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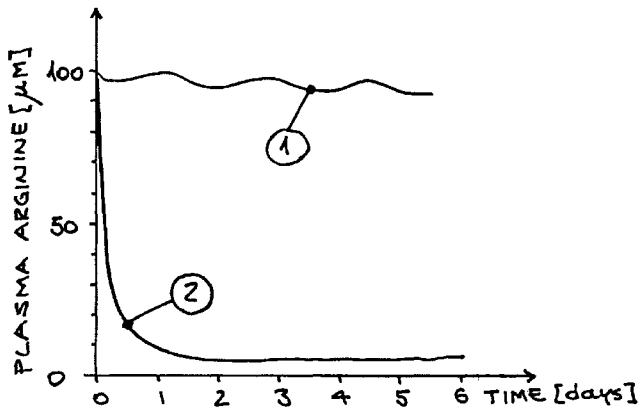
(54) Title: THERAPEUTIC COMPOSITION FOR TREATMENT OF CANCER BY ARGININE DEPLETION




(57) Abstract: A therapeutic composition and a method for the treatment of cancer by depletion of arginine without systemic complications comprising an arginine decomposing enzyme and protein breakdown inhibitors, a nitric oxide donor, a pressor peptide, and prostacyclin. The composition may further include an amino acid mixture lacking arginine, an antidote for cyanide, blood plasma or its derivatives, and/or a preparation of arginine. The arginine decomposing enzyme may modified to increase circulation half-life and can be type I liver arginase, or type II of human or animal, partially purified, or recombinant, or even bacterial origin. It may be administered as a drug or released from the patient's own tissue. Endogenous production of arginine, particularly via so-called intestinal-kidney axis, can be beneficially inhibited at several enzymatic steps, allowing for deeper reductions of circulating arginine. Different components of the composition may be administered separately, or in suitable mixtures, allowing for needed adjustments during the treatment.

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*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.*

# THERAPEUTIC COMPOSITION FOR TREATMENT OF CANCER BY ARGININE DEPLETION

## Field of the Invention

The present invention generally relates to a therapeutic composition and a method for the treatment of patients in the state of arginine depletion, and more particularly for the treatment of cancer patients depleted of arginine for therapeutic reasons.

In particular, the composition comprises a nitric oxide donor, a vasoconstricting peptide, and a prostacyclin analog, the combination of which prevents activation of platelets, while maintaining hemodynamic stability. Homeostatic response to arginine depletion is counteracted by inhibiting protein breakdown of expandable cellular proteins, mostly in muscles, by known modulators of protein turnover, such as insulin, which promotes protein synthesis, and inhibits lysosomal protein breakdown, and by, for example, a heme-containing compound which inhibits proteasomal protein breakdown.

## Background of the Invention

Treatment of cancer by limiting the supply of an amino acid has been known and practiced in clinical oncology since the seventies, following the discovery of the unusual sensitivity of the acute lymphocytic (lymphoblastic) childhood leukemia (ALL) to restriction of asparagine. The sensitivity is due to suppressed asparagine synthesis in ALL cells. However, usefulness of asparaginase remained limited to ALL, which also was found to become resistant to repeated treatments. Leukemia cells surviving the initial period of depletion eventually activate their own, normally latent, synthesis of asparagine, a non-essential amino acid. Asparaginase is thus usually used for induction in a multi-drug chemotherapy regimen for ALL, which at approximately 75% cure rate is one of the best treatments for any disseminated cancer.

Depletion of an essential amino acid would make it impossible for cells to become resistant, and a number of in vitro experiments performed over the last three decades have shown that withdrawal of an essential amino acid can in fact kill cancer cells more readily than healthy ones (J.M. Storr and A.F. Burton: "The Effects of Arginine Deficiency on Lymphoma Cells", Br. J. Cancer, 30, 50-59, 1974; L. Scott, J. Lamb, S. Smith, and D.N. Wheatley: "Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells", Br. J. of Cancer, 83(6), 800-810, 2000). Depletion of arginine, of all essential amino acids, is most effective and

most selective in eliminating cancer cells versus their healthy counterparts. While this unusual effectiveness of arginine depletion is not fully understood, it almost certainly is a consequence of the many different metabolic roles which arginine plays, on both systemic and cellular levels. Arginine is a substrate for a number of enzymes, widely distributed in different tissue and cell types (see generally the discussion in Wu G. and Morris S.M. Jr.: "Arginine metabolism: nitric oxide and beyond", Biochem J, 336 (Pt 1): 1-17, 1998).

Polyamine synthesis, present in all cells, and increased in proliferation, relies on conversion of arginine to ornithine by arginase (ARG) of so-called type II (U.S. Patent No. 5,912,159). The same reaction is catalyzed by arginase of type I within the urea cycle, localized in the liver (Haraguchi Y., Takiguchi M., Amaya Y., Kawamoto S., Matsuda I., Mori M.: "Molecular cloning and nucleotide sequence of cDNA for human liver arginase", Proc Natl Acad Sci USA, 84(2): 412-5, 1987). The urea cycle is responsible for conversion of ammonia into urea, the dominant path for elimination of excess nitrogen.

Arginine is also a substrate for nitric oxide synthase (NOS), which converts arginine into citrulline and nitric oxide (NO). There are several types of NOS, reflecting the wide-ranging and very important roles of nitric oxide (NO). One of these roles is maintenance of platelet inactive state, mostly by NO produced by the vascular endothelium. NO stimulates production of cGMP in platelets in their inactive state. Removal of NO signal leads to depletion of cGMP, and then, through a number of molecular events, to influx of calcium and platelet activation. Prostacyclin has a similar, synergistic, role of platelet inactivity maintenance by stimulation of cAMP production (Anfossi G, Russo I, Massucco P, Mattiello L, Balbo A, Cavalot F, Trovati M.:" Studies on inhibition of human platelet function by sodium nitroprusside. Kinetic evaluation of the effect on aggregation and cyclic nucleotide content", Thromb Res., 15; 102(4): 319-30, 2001; Anfossi G, Massucco P, Mularoni E, Cavalot F, Mattiello L, Trovati M.: "Organic nitrates and compounds that increase intraplatelet cyclic guanosine monophosphate (cGMP) levels enhance the antiaggregating effects of the stable prostacyclin analogue iloprost", Prostaglandins Leukot Essent Fatty Acids, 49(5): 839-4, 1993). The main source of prostacyclin is also vascular endothelium. Half-life of both NO and prostacyclin is extremely short, measured in seconds, explaining why even a

brief exposure of platelets to an environment lacking constant production of these two molecules, leads to platelet activation and rapid clot formation.

NO is also a potent vasodilator. Blood vessel lumen is under constant control by agonist/antagonist actions of NO and so-called pressor peptides all of which contain arginine (vasopressin, angiotensins). Normal function of the heart, and its impact on the circulation of blood, referred to as hemodynamics, depends on the appropriate volume of the blood and the peripheral resistance of the complete vascular system.

The heart's own feedback control will respond to a potential drop in pressure, due to either reduced blood volume, or reduced peripheral resistance, increasing the pulse rate, even before a measurable pressure drop is registered at the periphery. The early workers on essential amino acid depletion for cancer could have not understood multiple dependencies of hemodynamic stability on the substances, which either contain arginine, or are produced from it. NO is a "modern" molecule, perhaps the most intensely studied one in the last decade. However, medical literature remains silent on the problems facing in vivo arginine depletion as a modality for cancer treatment.

Arginine is also a substrate for arginine decarboxylase (ADC), which converts arginine to agmatine. ADC is present in the brain and kidneys of mammals, but the metabolic role of agmatine remains rather poorly understood.

In view of the amounts of these different enzymes and their activity in transformed cells, the most likely culprit for the high rate of arginine consumption by cancer cells in comparison to normal cells is arginase type II (see U.S. Patent No. 5,912,159). The rate at which arginine is used for production of polyamines in cancer cells appears to lead to local, intracellular depletion of arginine, which then affects overall protein synthesis and thus cells' viability. This may explain why the cancer-killing window for arginine depletion opens at between 10 and 1 micromolar (extracellular concentration), while other essential amino acids need a thousand-fold lower concentrations for a similar effect.

Effectiveness of arginine depletion in selective killing of cancer cells in vitro seems thus to be the result of the unique position which arginine occupies in the metabolic processes on cellular and systemic level. Transfer of arginine depletion from in vitro to in vivo conditions has required solving a number of puzzles and problems posed by arginine metabolic functions. The first problem, and a solution to it, disclosed in U.S. Patent No. 6,261,557, is caused by homeostatic responses at the systemic level,

initiating a massive protein breakdown, mostly of muscle tissue, in order to replenish arginine to its base-line level (of about 80 to 100 micromolar in plasma). Attempting to overcome this response by higher rates of arginine removal via enzymatic degradation, would lead to a life-threatening condition with ammonia accumulation. Since protein breakdown leads to influx of all of the amino acids, and only arginine is targeted for removal, concentrations of other amino acids tend to increase. In fact, the non-essential ones are allowed to increase, but most of the essential ones are catabolized to their respective base-line concentrations. This floods the body with ammonia, which is the final form in which nitrogen is released from each of the amino acid molecules degraded. Removal of ammonia so generated, however, requires an increased conversion rate by the urea cycle, which, for that, unfortunately, needs an elevated concentration of arginine. Whilst enzymes of the urea cycle hold pretty tight onto the cycle intermediaries (arginine, ornithine, citrulline, and arginino succinate), the cycle activity still depends on the general availability of these substrates. Hence the Catch-22 of arginine depletion. The solution to the problem, ultimately enabling arginine depletion, was found in inhibiting this homeostatic response of the protein breakdown by, for example, insulin.

However, depletion of arginine suppresses NO production. Ensuing platelet activation leads to clot formation, loss of platelets and subsequently, to internal bleeding. In experimental dogs, the bleeding usually starts by the second or third day of depletion. There is also an increased risk of generalized, rapid platelet activation; potentially leading to disseminated intravascular coagulation (DIC), a difficult-to-treat, life-threatening condition. Transfusion of platelet-enriched plasma is only a temporary measure, leading to a new round of clotting and further damage to vital organs. Kidneys, lungs and the liver are the primary sites of clot entrapment -- their failure a common cause of death. Therapeutic composition of this invention restores systemic levels of NO by continuous delivery of one of the known NO donors, preferably a direct one, such as sodium nitroprusside. However, restoring NO supply, in arginine depleted state, leads to excessive vasodilatation for the lack of normal pressor counterbalance (since all of pressor peptides contain arginine and thus cannot be produced at normal rates). Therapeutic composition of this invention resolves this difficulty by supplying a pressor peptide, for example vasopressin, or preferably, one of its more stable analogs, such as ornipressin, terlipressin or desmopressin.

