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(54) Title: NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE (NTPDASE 8) AND USES THEREOF

(57) Abstract: The present invention relates to a new ectohydroxynucleotidase, namely the NTPDase8, which allows to regulate platelet aggregation or activation involved in the formation of thrombosis and related diseases. The nucleic acid sequence and the corresponding amino acid sequence and uses thereof are described.



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**NUCLEOSIDE TRIPHOSPHATE
DIPHOSPHOHYDROLASE AND USES THEREOF**

TECHNICAL FIELD

5 The present invention relates to nucleic acid sequences and related proteins to modulate the adhesion of platelets in blood. More particularly, the invention allows for inhibiting partially or totally the formation of thrombosis and related physiological diseases of causes.

BACKGROUND ART

10 Nucleosides play significant roles in different body systems, including the nervous and the vascular systems. Their specific functions in these systems is determined by the presence of purine and pyrimidine receptors which react with triphosphate nucleosides and their derivatives at the surface of numerous cell types. The presence in the vascular system of both ectoATPase and ectoADPase activities,
15 that cleave tri- and diphosphate nucleosides respectively, has been known for many years. By their location and kinetic properties, these different catalytic activities could influence these systems. Both activities were previously attributed to two distinct enzymes. These activities were characterized and showed that, in bovine aorta, a single enzyme was responsible for the sequential hydrolysis of ATP and
20 ADP.

 The enzyme responsible for that sequential cleavage of the γ and β phosphate residues of polyphosphorylated nucleosides are commonly called nucleoside triphosphate diphosphohydrolases (NTPDase) or apyrases (EC 3.6.1.5). These enzymes are generally activated in the presence of divalent cations Ca^{+2} or
25 Mg^{+2} , while sodium azide inhibits their activity azide is an inhibitor of NTPDase1 and a few other NTPDase but not all of them. The catalytic site of these enzymes is generally exposed to extracytoplasmic spaces (ectoenzymes).

 In plants, ATPDase enzymes are found in the cytoplasm, in soluble or membrane-associated forms, and are generally more active in acidic conditions.

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Although their precise function remains unknown, some evidences suggest their implication in the biosynthesis of carbohydrates. At the opposite, the activity of NTPDases is higher at neutral or alkaline pH in invertebrates species, where they principally shown to act as antihemostatic agents in saliva and in salivary glands of
5 hematophagous insects.

In vertebrates, a limited number of studies have already defined a diversity of NTPDases. The first mammalian NTPDase has been isolated from pancreas and was further identified in other tissues, including bovine aorta. It is recognized in the art that NTPDase may be the object of other nomenclature. For
10 exaple, NTPDase1 may refers to vascular ATPDase or CD39.

Nucleosides and thus, NTPDases, contribute to the vascular system homeostasis. Extracellular nucleosides present in the blood due to, e.g., arterial vascular injury can influence cardiac function, vasomotor responses, inflammatory processes, thrombosis, and platelet activation. To maintain blood fluidity and flow,
15 the normal vascular endothelium inhibits coagulation and platelet activation and promotes fibrinolysis. Quiescent endothelial cells are considered to directly express natural anticoagulants and thromboregulatory factors, therefore preventing thrombosis, which usually develops as a consequence of overwhelming these antithrombotic mechanisms. This may occur following the heightened production
20 of locally produced mediators, including cytokines, activated complement components and particularly extracellular nucleotides. In the bovine aorta, NTPDase1 was found to be associated with smooth muscle cells and endothelial cells and could inhibit ADP-induced platelet aggregation. It was further showed that concurrent addition of a semi purified fractin of NTPDase and ATP to platelet-
25 rich plasma resulted in an immediate dose-dependent platelet aggregation caused by the accumulation of ADP, followed by a slow disaggregation attributable to its hydrolysis into AMP. In the absence of NTPDase, ATP does not induce any

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aggregation while ADP initiates an aggregation which extent is limited by the ADPase activity of the enzyme.

