kidney UTI has been discovered in the cytoplasma of proximal canal epithelial cells. which suggests its secretion into the urine by those cells. UTI has not been discovered in glomerules, distal canals or vascular walls. Moreover, also interstices did not contain UTI, while the tissue surrounding the tumor showed a level of UTI just like in a healthy kidney. It was, therefore, suggested that cancer cells obviously do not produce UTI.

[0030] According to the present invention the increase of the UTI level in body fluids, such like blood or, more preferably urine, may now be utilized for determining the onset of the development of diseases associated with AIDS or, more preferably predromal AIDS. In an HIV-infected patient, in whom the HIV virus is presently dormant and who does not suffer from the syndromes associated with AIDS the level of UTI in the urine is in the normal range of up to 3 μg/ml like in normal healthy people. However, when the diseases making up the syndrome are about to emerge due to the patient's immune system being weakened to an extent such that it may no longer defeat "normal" bacteriological, viral or oncological occurrences in the body so that the syndrome is

starting to emerge the level of UTI in urine markedly exceeds the normal level of up to 3 μg/ml.

[0031] This increase of UTI-level, measurable long before the symptoms of the diseases associated with the syndrome are noticeable, may be taken as an indication of the onset of the development of AIDS. This knowledge will allow the physician to start with an appropriate treatment of the patient before the respective person will effectively fall ill.

[0032] In addition by measuring the amount of UTI during a treatment of a patient with drugs, such as anti-HIV drugs or drugs specifically directed against the particular disease the patient has acquired, e.g. candidasis, the efficacy of said drug in an AIDS patient may be easily monitored. Consequently, when e.g. applying drugs, such as AZT or the so called "triple therapy", to a patient suffering from diseases associated with AIDS the decline of the disease may be monitored by determining the UTI level in the patient.

[0033] Moreover, the present invention also provides for the advantage that due to the possibility to monitor an HIV-infected person for the onset of the disease the amount of drugs administered to said patient before AIDS breaks out anyway may be reduced, so that the immense costs involved in such a therapy may be saved and in addition the possibility of creating resistance in the virus will be reduced as well.

[0034] Since the assay may also be carried out on a biological material easily available, i.e. urine, the assay is easy to perform. No skilled person, such as a physician or a medical technician taking the blood is necessary.

Examples

Example 1

Determination of UTI via antibodies

[0035] To detect the UTI, the following procedure was used: Three monoclonal antibodies, M2, B6, P1 were raised against UTI in a manner known per se and examined in the presence of the trypsin binding domain for their binding capacity. The M2 antibody was shown to have the highest affinity for this domain. On the basis of the mab M2, a competitive ELISA of UTI concentration in urine has been set up.

[0036] The methodology that was used to detect UTI with a view of determining its value in predicting the progress of HIV infection at different stages of the disease, has revealed the following:

Results

Groups of Patients

[0037] Seventeen HIV-1-infected patients and 30 healthy volunteers were examined; 4 patients had no clinical symptoms; 8 patients had different opportunistic infections, and 5 patients had already developed AIDS.

Materials and Methods

[0038] UTI ELISA (mab to UTI: secreting hybridoma were derived from a fusion of non-secreting mouse myeloma cells (clone P3 O1) with spleen cells of BALB/c mice that have been immunized with human UTI) level in urine, residual nitrogen and creatinin concentrations and a number of immunological parameter's (CD4, CD8, CD16, B-lymphocytes. HLA-DR), p24 concentration were tested.

[0039] The preparation of urinary protein concentrates:

1. Collection of urine (preferably, the morning portion); then treatment with acetone (5 ml of urine + 10 ml of acetone cooled down to +4°C);
2. The acetone precipitation of urinary proteins may be kept at +4°C for one month.
3. Spinning-down of protein precipitates (1.000 g, 10 min.).
4. Preparation of suspension of the precipitates obtained (precipitate +2,5 ml 0,05 M buffer, pH 7,8 the precipitate is triturated with a glass rod) the concentration and depigmentation of the urine.
5. The undissolved precipitate is centrifuged.
6. The supernatant thus obtained is used for the determination of the UTI.