However, after a day or two of strict arginine depletion, lack of another important molecule becomes a limiting factor in the maintenance of normal blood circulation. While arginine is not directly involved in synthesis of prostacyclin, arginine depletion suppresses prostacyclin production in an indirect way. In fact, it is generally expected that in such a metabolic stress, all cells will gradually revert to their own survival, dismantling production of all, but the most crucial "export" substances. Shortage of circulating prostacyclin also leads to platelet activation and all of the sequelae. The problem is solved by administration of prostacyclin, or preferably, one of its more stable analogs, such as iloprost.

Arginine is a semi-essential amino acid, i.e. the body is capable of producing some, but usually not all of the required arginine from non-essential amino acids as the precursors, namely from proline and glutamine. The relative need for endogenous sources of arginine varies with age and animal species. For example, milk of many mammals, including humans, is a poor source of arginine and neonates are very much dependent on endogenous production of arginine via so-called intestinal-kidney axis, whereby citrulline is produced by mostly small intestine and then converted to arginine by mostly kidneys. Use of glutamine as a substrate for production of citrulline decreases progressively in neonatal period, leaving proline as the dominant precursor for citrulline synthesis in adults. Tight control of circulating arginine is subject to restriction of all possible sources: (1) exogenous through food intake; (2) endogenous through protein breakdown; and (3) endogenous through conversion of mostly proline to arginine via pyrroline-5-carboxylate, ornithine and citrulline (in the intestine), and argininosuccinate (in the kidneys). A case report on a newborn with lactic acidaemia and hyperammonaemia (Byrd D J, Krohn H -P, Winkler L, Steinborn C, Hadam M, Brodehl J, and Hunneman D H, "Neonatal pyruvate dehydrogenase deficiency with lipoate responsive lactic acidaemia and hyperammonaemia", Eur J Pediatr (1989) 148:543-547) described the physiological abnormalities caused by deficiency of lactate conversion to pyruvate: acidosis (ultimately due to reduced production of bicarbonate from lactate); severe deficiency of citrulline and arginine (below detection in plasma); and hyperammonaemia (due to lack of arginine and thus ineffective, if intact urea cycle enzymes). Use of emergency measures, including dialysis to remove ammonia, in the first days of the baby's life kept her alive in spite of complete depletion of arginine. Supplementing other medications with arginine, initiated at day 9, led to normalization of

ammonia and she lived for a year and a half. Biochemical consequences of increased lactate were subsequently elucidated and reduced to inhibition of proline oxidase (Dillon E L, D. Knabe A, and Wu G, "Lactate inhibits citrulline and arginine synthesis from proline in pig enterocytes", AJP-Gastrointestinal and Liver Physiology, Vol. 276, Issue 5, G1079-G1086, May 1999). These discoveries have inspired the last and truly enabling intervention aimed at deep systemic depletion of arginine – administration of lactate as a controlled co-infusion of sodium lactate and lactic acid. Feedback control of the ratio of sodium lactate to lactic acid is based on the measurement of blood pH – a crucial, yet often disturbed, physiological parameter in many pathologies, including cancer.

Another possibility for inhibition of intestinal-kidney axis is by per os administration of a glycylglycine derivative of delta-N-(phosphonacetyl)-L-ornithine (Gly-Gly-PALO), a powerful and specific inhibitor of ornithine transcarbamylase, an enzyme responsible for intestinal production of citrulline (Hoogenraad N, Totino N, Elmer H, Wraight C, Alewood P, Johns RB, *Inhibition of intestinal citrulline synthesis causes severe growth retardation in rats*, Am.J.Physiol. 1985 Dec; 249(6 Pt 1): G792-9).

## Summary of the Invention

The present invention generally relates to a therapeutic composition and a method for the treatment of patients in the state of arginine depletion, and more particularly for the treatment of cancer patients depleted of arginine for therapeutic reasons.

In particular, the composition comprises a nitric oxide donor, a vasoconstricting peptide, and a prostacyclin analog, the combination of which prevents activation of platelets, while maintaining hemodynamic stability. Homeostatic response to arginine depletion is counteracted by inhibiting protein breakdown of expandable cellular proteins, mostly in muscles, by known modulators of protein turnover, such as insulin, which promotes protein synthesis, and inhibits lysosomal protein breakdown, and by, for example, a heme-containing compound which inhibits proteasomal protein breakdown. A number of more specific inhibitors of the proteasomal pathway have been discovered, and can be beneficially used in combination with arginine depletion.

Endogenous production of arginine can be inhibited at several of the enzymatic steps required to convert e.g. proline to arginine by the so-called intestinal-kidney axis. For example, inhibition of proline oxidase by lactate has been shown to allow for very deep reductions of circulating arginine.

The invention relates to a method for the treatment of cancer by depletion of arginine and the therapeutic compositions useful in such methods. The composition enables arginine depletion in the therapeutic window without systemic complications, which otherwise would ensue. The composition comprises, in addition to an arginine decomposing enzyme and protein breakdown inhibitor(s): a nitric oxide donor, such as sodium nitroprusside; a pressor peptide, such as vasopressin, or one of its analogs, such as ornipressin, desmopressin, or terlipressin; and prostacyclin, or one of its analogs, such as iloprost. Controlled co-infusion of sodium lactate and lactic acid inhibits endogenous production of arginine via intestinal-kidney axis. The ratio of sodium lactate-to-lactic acid infusions is adjusted as needed to maintain physiologically normal pH (measured in blood); the total infusion rate is adjusted to achieve the target value of lactate concentration (also measured in blood).

Additionally, the composition may include: an amino acid mixture lacking arginine; an antidote for cyanide, such as hydroxocobalamine, or sodium thiosulfate; blood plasma, or one or more of its derivatives, such as cryoprecipitate rich in clotting factors, or albumin. The composition may also include a preparation of arginine, such as a solution of arginine hydrochloride. Arginine decomposing enzyme may be arginase, decarboxylase, deiminase, or kinase. The enzyme may be pegylated, or otherwise modified to increase its half-life in circulation. Arginase may be type I liver arginase, or type II. It may be human or animal, partially purified, or recombinant. It may also be of bacterial origin, particularly a thermostable type. It may be administered as a drug, or it may be released from the patient's own tissue, particularly from the liver in case of hepatocellular carcinoma. Release may be effected by arterial occlusion of the liver, or by cryo, ultrasonic, or RF ablation of the tumor lesions. Protein breakdown inhibitors may be insulin, insulin-like growth factor I, IGF-I, growth hormones, protein breakdown inhibiting peptide aldehydes such as Cbz-Leu-Leu-Leucinal, lactacystin or its analogs, heme or its derivatives, and inhibitors of prostaglandin $E_2$ production, such as nonsteroidal anti-inflammatory drugs (NSAID's). These compounds effect different pathways of protein breakdown and can be combined in different ways. For example, insulin, which promotes protein synthesis and inhibits lysosomal breakdown, can be beneficially combined with heme, or hemin, which inhibits proteasomal breakdown, and one of NSAID's which inhibits $PGE_2$. If insulin is used, its effect on glucose metabolism is counteracted by a continuous infusion of glucose. Novel, specific and potent

proteasomal inhibitors have recently been developed and are in various stages of clinical testing for different pathologies, including cancer. These compounds, including a peptide boronate PS-341 (Adams J, "Proteasome inhibitors as new anticancer drugs", Curr Opin Oncol, 2002 Nov; 14(6):628-34) and epoxomicin (Hanada M, Sugawara K, Kaneta K, Toda S, Nishiyama Y, Tomita K, Yamamoto H, Konishi M, Oki T, "Epoxomicin, a new antitumor agent of microbial origin", J Antibiot (Toyo) 1992 Nov; 45(11):1746-52), which has also been synthesized (Sin N, Kim KB, Elofsson M, Meng L, Auth H, Kwok BH, Crews CM, "Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology", Bioorg Med Chem Lett 1999, Aug 2; 9(15):2283-8) can be used in conjunction with all of above.

Different components of the composition may be administered separately, or in suitable mixtures, allowing for needed adjustments during the treatment. The various embodiments of the therapeutic composition and method for treatment of cancer by depletion of arginine are set forth in the claims and described in detail herein.

**Brief Description of the Drawing Figures**

Figure 1 is a graph depicting the change in arginine plasma concentration over time in experimental dogs being treated with arginine depletion with curve 1 showing arginine plasma concentration without an insulin/glucose clamp and curve 2 showing arginine plasma concentration with an insulin/glucose clamp.

Figure 2 is a graph depicting platelet loss over time in experimental dogs comparing the platelet loss where SNP/terlipressin/iloprost was not administered (curve 1) and where it was administered (curve 2).

Figure 3 is a graph depicting plasma concentration in experimental dogs being treated with bolus i.v. injections of a crude liver extract alone or in combination with insulin/glucose/SNP/terlipressin/iloprost.

Figure 4 is a schematic diagram showing plasma concentration of arginine in experimental dogs treated with or without infusion of sodium lactate/lactic acid in addition to pegylated recombinant human liver arginase / insulin / glucose / SNP / terlipressin / iloprost.

Figure 5 is a chart depicting the dosages derived from the experiments discussed in the present application.

**Definition of Terms**

**Acute lymphocytic leukemia** (**ALL**) – a type of childhood leukemia, which responds well to asparaginase treatment.

**Alpha-fetoprotein** (**AFP**) – a tumor marker useful for diagnostic and follow-up procedures in hepatocellular carcinoma.

**Arginase** (**ARG**) – converts L-arginine and $H_2O$ to L-ornithine and urea; found at high concentrations in the liver; other isoforms widely distributed in basically all tissues of all animals, but also in plants and bacteria.

**Arginine** – an essential amino acid (L-arginine); by some accounts considered semi-essential, since it can be produced from for example citrulline, which in turn can be produced from praline or glutamine. All amino acids have optical (or stereo) isomers, D and L, and so does arginine. Proteins consist exclusively of L-amino acids, and in this text, if not specified, L-form is implied.

**Arginine decarboxylase** (**ADC**) – converts L-arginine to agmatine and $CO_2$.

**Arginine deiminase** (**ADI**) – converts L-arginine and $H_2O$ to L-citrulline and $NH_3$.

**Arginine kinase** (**AK**) – converts L-arginine and ATP to $N^{\omega}$-phosphor-arginine and ADP.

**Arterial occlusion** – a procedure frequently used to induce partial tumor necrosis, usually performed by controlled injection of occluding substances, e.g. an oil emulsion and/or collagen particles (leading to embolization), but can also be performed by mechanical / surgical means (e.g. ligation).

**Asparaginase** – converts L-asparagine and $H_2O$ to aspartate and $NH_3$ (drug approved for the treatment of **ALL**).

**Asparagine** – L-asparagine, a non-essential amino acid.

**Catch-22** – a 1961 book by Joseph Heller, which also became a widely excepted English expression defining a problem with no solution, a difficulty defying an exit strategy.

**cAMP** – cyclic adenosine monophosphate; plays an important role in prevention of calcium influx into thrombocytes; its production is stimulated by prostacyclin.

**cGMP** – cyclic guanosine monophosphate; plays an important role in prevention of calcium influx into thrombocytes; its production is stimulated by **NO**.

**Desmopressin** – an analog of vasopressin (approved drug).

**Disseminated intravascular coagulation (DIC)** – an extreme form of pathological coagulation, leading to massive clot formation, and, subsequently to platelet depletion and to increased risk of (internal) bleeding.

**Heme** – porphyrin ring containing iron at its core; central to oxygen transport and biochemistry; a part of hemoglobin, the most important molecule in red blood cells.

**Hemin** – or hemin chloride – heme with a chlorine ion bound to iron.

**Hematin** – hemin with the chlorin ion replaced by hydroxyl ion.

**Hepatocellular carcinoma** – a predominant type (about 90% of all cases) of primary liver cancer. Hepatitis B and C are considered main causative factors. Endemic in many Asian and African countries, accounting for perhaps as many as 0.5 to 1 million annual cancer deaths worldwide (according to WHO, 25'000 in Europe; 5'000 in the US; 30'000 in Japan). Hepatocellular carcinomas are very responsive to arginine depletion.

**Iloprost** – an analog of prostacyclin (approved drug).

**Insulin / glucose-clamp** – a therapeutic composition, most frequently used as a diagnostic tool for determining insulin resistance in diabetes; a dose of insulin (usually administered as a primed, fixed-rate continuous infusion) is balanced by a variable dose of glucose required to maintain its normal plasma concentration (by feedback control); hence the name: glucose-clamp (also euglycemic insulin clamp).

**Melanoma** – a highly invasive cancer, usually of cutaneous origin. Excessive exposure to sunlight is a major risk, and the most important factor in the rapidly rising incidence of melanoma. Early surgical treatment is very effective; overall 5-year survival rate is about 80%. Melanomas are very sensitive to arginine depletion, and generally unable to substitute citrulline for arginine because they fail to produce the required enzymes.

**Nitric oxide (NO)** – a ubiquitous signaling molecule with different physiological functions, including vasodilation and inhibition of platelet activation.

**Nitric oxide synthase (NOS)** – converts L-arginine and oxygen to citrulline and NO (the stoichiometry not clear), found in different forms and in different cell types.

**Nitric oxide donor** – compounds which release NO, either directly, or via metabolic conversions of certain substrates.

**Nitroglycerin** – an indirect NO donor; a drug approved for treatment of heart disease, particularly angina pectoris.

**Nonsteroidal Anti-Inflammatory Drugs** – NSAID's a class of compounds inhibiting production of inflammatory mediators, including prostaglanding $E_2$.

**Ornipressin** – an analog of vasopressin with ornithine substituted for arginine, vasoconstrictor (approved drug).

**Pegylation** – process of covalent attachment of **PEG** molecules to selected amino acid side chains of a protein, which leads to increased circulation time and reduced immunogenicity.

**Polyethyleneglycol (PEG)** – polymer of ethyleneglycol, characterized by high affinity for water.

**Proteasome** – an ATP-dependent proteolytic complex, which degrades many cellular proteins tagged for destruction by ubiquitin.

**Prostacyclin** – prostaglandin $I_2$, potent inhibitor of platelet aggregation.

**Prostaglandin E2 – ($PGE_2$)** an important inflammatory mediator; promotes protein breakdown

**Sodium nitroprusside (SNP)** – decomposes to NO and cyanide; a direct NO donor; a drug approved for treatment of hypertension.

**Terlipressin** - an analog of vasopressin (approved drug).

**Therapeutic composition** – a multi-component drug, whereby single components (active ingredients) are either mixed together by the producer, or by the physician prior to application, or kept and delivered separate to the patient during a given treatment session.

**Urea cycle** – a set of enzymes, residing in liver, which convert arginine to ornithine to citrulline back to arginine via arginino-succinate; its physiological function is conversion of excess nitrogen to urea, which is then excreted by kidneys.

**Vasopressin** – posterior pituitary hormone, anti-diuretic hormone, an arginine-containing peptide, also an approved drug.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a therapeutic composition and a method for the treatment of cancer, the composition comprising an arginine-degrading enzyme, which is either released endogenously, particularly in the treatment of hepatocellular carcinoma, by any one of several conventional tumor destruction methods, or infused together with the other composition constituents, namely: a protein breakdown inhibitor, preferably insulin/glucose; a heme derivative; a nonsteroidal anti-inflammatory drug,

preferably a cyclo-oxygenase-2 (COX-2) specific; a nitric oxide donor, preferably sodium nitroprusside; a vasoconstricting substance, preferably an analog of vasopressin peptide; and an analog of prostacyclin, preferably iloprost. Additionally, co-infusion of sodium lactate and lactic acid causes a systemic elevation in lactate, which via inhibition of proline oxidase depresses endogenous production of arginine.

Administration of a protein breakdown inhibitor(s) allows reduction of plasma arginine into micromolar range without the influx of other amino acids, which would otherwise result in a deadly ammonia accumulation.

Sodium nitroprusside (SNP) is a direct donor of nitric oxide (NO), i.e. SNP releases NO without any intervening metabolic step. The drawback of this simple reaction is release of cyanide. Infusion rates of SNP needed in this protocol are well below standard therapeutic dosage for treatment of hypertension. The risks of cyanide poisoning can be completely offset by concurrent administration of either B12a (hydroxocobalamine, precursor of vitamin B12) or sodium thiosulfate, both of which are approved as antidotes in cyanide poisoning. There are many novel NO donors being developed, which could be used for this purpose (Zampolli A., Basta G., Lazzerini G., Feelisch M., De Caterina R.: "Inhibition of endothelial cell activation by nitric oxide donors", J Parmacol Exp Ther, 295(2):818-23, 2000). Scientific and medical literature is so vast that any review would add more material than considered relevant for this application. The preference is for direct, cysteine-containing, NO donors (such as SP/W 3672 reviewed in the above citation). SNP was used in animal experiments and is given here as an example since it the oldest, and universally available drug of this class. Modern donors may of course be used, once clinically tested and approved, and hopefully with reduced risks of toxicity.

In a preferred embodiment of the invention, the therapeutic composition is administered to a patient in need of treatment through infusion, an aerosol nasal spray, or in other methods known to one of ordinary skill in the art. In another preferred embodiment of the present invention, the therapeutic composition is administered by means of an extracorporeal blood treatment characterized by molecular exchange between the blood and a dialyzing fluid across a molecular sieve membrane, whereby the conventional dialyzing fluid is supplemented by a plurality of low molecular weight organic and inorganic substances at concentrations essentially equal to those found in the normal blood plasma with the exception of at least one essential nutrient, preferably an

essential amino acid, which is either not present, or is present at a substantially lower concentration.

In further preferred embodiments of the present invention, the therapeutic composition is administered, either simultaneously or in series with, molecular factors, at normal or at elevated concentrations, involved in the cellular processes of protein synthesis and degradation in order to limit the release of amino acids from non-essential cellular proteins, mainly fibrillar muscle proteins. These factors are branched side chain amino acids (leucine, isoleucine and valine, glutamate), insulin, insulin like growth factors and growth hormones. Insulin was found to be the most effective. Delivery of insulin must be balanced by an appropriate rate of delivery of glucose in order to avoid hypoglycemia. Chemical inhibitors of the protein degradation pathways may also be administered, as well as antibiotics needed to reduce the risk of infection.

These factors and inhibitors may be separately administered by any suitable means such as injection, i.v., aerosol nasal spray, or any other suitable means known to one of ordinary skill in the art, either at the same time as administration of the therapeutic composition of the present invention or sometime before or after administration of the therapeutic composition.

In a further embodiment, when a dialyzing fluid is used, these factors and inhibitors may be contained in the dialyzing fluid or may be separately administered by any suitable means such as injection, i.v., aerosol nasal spray, or any other suitable means known to one of ordinary skill in the art.

In still further embodiments of the invention, the temperature of the patient is controlled during or following administration of the therapeutic composition of the present invention. In particular, the temperature of the patient is preferably controlled to subnormal levels in order to reduce the muscle protein breakdown in response to removal of the targeted essential amino acid.

In yet another preferred embodiment of the present invention, a continuous treatment is carried out over a period of several days, leading to selective killing of the tumor cells. This result can be accomplished due to the relaxed cell cycle control mechanisms found in all tumor cells. Upon deprivation of an essential amino acid, healthy, normally cycling cells exit the cycle and are kept in the rest phase where they can easily survive the harsh conditions of deprivation. In contrast, tumor cells are less restricted and will proceed into the cycle finding themselves vulnerable to conditions of

deprivation. During the first cycle of deprivation, a majority of cycling tumor cells proceed over the restriction point into the S-phase (DNA synthesis) and are readily killed after, for example, no more than 72 hours of arginine deprivation. The few survivors among the cycling tumor cells can again be sent across the restriction point by re-supplying the deprived essential amino acid during a time which is too short for the normal cells to enter the cycle, and then eliminated by repeated deprivation. Programming of such cycles is preferably achieved by administration of appropriate concentrations of the therapeutic composition over the period of treatment. In the case of extracorporeal blood treatment, equilibrating mass transport between the blood and the appropriate dialyzing fluid results in a sufficiently powerful systemic-level control with sustainable extracorporeal blood flow rates and can be achieved by switching between the appropriate concentration formulations of the dialyzing fluid.

This method of treatment can be readily combined with a suitable protocol of chemotherapy. Deprivation of arginine causes most tumor cells to crowd into and to get arrested in the S-phase, while most normal cells manage to complete their cycle and exit into the rest phase (G.sub.0). S-phase-specific drugs can thus be used in significantly escalated doses. The preferred mode of drug delivery is by loading the drug into (or mixing it with) the therapeutic composition, thus avoiding any risk of overdose. In the case of extracorporeal blood treatment, this allows the drug to be readily removed from circulation by simply switching to a drug-free dialysate at the end of the drug treatment, before the healthy cells are allowed back into the cycle. Alternatively, drugs can be infused directly into the blood, taking into account kinetics of removal by the continuous dialysis. As soon as the infusion is stopped, dialysis will quickly reduce the concentration of the remaining drugs.

It has been determined, by performing in vitro work on seven different human cancer lines, that arginine is the best target for amino acid deprivation because arginine is used in disproportionate amounts by all cancer cells tested for production of proteins, but also of polyamines. The "killing window" is defined by concentrations below 10 micro molar and by deprivation time longer than 72 hours. Normal cells exit the cycle and reemerge from the rest phase apparently undamaged after even ten days of arginine deprivation.

It has also been discovered, by performing in vivo experiments on healthy large dogs, that the cancer killing concentrations of arginine are attainable and sustainable for

up to six days without major systemic complications. This resultant time period was achieved by the application of the continuous dialysis described above and by controlled infusion of both insulin and glucose.