ELISA-Test

[0040]

1. The UTI solution is adsorbed on a plate having 96-wells for one night at a temperature of 4°C;
2. The plate is rinsed with water and knocked out;
3. The filling of vacant binding-sites with albumin (in each well +200μl of 1% albumin solution in 0,05 M phosphate buffer, pH 7,4 for one-hour incubation;
4. The plate is rinsed with water and knocked out.
5. The titration into the plate's well of: (a) urine specimen (see point 6 above; (b) the standard UTI solution for the calibration curve; and (c) the control sample with the known UTI content; thereto the solution of the UTI monoclonal antibodies conjugate with horse-radish peroxidase is added and one-hour incubation; the binding of UTI from the sample and that adsorbed on the plate with the antibodies.
6. The plate is rinsed with the buffer (with 0,1% albumin and Tween®-20), and with water, and knocked out.
7. Development: addition of the substrate (ortho-phenylene-diamine in the citrate-phosphate buffer); incubating for 10 minutes.
8. The stopping of the reaction: +10% sulphuric acid.

9. The measurement of optical density with an ELISA-reader at 492 nm.
10. Drawing a calibration curve with known amounts of UTI; the standard curve was constructed using 2-fold dilution of UTI in the wells of the microtiter plate; the linear segment of the standard curve is to cover the working concentration range (see Fig. 1);
11. Determination of the UTI concentration of the specimen therefrom.

[0041] In order to determine the UTI concentration in blood, the following equipment may be used:

1. A 80-chanel pipette, 40 x 200 µl.
2. An Eppendorf® centrifuge for test-tubes (microfuge).
3. An ELISA-reader with a filter for 492 nm for plates.
4. Plates for the agents.
5. The measuring of the UTI activity, in addition to concentration, would require a photometer with a 405-nm filter.
6. A washer.

Example 2

Quantitative ATA determination

[0042] The urine sample was obtained as described in example 1.

[0043] Reagents used:

1) Trypsin concentrate: 1 mg trypsin was dissolved in 1 ml of 0.0025 M HCl with 0.1 M $CaCl_2$,; the solution may be stored at 4 °C up to 10 days;
2) working trypsin solution; dilute trypsin concentrate 50 times by 0.0025 M HCl with 0.1 M $CaCl_2$;
3) 0.2 M TRIS-HCl pH 7.8 - 8.0;
4) BAPNA concentrate (p-nitroanilide N-benzoyl-DL-arginine); dissolve 11.4 mg BAPNA in 6 ml dehydrated DMSO; store in darkness:
5) BAPNA working solution; dilute BAPNA concentrate with distilled water in a ratio of 1 : 2;

ATA measurements in the urine sample

[0044]

1)add 40 µl of trypsin working solution and 2 - 80 µl of the sample to be tested into a spectrophotometer cuvette;
2) add 0.2 M TRIS-HCl, pH 7,8 - 8,0 for a total volume up to 160 µl;
3) mix solution and incubate for 5 min at 37 °C;
4) add 40 µl of BAPNA working solution;
5) mix solution and measure the initial optical density (A_1) at 405 or 410 nm in the spectrophotometer;
6) incubate the solution for 30 min at 37 °C;
7) mix solution and measure the final optical density (A_2) at 405 or 410 nm in the spectrophotometer.

Processing of the results obtained

[0045] The quantity of ATA is calculated with the following formulae:

$$ATA = \varepsilon (B_2 - B_1 - A_2 + A_1) / (t\ V),$$

wherein A_1 und A_2 are the initial and final optical density in the presence of a tested sample. B_1 and B_2 are the initial and final optical density in the absence of the sample. V is the volume of the urine sample added, t is the reaction time and ε is a tabulated extinction coefficient of p-nitroaniline in dependence of the wavelength used and the total solution volume.

[0046] The most accurate results are obtained if

$$0.4\ (B_2 - B_1) < A_2 - A_1 < 0.6\ (B_2 - B_1)$$

Claims

1. Use of urinary trypsin inhibitor (UTI) for the in vitro diagnosis of the onset of AIDS.

2. The use according to claim 1, wherein the amount of UTI is determined in urine or blood samples.

3. The use according to any of the claims 1 or 2, wherein the amount of UTI is determined by means of polyclonal or monoclonal antibodies or antibody containing sera.

4. The use according to any of the claims 1 or 2, wherein the amount of UTI is determined via the measurement of the antitryptic activity.