Arginine has a special role in the physiology of mammals. The main pathway for elimination of excess nitrogen is the urea cycle, whereby liver cells use a set of enzymes which turn arginine into ornithine, ornithine into citrulline, and citrulline back into arginine with the net effect of releasing nitrogen from ammonia (which is produced by ultimate degradation of amino acids) as a constituent of urea. Should this process be inhibited by the lack of arginine, the predictable outcome is accumulation of highly toxic ammonia. This phenomenon seems to have escaped the attention of many investigators who have worked on arginine degrading enzymes.

Any suitable arginine degrading enzyme can be used in the present invention. A preferred choice for an arginine degrading enzyme is co-called biosynthetic arginine decarboxylase (bADC) of E. coli, which in contrast to a similar enzyme, so-called biodegradative arginine decarboxylase (dADC) of E. coli, has very favorable kinetic properties at normal physiological conditions (Wu WH, Morris DR, "Biosynthetic arginine decarboxylase from Escherichia coli. Purification and Properties", J Biol Chem 1973 Mar 10;248(5):1687-95; Wu WH, Morris DR, "Biosynthetic arginine decarboxylase from Escherichia coli. Subunit interactions and the role of magnesium ion", J.Biol.Chem. 1973 Mar 10;248(5):1696-9). bADC has been cloned (Moore RC, Boyle SM, "Nucleotide sequence and analysis of the speA gene encoding biosynthetic arginine decarboxylase in Escherichia coli", J Bacteriol 1990 Aug;172(8):4631-40) and produced by S. Boyle as a highly purified recombinant enzyme (expressed in E. coli) for our experimental work.

Another enzyme of choice is a thermostable arginase (Patchett ML, Daniel RM, Morgan HW, "Characterisation of arginase from the extreme thermophile 'Bacillus caldovelox'", Biochim Biophys Acta 1991 Apr 29; 1077(3):291-8), which has high specific activity and can be produced and purified very economically (Bewley MC, Lott JS, Baker EN, Patchett ML, "The cloning, expression and crystallisation of a thermostable arginase", FEBS Lett 1996 May 20; 386(2-3):215-8).

It has been discovered that the accumulation of highly toxic ammonia in the administration of a therapeutic composition comprising an arginine decomposing enzyme

can be avoided by the deployment of protein breakdown inhibitors, dialysis in order to remove ammonia, or combinations thereof.

The arginine decomposing enzyme is administered by any suitable means including intravenously (i.v.), intraperitoneally (i.p.), intramuscularly (i.m.), nasally, or extracorporeally. In a preferred embodiment of the present invention, the arginine decomposing enzyme is administered intravenously. In another preferred embodiment, the arginine decomposing enzyme is inhaled as an aerosol, which may allow minimization of immunological side effects caused by i.v. or i.m. injections of enzymes.

All known arginine decomposing enzymes are large proteins which cannot enter blood circulation through the respiratory membrane. Instead, the amino acids, specifically arginine, will diffuse out from the blood capillaries of the lungs and decompose within the fluid layer which coats the lungs, and which contains the enzyme. This mode of operation has the potential for a much higher efficiency than hemodialysis since the artificial membrane of dialyzing filters is typically 1.8 square meters and the extracorporeal blood flow is up to 0.5 liters/minute, while the surface of the lungs is about 50 square meters and the blood flow through the lungs is equal to the total heart output of about 5 liters/minute. For a further reduction of the risks of immune response the enzyme can be encapsulated into a suitable polymer or conjugated with PEG. In a preferred embodiment of the present invention, the arginine decomposing enzyme is PEG-ilated (covalently bound to a number of molecules of polyethylene glycol or polyethylene glycol derivatives such as methoxypolyethylene). As the enzyme degrades and loses its activity, it is eliminated from the lungs by a natural process of mucosal excretion.

In another embodiment, this potential toxicity is avoided by the concurrent removal of ammonia by hemodialysis. A further advantage of the dialysis of the present invention is the possibility of removal of citrulline and ornithine which are precursors of arginine (these metabolic processes are not confined to liver). Alternatively, dialysis can be performed using conventional dialyzing solutions, while some, or all, of these substances, as well as any necessary adjuvants (e.g. glucose with insulin), can be delivered by a controlled infusion into the return line of the extracorporeal circuit. This embodiment constitutes a simple controller of the systemic concentration of these substances. The performance of the controller is dependent on the blood flow and the

efficiency of the filter, which is predictable, can be monitored essentially on line, and the necessary adjustments of the infusion rate are easily implemented.

The need for these pharmacological interventions has been established in a series of experiments on animals (mostly dogs), which ultimately led to a treatment protocol for humans, including the recommended dosages for individual drugs. Dosage calculation for human was done with the usual conversion factor of 0.5, accounting for the difference in the ratio of the body weight to the body surface area of dogs vs. human. A brief description of some of the animal experiments is given herein to illustrate the process by which the protocol has been developed.

**Experiment 1:** ***Inhibition of protein breakdown by insulin***

Figure 1 is a schematic depiction of the effect of insulin/glucose on the concentration of plasma arginine in dogs being exposed to depletion of arginine by extracorporeal means (U.S. Patent No. 5,851,985) - if the homeostasis is allowed to develop unperturbed, arginine plasma concentration is maintained at near normal level of 100 micromolar, depicted by curve 1. However, if an insulin/glucose clamp is deployed, arginine can be readily lowered to below 10 micromolar, as shown by curve 2. This effect has been consistently observed in a dozen of experimental sessions lasting up to 6 days.

**Experiment 2:** ***Inhibition of platelet activation by NO and iloprost***

In all cases, where plasma arginine concentration was reduced to below 10 micromolar (with the aid of insulin/glucose), a rapid loss of platelets has limited the duration of any single session to about 3 days, as shown again by a schematic depiction of Figure 2, curve 1. At the time the platelet count dropped to less than 20'000 per microliter, the risk, or signs of internal bleeding forced an end to the experiment. Administration of SNP alone did not prevent the loss of platelets, but SNP and iloprost, both dosed well within normal therapeutic ranges, completely inhibited platelet activiation and subsequent loss, curve 2. Administration of SNP was balanced by a vasopressin analog. Without vasopressin, SNP administration led to a very high pulse rate (up to 200 per min, double of normal for a medium-sized dog) which prevented continuation of the experiment.

**Experiment 3:** ***Systemic depletion of arginine by crude liver extract***

Utility of liver-type arginase for systemic depletion of arginine was demonstrated by an acute pre-terminal experiment on dogs. Dogs were kept under anesthesia continuously for 24 hours. First, one (of six) liver lobe was surgically removed, and the abdomen closed as for a normal surgical intervention on liver. The approximately 100 g of liver tissue so obtained, was then homogenized, partially purified, and sterile-filtered before being returned to the animal by i.v. infusion during the next 18 to 20 hours. Bolus injections (0.4g of liver tissue / kg body weight) at 3 hour intervals resulted in a rapid drop of plasma arginine concentration, followed by an equally rapid recovery, for every injection given, if no insulin was used, Figure 3, curve 1. However, with insulin/glucose clamp, arginine was reduced and maintained in the micromolar range, curve 2. Dogs which were given no SNP lost about a half of their platelets during 24 hours; those on SNP, vasopressin and iloprost lost no platelets.

**Experiment 4:** ***Systemic depletion of arginine by pegylated, recombinant, human liver arginase supplemented by infusion of lactate***

Pre-terminal, 24-hour experiments were carried out on healthy Beagle dogs in order to test our ability to reduce plasma arginine into micromolar range, preferably below one micromolar, the detection limit of our amino acid analyzer. Adult dogs of 10 to 15 kg body-weight were maintained in anesthesia throughout the experiment. Continuous infusion of PEG-arginase at 4mg/kg/day provided for removal of residual / released / produced arginine. In all cases, infusions of SNP, iloprost and glypressin were additionally administered to control the side effects of reduced arginine.

Different combinations of protein breakdown inhibitors were tested, including insulin/glucose, but in no case could the concentration by lowered and maintained much below 10 micromolar during 24 hours. Usually, arginine concentration reached its lowest levels in about 12 hours and thereafter started to rise again, often finishing at 40 or 50 micromolar, Figure 4, curve 1. However, with co-infusion of sodium lactate and lactic acid arginine was brought down to the level of detection within about 8 hours and kept there for the duration of the experiment, curve 2. Infusing sodium lactate alone results in alkalosis, since higher levels of lactate result in higher rates of its ultimate conversion to bicarbonate. By contrast, infusion of lactic acid alone results in acidosis. In order to maintain normal pH both were infused together, their ratio being adjusted so

as to keep blood plasma pH at approximately 7.4, while the sum of the two rates is adjusted so as to maintain plasma lactate at approximately 10 millimolar. Normal lactate concentration in blood plasma is 1 to 2 millimolar. Both, sodium lactate and lactic acid solutions were prepared as 1 molar.

In this dog of 12kg body weight the total infusion rate required to maintain the targeted plasma lactate of 10 millimolar was approximately 100ml/h, with the final ratio of lactic acid to lactate of approximately 6. Both solutions were started at infusion rates of 25ml/h, with lactic acid ramping up and sodium lactate ramping down. Plasma pH and lactate were measured in hourly intervals, adjusting the infusion rates as needed. Both ornithine and citrulline were significantly lowered in comparison to all of the experiments performed without elevated lactate.

In this context, it should be noted that the superior responses obtained with partially purified liver extract, referred to above, as compared with pure preparations of recombinant enzyme were probably due to inadvertently caused elevation of lactate. It is known that surgical interventions on liver, such as the partial resections we performed in order to harvest the tissue for the extract, can cause an increase in plasma lactate. At the time those experiments were performed, we did not suspect this would have an effect on arginine depletion.

**Dosage of Drugs in arginine depletion for cancer treatment**

Drug dosages presented in this document are based on dog and mice experiments carried out at the School of Veterinary Medicine, University of Zurich, from 1995 until the end of 2000.

Rationale for use of a particular drug to control observed side effects of arginine depletion is given in brief – in all cases there is ample scientific literature to support the choices made. Since arginine depletion to the extent and duration needed to effect significant cancer reduction (deduced from in vitro experiments) has never been achieved outside the experimental work of the Zurich group, side effects of the depletion could only be anticipated, but have not been actually observed by any other research team. Exceptions are certain unintended conditions of arginine depletion, such as in incidental release of liver arginase due to injury or therapeutic intervention (e.g. cryoablation of liver metastasis). The Zurich experience has facilitated explaining the causes of some of

the side effects of such incidents / interventions, and has provided clues and guidelines for corrective measures.

Dosage of most of the drugs used to correct the side effects of arginine depletion was determined by rudimentary search for an effective dose, always respecting the limits of suggested pharmacological dosage for the approved indications. Limits for use in animals were calculated from those recommended for humans, using the generally accepted rules of dose adjustment for body surface area. For example, all dosages for humans are multiplied by factor 2 when recalculating for dogs, and by factor 12 when recalculating for mice (DeVita VT, Hellman S, Rosenberg SA, "Cancer – principles & practice of oncology", 4th ed., pg. 288). Conversely, when recalculating from dogs to humans all dosages were divided by factor 2; from mice to humans by factor 12.

Limited dose searches performed for the required drugs in most cases did not produce the minimal effective dose – as long as the side effects could be well controlled using a given dose, which was well within the drug's nominal pharmacological range, the dose was accepted for further use. Since some of the drug effects are interrelated, it is quite obvious that such a pragmatic approach was the only one feasible, given the number of drugs needed and the constraints on animal testing resources.

Ultimately, fine-tuning of the dosages will have to be performed on human patients – interspecies differences in the basic physiological process involved are too large to allow for direct translation of the results from animals to humans.

This is particularly true of the main therapeutic component – (recombinant) human liver arginase. It is well established that for products of this type, e.g. asparaginase, effective *in vivo* dosages may be hundred times higher than those predicted from *in vitro* experiments. The dose ultimately required will depend on the rates of supply of arginine from different endogenous sources, which again, have been shown to greatly vary from species to species.

This document provides only the guidelines based on the limited amount of animal data collected by the inventor and his collaborators – no warranty of effectiveness and safety is to be implied by anyone attempting to use these guidelines for humans.

For the optimal control and utilization, all of the drugs are to be continuously infused. In general, where possible, the drugs with shorter half-life are preferable, so that, should the need arise, the treatment could be interrupted quickly. Some of the drugs are to be given in a fixed dose; others are to be adjusted according to the measured response.

Care must be exercised that the dose adjustments based on feedback are carried out in a measured way and within appropriate time intervals in order to avoid excessive swings in the delivery rates and responses.

**(1) Arginase**

In acute experiments on dogs carried out at the University of Zurich (beagle dogs, treated under total anesthesia during 18 to 24 hours following a liver lobe resection), liver extract was continuously infused i.v. at 4 different rates, delivering approximately 900, 300, 90 or 30 I.E./kg/day of arginase. The two highest rates lowered plasma arginine into sub-micromolar range. However, even with the highest rate of infusion, lymphatic system remained after 18 hours above therapeutic level at 15 micromolar (normal plasma concentration of arginine is about 150 micromolar). Circulation between the vascular and lymphatic systems would have been much higher if the animals were not under anesthesia, but this result points to a crucially important issue: plasma concentration is only a crude measure of arginine depletion, which for the full effect on a disseminated cancer should be effected in all of extracellular fluid. It should thus be anticipated that truly effective *in vivo* doses would be much higher than could be calculated based on enzymatic activity and say the total volume of extracellular compartment. In case of asparaginase, for example, the most similar approved and practiced treatment, the effective doses ultimately determined were 100 times higher than the original estimates.

The real limitation, and the most difficult estimate to produce from the available literature, is the net flux of arginine in a human patient undergoing enzymatic treatment and given insulin at recommended rates. There are two endogenous sources of arginine: protein turnover and synthesis via intestinal-kidney axis. No measurements of the rates of the two in an arginine-depleted state are available. In fact no such state has ever been achieved in a human. The work of Zurich group demonstrated extraordinary capacity of muscle protein mobilization in dogs: up to 10% of total body protein was catabolized per day in order to keep circulating arginine near normal. Insulin not only neutralizes, but reverses the turnover into anabolic state.

There are major inter-species differences in the rates of arginine supply from these different sources in the normal nutritional state. This is a serious limitation on our capacity to predict the dose that ultimately will be found effective in humans.

The minimal effective dose in acute dog experiments of 300 I.E./kg/day would translate into 150 I.E./kg/day for a human. With the current batch of recombinant human

liver arginase from the University of Kyoto, the activity is about 1800 I.E./mg, so the activity-based dose would translate into 0.08 mg/kg/day, or approximately 6 mg/day for a 70 kg patient.

However, infusion of the recombinant human liver arginase Beagle dogs did not reduce arginine as effectively as the liver extract – a rough estimate suggests that 8000 I.E. of recombinant arginase was only as effective as 1500 of I.E. of dog liver extract. This, of course, is not unusual, since the ultimate *in vivo* activity depends on many factors such as clearance from circulation and inactivation.

In view of these serious limitations on our ability to predict an effective dose, it seems prudent to use other means of determining an initial dose and an escalation schedule for the phase I study.

In case of asparaginase, which is the closest related clinically used product, for induction treatment of ALL, bolus injections of 10'000 I.E./m2 are given at 3-day intervals, typically four times. For an average patient, the body surface area is 2 m2, i.e., a typical bolus injection is 20'000 I.E., which corresponds to 100 to 200 mg of protein.

Higher doses of asparaginase of 25'000 I.E./m2 are also not uncommon, resulting in bolus injections of up to 500 mg of protein. Other than their functional (aimed for) effects, therapeutic proteins may cause serious side effects just due to their un-physiological presence in circulation, such as provoking a non-specific (in early use) immune response.

Current dose used in mice experiments would correspond to 90 mg/day of arginase for a 70 kg human patient. At this time there are no results as to the effectiveness of this dose in treating implanted tumors. However, the route of application is also different – i.p. instead of i.v.– and will thus make it difficult to make any rational scaling to humans.

In face of all of these limitations, the following escalation schedule is proposed for the human phase I study: 50 mg/m2/day for the initial dose, and then a standard (modified Fibonacci) escalation to 100 mg/m2/day; 165 mg/m2/day; and 250 mg/m2/day.

While plasma levels of arginine must be lowered to sub-micromolar range, the ultimate effective dose (which may be several-fold higher) is to be determined by the tumor response only.

**(2) *Insulin***

Insulin selected must be suitable for i.v. infusion. In all animal experiments at the University of Zurich the product used was Insulin, ACTRAPID® HM (human, monocomponent), 100 I.E./ml, from Novo Nordisk.

The purpose of insulin is to inhibit the normal physiological response the body would otherwise mount to depletion of arginine – initiation of a (potentially) massive protein breakdown (mostly in muscles) to normalize concentration of free arginine. Animal experiments have clearly demonstrated the essential need for inhibition of this response in arginine depletion. Insulin, which is a very potent natural anabolic hormone, was selected for its widespread use, familiarity, availability and safety record in treatment of diabetes, but also for its well demonstrated, if less appreciated, effectiveness in controlling protein wasting in cancer patients.

In acute experiments on dogs insulin dose of 0.5 I.E./kg/h was found to be sufficient to allow for reduction of plasma arginine to micromolar level; source of arginase for arginine decomposition was dog liver extract (autologous). This was determined by the calculation (1.6 ml insulin x 100 I.E./ml / 100 ml saline ) x 5 ml/h / 16 kg of body weight = 0.5 I.E./kg/h, or 12 I.E./kg/day.

For human application this would translate into 6 I.E./kg/day , or 4 mI.E./kg/min (i.v. delivery).

In a single patient dog with lymphoma, this dose was reduced by factor two (for human, this would translate into 3 I.E./kg/day) and was still found effective.

Euglycemic diagnostic clamps are usually administered with insulin doses of 0.5 mI.E./kg/min (low physiologic); 1.0 mI.E./kg/min (high physiologic); 4.0 mI.E./kg/min (supraphysiologic), during 2 hours.

Thus, the dose used in acute dog experiments, corresponds to supraphysiologic dose in diagnostic euglycemic clamps.

In tumor bearing rats Tomas et al. (Tomas FM et al., "Effects of insulin and insulin-like growth factors on protein and energy metabolism in tumor-bearing rats", Biochem. J, 1994, 301; 769-775), used a dose of 100 micrograms/day during 7 days, delivered s.c. by an osmotic micro pump.

This translates into human dose (factor 7 lower; with a typical insulin activity of 24 I.E./mg; and 200 g rats) of 1.7 I.E./kg/day.

Currently, in our experiments at the University of Zurich, two doses are being tested in mice (s.c. osmotic micro pump delivery; 20g mice): 0.8 I.E./day and 1.6 I.E./day. This translates (with factor 12 reduction) into human doses of 3.3 I.E./kg/day, and 6.6 I.E./kg/day, respectively.

In the most relevant clinical publication, Pearlstone et al. (Pearlstone D B, Wolf R F, Berman R S, Burt M, Brennan M F, "*Effect of systemic insulin on protein kinetics* in postoperative cancer patients", Ann Surg Oncol, 1994, Jul; 1(4):321-32), describe the positive experience with a standard parenteral nutrition supplemented with an insulin infusion during 4 days in postoperative cancer patients, with 1.44 I.E./kg/day.

In summary, then, a continuous insulin infusion of 1.5 I.E./kg/day is a well-documented precedent with a beneficial effect on cancer patients' nutritional (protein balance in particular) status, and is the recommended starting dose.

Should this dose be insufficient to allow for reduction of plasma arginine (to a micromolar level), escalation up to 3 I.E./kg/day, and to maximum of 6 I.E./kg/day of insulin could be attempted. This should be done with 12 hours observation periods (between stepping up) and a fixed dose of arginase. Ultimately, if arginine is not reduced in spite of the highest dose of insulin being used, the dose of arginase should be considered insufficient.

At 1.5 I.E./kg/day, for a 70 kg patient, daily dose would be 105 I.E., or 1ml/day of e.g. ACTRAPID® from Novo Nordisk. Appropriate dilution in physiological saline should be prepared for infusion by, preferably, a syringe pump. Infusion of insulin must be balanced with a controlled infusion of glucose, aiming at keeping plasma concentration in the range of normal. Once the infusion of insulin is terminated, glucose infusion must be continued as long as required to maintain normal plasma glucose. It is possible that arginine depletion prolongs this period due to general disturbances in protein clearance and homeostasis.

**(3) Glucose**

Glucose (dextrose) infusion is deployed in order to avoid hypoglycemia which otherwise would be induced by infusion of insulin. High concentration (50%) glucose solution suitable for i.v. infusion is recommended in order to avoid over-hydration of the patient. In general, relatively high rates of glucose infusion should be anticipated in order to balance out the effects of insulin.

In acute dog experiments, with 12 I.E./kg/day insulin infusion, maintenance of normal plasma glucose levels (in dogs 4.5 to 6.0 mmol/l) required approximately (25 ml/h x 50 g / 100 ml ) x 24 h/day / 15 kg = 20 g/kg/day. For a human this would translate into 10 g/kg/day.

In Pisters et al. (Pisters P W, Pearlstone D B, "Protein and amino acid metabolism in cancer cachexia: Investigative techniques and therapeutic interventions", Crit Rev Clin Lab Sci, 1993; 30(3):223-72), and Cerosimo et al. (Cerosimo E, Pisters P W, Pesola G, Rogatko A, Vydelingum N A, Bajorunas D, Brennan M F, "The effect of graded doses of insulin on peripheral glucose uptake and lactate release in cancer cachexia", Surgery, 1991, Apr; 109(4):459-67), normal human subjects required 13.7 mg/kg/min, or 20 g/kg/day, for supraphysiologic euglycemic clamp with 4 mI.E./kg/min insulin infusion (or approx. 6 I.E./kg/day).