5. The use according to any of the claims 1 or 2, wherein the amount of UTI is determined via the measurement of the antichymotryptic activity.

6. The use according to claim 3, wherein the antibodies are labeled with a marker.

7. The use according to claim 6, wherein the marker is a dye, a fluorescent or a radioactive marker.

8. The use according to claim 7, wherein the antibodies are applied in an ELISA assay.

9. Use of UTI in an in vitro method for determining the efficacy of an anti-HIV drug.

Patentansprüche

1. Verwendung von Urintrypsininhibitor (UTI) zur in-vitro Diagnose des Einsetzens von AIDS.

2. Verwendung nach Anspruch 1, wobei die Menge von UTI in Urin- oder Blutproben bestimmt wird.

3. Verwendung nach einem der Ansprüche 1 oder 2, wobei die Menge von UTI mittels polyklonaler oder monoklonaler Antikörper oder Antikörper-enthaltenden Seren bestimmt wird.

4. Verwendung nach einem der Ansprüche 1 oder 2, wobei die Menge von UTI durch die Bestimmung der anti-tryptischen Aktivität bestimmt wird.

5. Verwendung nach einem der Ansprüche 1 oder 2, wobei die Menge von UTI durch die Bestimmung der anti-chymotryptischen Aktivität bestimmt wird.

6. Verwendung nach Anspruch 3, wobei die Antikörper mit einem Marker markiert sind.

7. Verwendung nach Anspruch 6, wobei der Marker ein Farbstoff, ein fluoreszierender oder ein radioaktiver Marker ist.

8. Verwendung nach Anspruch 7, wobei die Antikörper in einem ELISA-Assay verwendet werden.

9. Verwendung von UTI in einem in-vitro Verfahren zur Bestimmung der Wirksamkeit eines anti-HIV Arzneimittels.

Revendications

1. Utilisation d'inhibiteur de trypsine urinaire (UTI) pour le diagnostic in vitro du début SIDA.

2. L'utilisation selon la revendication 1 dans laquelle la quantité d'UTI est déterminée dans des échantillons d'urine ou de sang.

3. L'utilisation selon l'une quelconque des revendications 1 ou 2 dans laquelle la quantité d'UTI est déterminée au moyen d'anticorps polyclonaux ou monoclonaux ou de séra contenant des anticorps.

4. L'utilisation selon l'une quelconque des revendications 1 ou 2 dans laquelle la quantité d'UTI est déterminée par la mesure de l'activité antitryptique.

5. L'utilisation selon l'une quelconque des revendications 1 ou 2 dans laquelle la quantité d'UTI est déterminée par la mesure de l'activité antichymotryptique

6. L'utilisation selon la revendication 3 dans laquelle les anticorps son étiquetés avec un marqueur.

7. L'utilisation selon la revendication 6 dans laquelle le marqueur est un colorant, un marqueur fluorescent ou radioactif.