At high physiologic rate of 1 mI.E./kg/min, or 1.5 I.E./kg/day, glucose requirement was 8.7 mg/kg/min, or 12.5 g/kg/day. In cancer patients, however, glucose rates were lower (10.9 and 5.3 mg/kg/min, respectively), so that an average glucose infusion rate of 7 g/kg/day could be anticipated at the suggested insulin infusion of 1.5 I.E./kg/day. It is believed that a maximum rate of 16 g/kg/day at 6 I.E./kg/day of insulin should be more than sufficient. In the event that this high rate of glucose infusion fails to normalize plasma glucose concentration, insulin infusion should be appropriately reduced.

**(4) Sodium nitroprusside**

Sodium nitroprusside is a direct donor of nitric oxide (NO). Its use in arginine depleted patients is essential in order to avoid loss of thrombocytes – NO stimulates production of cGMP within platelets, the lack of which leads to their activation. Depletion of NO is an eminently predictable consequence of arginine depletion – arginine is the only known substrate for NO production.

In all animal experiments, SNP used was purchased from Sigma Co., St. Louis, since the only approved SNP on the Swiss market, NIPRIDE® from Roche, was discontinued several years ago (in the United States there are three FDA-approved SNP products from: Abbott, Elkins Sinn and Gensia Sicor Pharms).

In acute dog experiments (beagle dogs, treated under total anesthesia during 18 to 24 hours following a liver lobe resection) SNP i.v. infusion rate of 1.6 mg/kg/day, or 1

microgram/kg/min, was sufficient to avert any platelet loss during the observation period (in contrast to the average 50% platelet loss in control animals). For humans, this would translate into 0.5 microgram/kg/min.

Generally recommended dose for long term (days to weeks) application to treat hypertension in humans is 2.5 microgram/kg/min.

Currently, in mice experiments, delivery of 10 microgram/kg/min, s.c., is being used apparently avoiding any risk of platelet loss. This would translate into human dose of 0.9 microgram/kg/min. Since the planned delivery in human patients will also be i.v., the recommended dose is 0.5 microgram/kg/min.

Should any loss of platelets be observed after a period of 12 hours of arginine depletion, this dose could be escalated to the maximum recommended dose of 1.0 microgram/kg/min (in increments of 0.25 microgram/kg/min for every 12 h).

Dose of prostacyclin (iloprost) should be increased together with SNP, as well as that of vasopressin (which is continually adjusted to balance out vasodilatation due to SNP and prostacyclin).

Use of hydroxocobalamin (B12a, or precursor to vitamin B12), an antidote for cyanide (which is the product of SNP decomposition) is recommended at a double molar rate to that of SNP. No signs of cyanide poisoning were seen in any of more than 20 dogs which have been given SNP infusions at comparable rates, for between 1 and 6 days, with or without hydroxocobalamin.

**(5) Vasopressin**

The need for vasopressin replacement became apparent in first dogs depleted of arginine (in those cases by selective dialysis; later be enzymatic decomposition) and given SNP in order to maintain platelets. Since all of the pressor peptides contain arginine and are short lived molecules, in arginine depleted state there will be a general suppression of vasoconstricting signals. Replenishing NO (with SNP), which is also a strong vasodilator, leads to excessive vasodilatation and hemodynamic disturbance (an increase in pulse rate and eventually a drop in blood pressure). It is thus essential to balance NO effects with an appropriate pressor molecule.

There are several approved analogs of the natural vasopressin peptide. In most of the experiments on dogs, glypressin from Ferring was used; for the last dog and now for mice, ornipressin (POR 8®), also from Ferring, was selected for its generally more

effective pressor function in comparison to glypressin (which has a relatively stronger anti diuretic effect).

POR 8® is supplied in solution with 5 I.E./ml. Due to limited capacity of the osmotic micro pump, for the mice experiments, POR 8® is concentrated factor two (by evaporation) and delivered at the rate of (12 microliters/day x 10 I.E./1000 microliters) / 0.02 kg of body weight = 6 I.E./kg/day.

For the human this would translate into 0.5 I.E./kg/day. This is delivered with the dose of SNP of 0.9 micrograms/kg/min, so for the planned dose of SNP of 0.5 microgram/kg/min the rate of ornipressin should be approximately 0.3 I.E./kg/day. For a 70 kg patient this would be 20 I.E./day.

In dogs glypressin was used at typically 0.13 mg/kg/day, which for humans would translate into 0.6 mg/kg/day. Since 1mg of glypressin corresponds to approximately 5 I.E. of ornipressin, this would be equivalent to the dose used in mice.

For comparison, for i.v. infusion in esophageal bleeding, 20 I.E. of ornipressin are diluted in 100 ml of saline and infused over 20 minutes. This can be repeated several times, as needed. The suggested dose of 20 I.E./day for a 70 kg patient seems reasonable. At any rate, administration of ornipressin (or possibly another one of vasopressin analogs) is to be adjusted as needed to balance vasodilatory effects of SNP and is best adjusted by keeping the pulse rate normal: SNP will raise the pulse; ornipressin will lower it. It is recommended to increase the SNP delivery rate in several steps, adjusting ornipressin infusion at each step to lower the pulse back to normal.

**(6) Prostacyclin**

*Prostacyclin is another molecule required by platelets to stay inactive.* Prostacyclin stimulates production of cAMP which, like cGMP, is essential in preventing activation. In contrast to NO, prostacyclin synthesis does not directly depend on arginine. It appears though from both the dog and mice experiments, that after 2 to 3 days of arginine depletion, production of prostacyclin is also suppressed leading to activation of platelets, with increased risk of DIC. When dialysis was used as means of arginine depletion, replacement of prostacyclin from the very beginning was essential to avert the risk of platelet loss.

Iloprost is a more stable analog of prostacyclin and is available from Schering, Berlin, under the trade name Ilomedin®. Recommended dose for humans is 0.5 to 2 ng/kg/min.

In mice the dose currently used is 12 ng/kg/min, which appears sufficient to prevent DIC. This would correspond to human dose of 1 ng/kg/min.

Prostacyclin and NO have a synergistic action on platelets, so should any loss of platelets be observed within the first 12 hours of depleted arginine, both SNP and iloprost delivery rates should be increased proportionally. This will in turn require a measured increase in vasopressin dose in order to prevent vasodilation (prostacyclin is also a vasodilatator), i.e. an increase in the pulse rate and a drop in blood pressure.

**(7) Nutritional support – amino acids**

Pearlstone et al. describe the benefits of insulin-supplemented parenteral nutrition in postoperative cancer patients. Infusion of insulin alone would lead to a significant reduction of all amino acids in absence of replenishment. This may have unwanted side effects, but also may diminish the effectiveness of arginine depletion attack on cancer – *in vitro* work suggests that removal of all of amino acids prolongs the survival of cancer cells. Sterile, arginine-free food would probably be preferable, but parenteral support with arginine-free amino acid mix of 1g/kg/day delivered as a continuous infusion is a good alternative.

**(8) Nutritional support – lipid**

As above, lipid infusion of 100 g/day, or 1.5 g/kg/day should help maintenance of the patient nutritional status during the several days of arginine depletion.

**(9) Plasma products**

Suppressed production of clotting factors due to lack of arginine is a major risk of the treatment. Currently in mice experiments, a half of plasma volume is given daily (i.p.) as a prophylactic measure. This is probably excessive, but was chosen since no means are available to monitor blood clotting on regular basis and frequently enough. In human patients prophylactic use of cryoprecipitate should be adjusted as needed based on clotting parameters.

The results of the foregoing experiments are summarized in the chart in Fig. 5 in which depicts the dosages derived from the experiments discussed in the present application.

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All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application had been specifically and individually indicated to be incorporated by reference. The discussion of the background to the invention herein is included to explain the context of the invention. Such explanation is not an admission that any of the material referred to was published, known, or part of the prior art or common general knowledge anywhere in the world as of the priority date of any of the aspects listed above.

Claims:

1. A therapeutic composition comprising an arginine decomposing enzyme and an inhibitor of endogenous production of arginine.

2. A therapeutic composition comprising an arginine decomposing enzyme and an inhibitor of intestinal-kidney axis.

3. A therapeutic composition comprising an arginine-decomposing enzyme and a nitric oxide donor.

4. The therapeutic composition of any of claims 1 to 3, wherein said therapeutic composition is used in a treatment process involving the depletion of arginine.

5. The therapeutic composition of any of claims 1 to 4, wherein said therapeutic composition is used in the treatment of cancer.

6. The therapeutic composition of any of claims 1 to 5, wherein said arginine decomposing enzyme is selected from the group consisting of arginase, arginine decarboxylase, arginine deiminase and arginine kinase.

7. The therapeutic composition of claim 6 wherein said arginase is selected from the group consisting of arginase type I, arginase type II, animal liver arginase, recombineant animal liver arginase, human liver arginase, recombinant human liver arginase, recombinant human arginase of type I, recombinant human arginase of type II, microbial arginase, such as thermostable arginase of Bacillus caldovelox and modifications thereof.

8. The therapeutic composition of claim 6 wherein said arginase decarboxylase is biosynthetic decarboxylase of E. coli and modifications thereof.

9. The therapeutic composition of any of claims 1 to 8, where the arginine-decomposing enzyme is modified to extend its circulation half-life.

10. The therapeutic composition of any of claims 1 to 9, where the arginine-decomposing enzyme is modified to reduce its antigenicity.

11. The therapeutic composition of any of claims 1 to 10 wherein said arginine decomposing enzyme is modified by pegylation.

12. The therapeutic composition of any of claims 1 to 11, where the arginine-decomposing enzyme is administered exogenously.

13. The therapeutic composition of any of claims 1 to 12, where the arginine-decomposing enzyme is released from the patient's own tissue.

14. The therapeutic composition of claim 13 where the release of the arginine-decomposing enzyme is effected by one of the following procedures: arterial occlusion of the liver, cryo-ablation, ultrasonic ablation or radio-frequency ablation of the tumor lesions in the liver.

15. The therapeutic composition of any of claims 2 and 4-14, wherein said inhibitor of intestinal-kidney axis is a mixture of lactic acid and a salt of lactic acid, such as sodium lactate.

16. The therapeutic composition of any of claims 2 and 4-14, wherein said inhibitor of intestinal-kidney axis is gly-gly-PALO.

17. The therapeutic composition of any of claims 1 to 16 further comprising at least one protein breakdown inhibitor.

18. The therapeutic composition of claim 17 wherein said protein breakdown inhibitor is selected from the group consisting of insulin, insulin-like growth factors, lactacystin, epoxomicin, peptide boronates, such as PS-341, heme and modifications thereof, hemin, NSAID, or mixtures thereof.

19. The therapeutic composition of claims 1 to 18 additionally comprising a vasoconstricting composition.

20. The therapeutic composition of claim 19, where the vasoconstricting composition comprises a peptide.

21. The therapeutic composition of claim 19, where the vasoconstricting composition comprises vasopressin, an analog of vasopressin, or mixtures thereof.

22. The therapeutic composition of claim 21 wherein the vasoconstricting composition is selected from the group consisting of vasopressin, ornipressin, terlipressin, desmopressin, and mixtures thereof.

23. The therapeutic composition of any of claims 1 to 22, additionally comprising prostacyclin, an analog of prostacylin, or mixtures thereof.

24. The therapeutic composition of claim 23, where the analog of prostacyclin is iloprost.

25. The therapeutic composition of any of claims 1, 2, or 4-25, further comprising a nitric oxide donor.

26. The therapeutic composition of either claim 3 or claim 25 wherein the nitric oxide donor is selected from the group consisting of sodium nitroprusside, nitroglycerin, cysteine-containing direct nitric oxide donors and mixtures thereof.

27. The therapeutic composition of any of claims 1-26 further comprising an antidote, such as thiosulfate, Vitamin B12A or analogs or mixtures thereof.

28. The therapeutic composition of any of claims 1-27 further comprising the independent administration of mixture of amino acids free from arginine.

29. The use of any of the therapeutic composition of any of claims 1-28 wherein said arginine decomposing enzyme is administered exogenously.

30. The use of any of the therapeutic composition of any of claims 1-28 wherein said arginine decomposing enzyme is released endogenously from the patient's own tissues, such as liver.

31. The use of therapeutic composition according to any of claims 1-30 for cancer therapy, wherein the cancer therapy comprises a depletion of arginine effected by an arginine-decomposing enzyme.

32. A pharmaceutical composition for the reduction of side-effects in cancer therapy comprising:

(a) an agent for the depletion of arginine, particularly an arginine-depleting enzyme;

(b) a NO donor;

(c) optionally an antidote;

(d) a protein-breakdown inhibitor, such as insulin, insulin-like growth factors, lactacystin, epoxomicin, peptide boronates, such as PS-341, heme and modifications thereof, hemin or NSAID, and mixtures thereof;

(e) optionally a vascular tonus controlling agent;

(f) optionally prostracyclin or an analog thereof;

(g) optionally a mix of amino acids free from arginine; and

(h) optionally arginine.

33. The pharmaceutical composition of claim 32 wherein components (a)-(g) if present are administered separately or in appropriate combinations during the therapy and component (h) if present is administered after finishing the therapy.

34. The use of NO donor for the manufacture of a medicament for the therapy of cancer.

35. The use of claim 34, wherein the cancer therapy comprises a depletion of arginine in the subject to be treated.

36. The use of claim 35, wherein the depletion of arginine is effected by an arginine-decomposing enzyme.

37. The use of claim 36, wherein the arginine-decomposing enzyme is administered exogenously.

38. The use of claim 36, wherein the arginine-decomposing enzyme is released from the tissue of the subject to be treated.

39. The use of any of claims 34 to 38, wherein the cancer therapy further comprises the administration of a protein-breakdown inhibitor, e.g. insulin.

40. The use of any one of claims 34-39 for the reduction of side-effects in cancer therapy.

41. The use of claim 40, wherein the side-effects comprise a systunction of endogenous platelet formation.

42. The use of any of claims 34-41 further comprising the use of antidotes, eg. thiosulfates or vitamin B12 A or analogs.

43. The use of any of claims 34-42 further comprising the use of vasoconstrictors.

44. The use of any of claims 34-43 further comprising the use of prostacyclin or analogs thereof.

45. The use of any of claims 34-44 further comprising the administration during therapy of a mixture of amino acids free from arginine.

46. The use of any of claims 34-45 further comprising the administration of arginine after completion of the cancer therapy.