8. L'utilisation selon la revendication 7 dans laquelle les anticorps sont mis en oeuvre dans un test ELISA.

9. Utilisation d'UTI dans une méthode in vitro pour la détermination de l'efficacité d'une drogue anti-HIV.

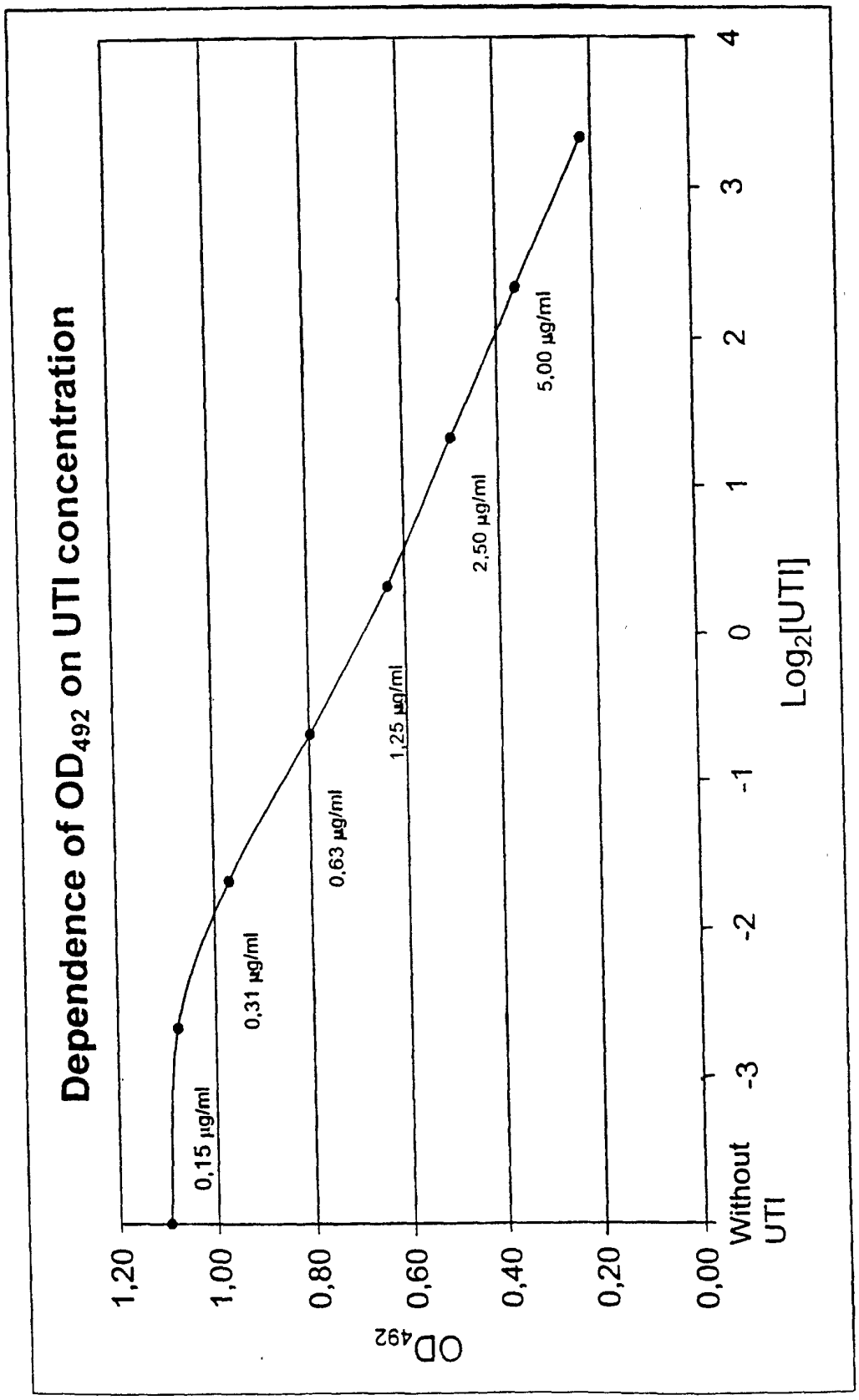
Dependence of OD492 on UTI concentration
OD492
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,15 µg/ml
0,31 µg/ml
0,63 µg/ml
1,25 µg/ml
2,50 µg/ml
5,00 µg/ml
Without UTI
-3
-2
-1
0
1
2
3
4
Log2[UTI]

FIG. 1

专利名称(译)	尿胰蛋白酶抑制剂诊断艾滋病		
公开(公告)号	EP1147420B1	公开(公告)日	2004-07-14
申请号	EP2000902627	申请日	2000-01-24
申请(专利权)人(译)	TECHNOLOGIE INTEGRALE LTD.		
当前申请(专利权)人(译)	TECHNOLOGIE INTEGRALE LTD.		
[标]发明人	PAPUASHVILI MARINA N		
发明人	PAPUASHVILI, MARINA, N.		
IPC分类号	G01N33/53 A61K38/00 A61K38/55 C12Q1/00 C12Q1/37 C12Q1/70 G01N33/543 G01N33/569 G01N33/573 G01N33/577 G01N33/68		
CPC分类号	G01N33/56988 G01N2333/16 G01N2333/811 Y10S435/975		
优先权	19903034 1999-01-26 DE 19920704 1999-05-05 DE		
其他公开文献	EP1147420A1		
外部链接	Espacenet		

摘要(译)

本发明涉及尿胰蛋白酶抑制剂用于诊断AIDS发作的用途和用于进行这种测定的试剂盒。本发明还涉及尿胰蛋白酶抑制剂在测试抗HIV药物功效中的用途。