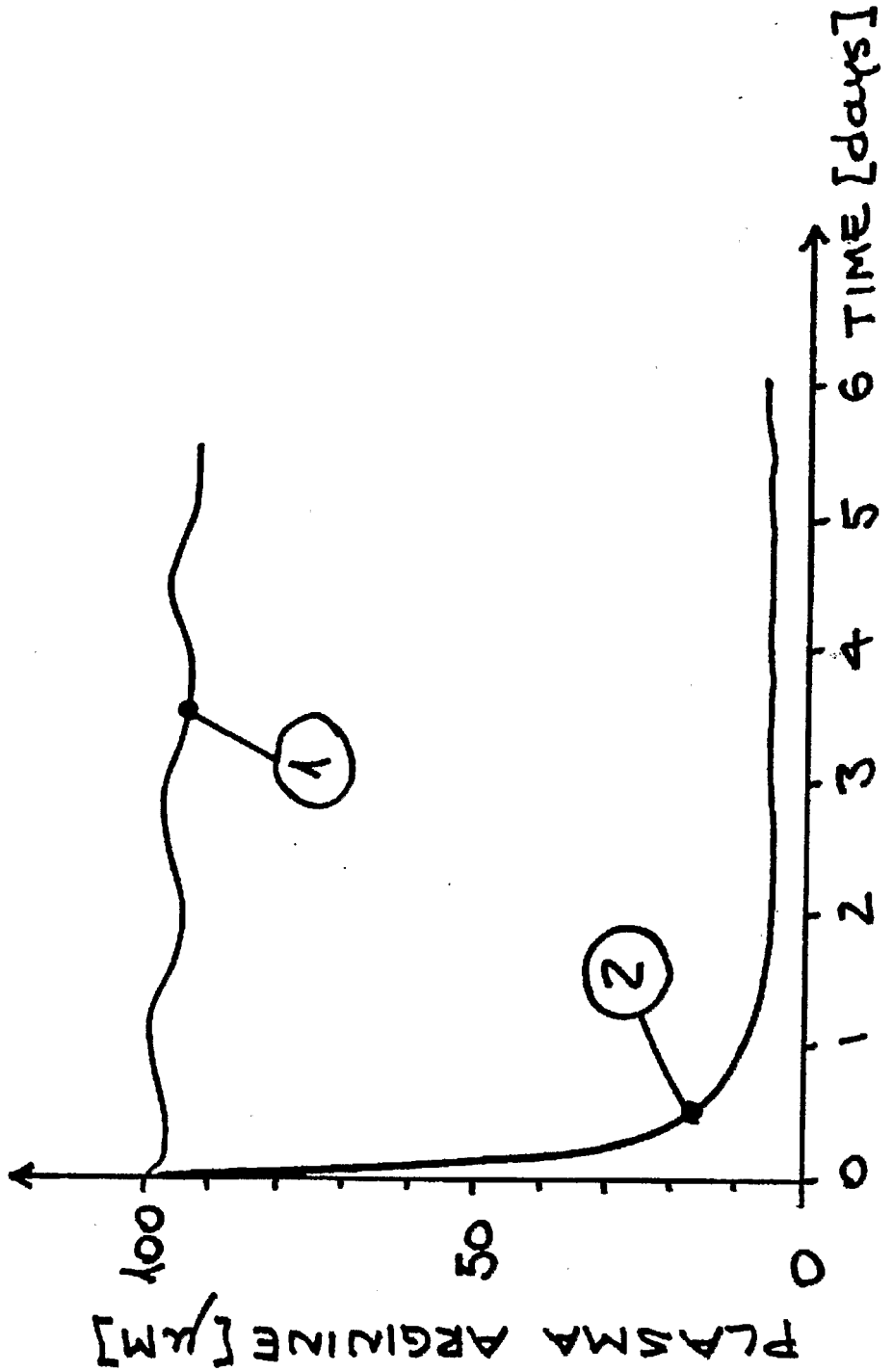


FIGURE 1

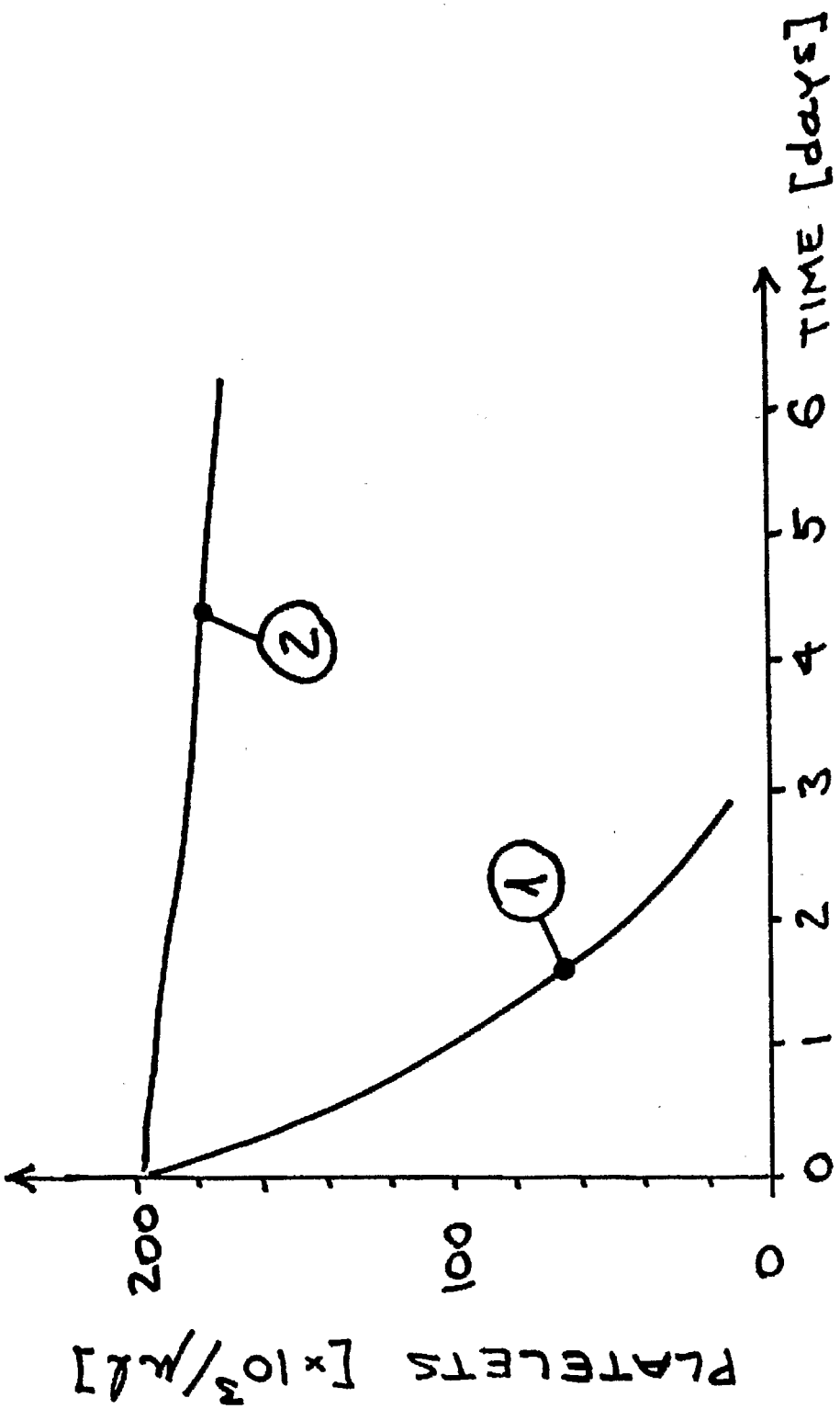


FIGURE 2

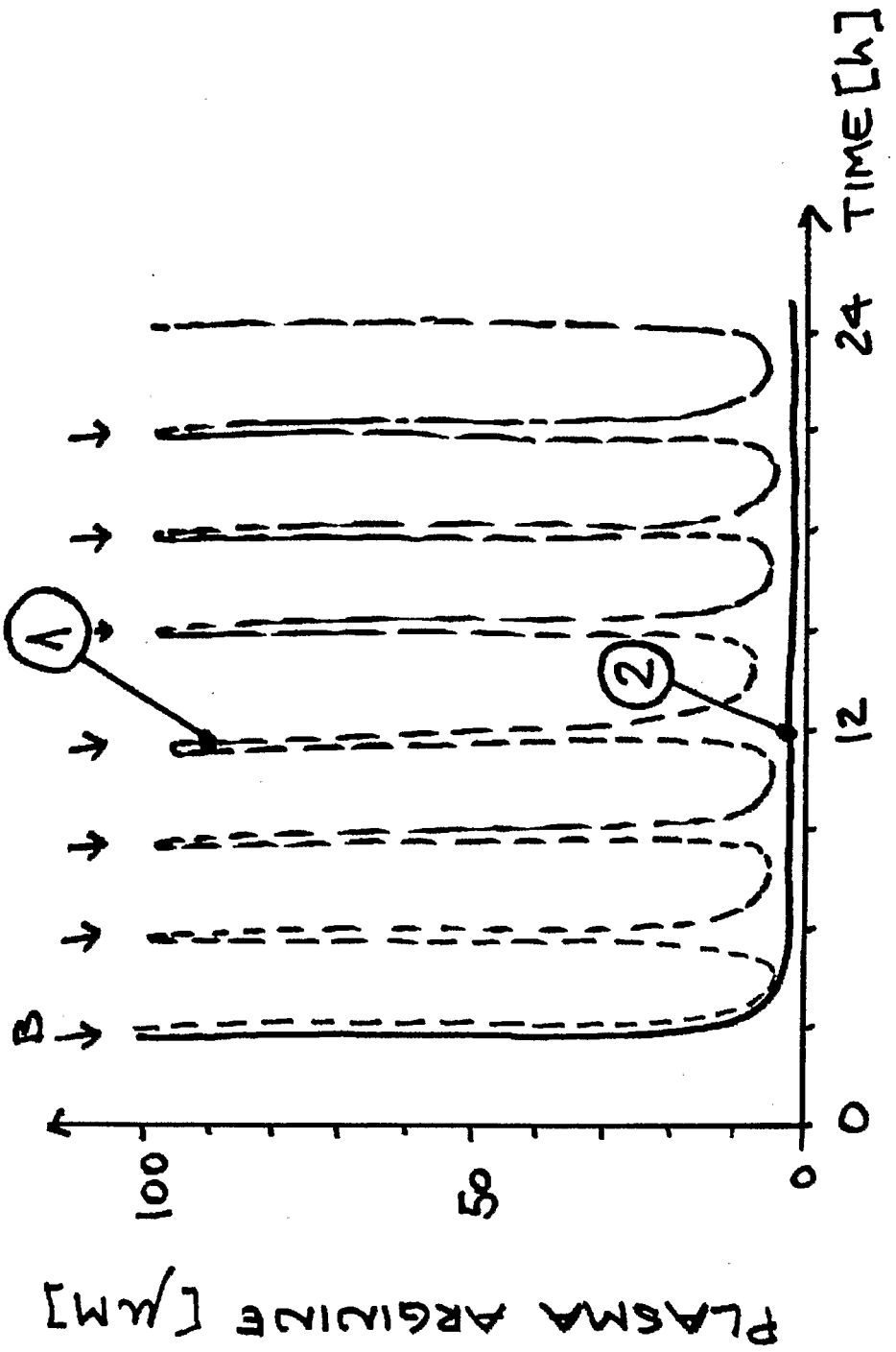


FIGURE 3

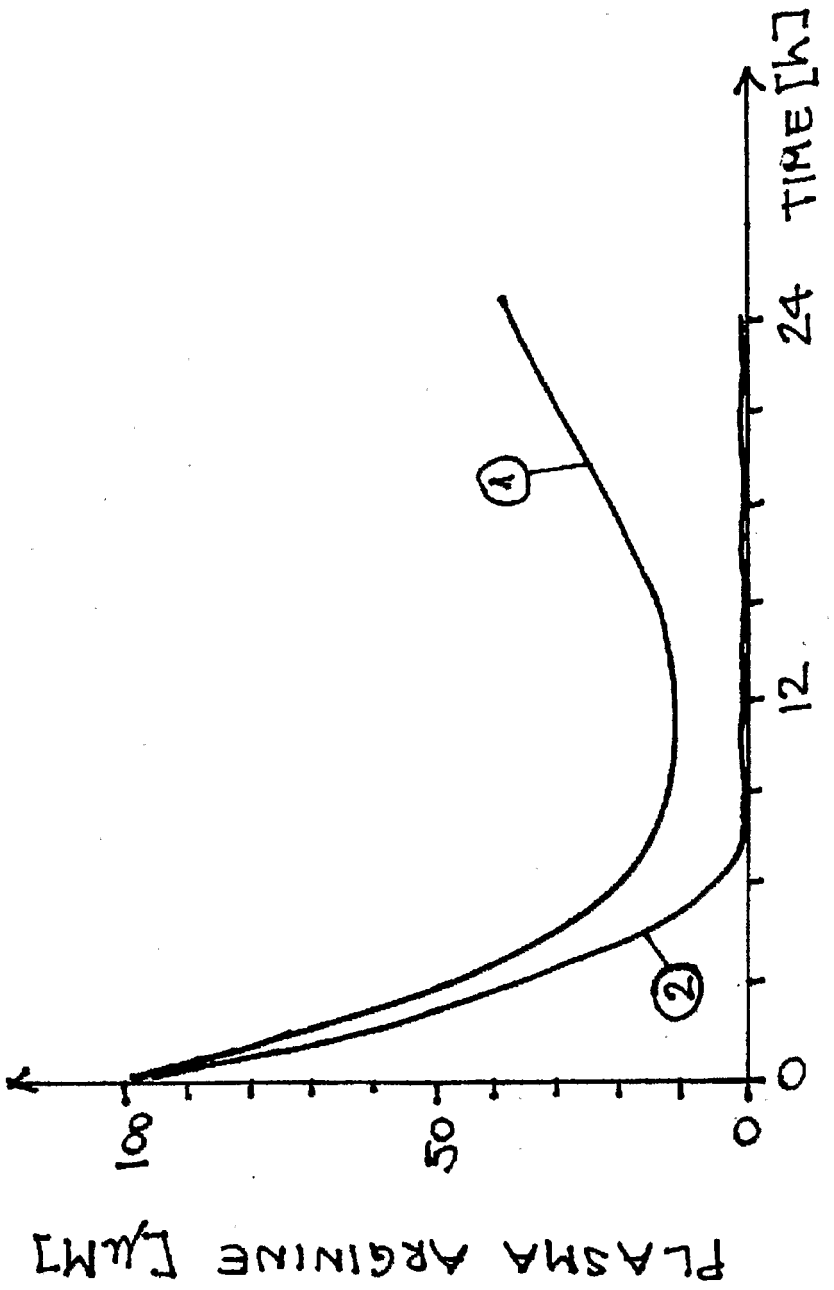


FIGURE 4

# FIGURE 5

| | | | initial rate | max rate | comment |
|---|---|---|---|---|---|
| 1 (s) | arginase | recombinant human liver arginase, University of Kyoto | 50 mg/m2/d<br>100 mg/m2/d<br>165 mg/m2/d<br>250 mg/m2/d | | keep the rate fixed in a given patient |
| 2 (s) | insulin | recombinant, human, actrapid hm, novo | 1.5 I.E./kg/d | 6 I.E./kg/d | if after 12 h plasma arginine >1microm, escalate; if max rate not sufficient, then arginase rate is low |
| 3 (p) | glucose | 50% solution | 7 g/kg/d | 16 g/kg/d | adjust to maintain normal plasma level; if max rate not enough, reduce insulin |
| 4 (s) | SNP | sodium nitroprusside | 0.7 mg/kg/d | 3.5 mg/kg/d | if any platelet loss after 12 h, increase in steps of 0.7 |
| 5 (s) | ornipressin | vasopressin analog por 8, ferring | 0.3 I.E./kg/d | 3 I.E./kg/d | adjust to maintain pulse and pressure |
| 6 (s) | iloprost | prostacyclin analog Ilomedin, schering | 1.4 μg/kg/d | 2.8 μg/kg/d | if platelet loss after 12 h, increase with SNP |
| 7 (p) | amino acids | arginine-free mix | 1 g/kg/d | 1.5 g/kg/d | maintain plasma amino acids levels; monitor ammonia !! |
| 8 (p) | lipid | | 1.5 g/kg/d | | |
| 9 (p) | plasma | cryoprecipitate, platelets if indicated | | | as needed to maintain normal clotting times |

(s) denotes preferable use of a syringe pump; (p) of a peristaltic pump.

| | | | |
|---|---|---|---|
| 专利名称(译) | 用于通过精氨酸消耗治疗癌症的治疗组合物 | | |
| 公开(公告)号 | EP1499342A2 | 公开(公告)日 | 2005-01-26 |
| 申请号 | EP2003735013 | 申请日 | 2003-01-27 |
| [标]申请(专利权)人(译) | 癌症治疗 | | |
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| IPC分类号 | A61K38/45 A61K38/50 A61K38/51 A61K45/06 A61K38/43 C12N9/00 C12N9/88 C12N15/09 G01N33/53 | | |
| CPC分类号 | A61K45/06 A61K38/45 A61K38/50 A61K38/51 | | |
| 优先权 | 60/350971 2002-01-25 US | | |
| 外部链接 | Espacenet | | |


摘要(译)

一种治疗组合物和通过消除精氨酸治疗癌症而没有全身并发症的方法，包括精氨酸分解酶和蛋白质分解抑制剂，一氧化氮供体，加压肽和前列环素。该组合物可进一步包括缺乏精氨酸的氨基酸混合物，氰化物的解毒剂，血浆或其衍生物，和/或精氨酸的制剂。精氨酸分解酶可以被修饰以增加循环半衰期，并且可以是I型肝精氨酸酶，或人或动物的II型，部分纯化的，或重组的，或甚至细菌来源的。它可以作为药物施用或从患者自身组织中释放。精氨酸的内源性产生，特别是通过所谓的肠 - 肾轴，可以在几个酶促步骤中被有益地抑制，允许循环精氨酸的更深的减少。组合物的不同组分可以分开给药，或以合适的混合物给药，允许在治疗期间进行所需的调节。