Mechanism of nucleosides actions in blood vessels implicates, between others, the binding to and stimulation of purinergic/pyrimidinergic type-2 (P2) receptors. This stimulation P2Y receptors initiates G protein-coupled signaling pathways and results in activation of platelets, endothelial cells (ECs), monocytes/macrophages, and leukocytes and could culminate in vascular thrombosis and inflammation *in vivo*. ATP and ADP regulation of platelet aggregation appear to occur through the concomitant activation of platelet P2Y₁ and P2Y₁₂ receptors. Indeed ADP is a major platelet recruiting and activating factor, whereas ATP acts as a weak competitive antagonist of ADP for platelet P2 receptors. This latter protective action of ATP may limit the formation of intravascular platelet aggregation and help localize thrombus formation to areas of vascular damage. NTPDases also attenuate the aggregation elicited collagen and low level of by thrombin but not by the platelet activating factor (PAF), the first two agonist effects being caused by a release of platelet ADP. It has therefore been suggested that NTPDase had a dual role in regulating platelet activation. By converting ATP released from damaged vessel cells into ADP, the enzyme induced platelet aggregation at the sites of vascular injury. By converting ADP released from aggregated platelets and/or from hemolyzed red blood cells to AMP, the NTPDase could inhibit or reverse platelet activation, and consequently limit the growth of platelet thrombus at the site of injury.

Platelets are particles found in whole blood that initiate and provide the structural basis for the haemostatic plug necessary to stop bleeding. Platelets depend on adhesive interactions with extracellular proteins and other cells for proper function. The external platelet plasma membrane surface is covered with a variety of membrane bound glycoproteins, many of which have adhesive functions. Perhaps the most abundant platelet membrane adhesive proteins belong to the

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integrin superfamily which include the glycoproteins; GP I_b III_a, GP I_a II_a, GP I_c II_a, GP I_b IX, and the fibronectin and vitronectin receptors. Each integrin receptor is an heterodimer displaying characteristic affinity and specificity toward various extracellular matrix proteins such as; von Willebrand factor (vWF), collagen, entactin, tenascin, fibronectin (Fn), vitronectin (Vn), and laminin, as well as fibrinogen (Fg) and thrombospondin. The most abundant integrin found on normal platelet surfaces is GP II_b et GPIII_a comprising about 50,000 molecules per platelet, representing about 2% of the total platelet protein. GP II_b III_a is a non-covalent, calcium ion dependent heterodimer complex and restricted in distribution to platelets and other cells of the megakaryocytic lineage. On activated platelets, GP II_b III_a binds a number of adhesive proteins with varying affinities; fibrinogen, fibronectin, von Willebrand factor, vitronectin and thrombospondin. It is believed the most important interactions mediating platelet aggregation involve GP II_b III_a binding with the trinodular fibrinogen and, to a lesser extent, with the filamentous von Willebrand factor.

Platelets are key components of all blood clots propagating within the arterial circulation and thus are an obvious therapeutic target in attempts to inhibit coronary artery thrombosis. Despite currently available therapies, a significant number of ischemic events, such as myocardial infarction, stroke, and death, occur each year. These events are generally the result of blood clots blocking the arteries supplying oxygen to heart or brain tissue. Therefore, there exists a need for therapeutics that effectively regulate platelet activation for the purpose of controlling platelet aggregation.

Angiogenesis is also a highly complex multistep phenomenon that incorporates both formation of new capillaries and expansion or extension of existing blood vessels. An associated increased permeability to plasma solutes results in the deposition of a provisional matrix in which fibrin is a major component. New vessel growth may be modulated by monocyte/macrophages that

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secrete angiogenic factors and metalloproteases that facilitate endothelial cell migration. Supporting cells are also essential for new vessel growth and angiogenesis, for example, smooth muscle cells in vascular maturation and arteriogenesis and pericytes in the protection of newly developing endothelial cell-lined tubes from rupture and regression.

In this context, UTP has been shown to be mitogenic and chemotactic for endothelial cells *in vitro*. Interestingly, binding of angiostatin, a proteolytic fragment of plasminogen and potent antagonist of angiogenesis, to ATP synthase expressed on endothelial cells, has been shown to mediate antiangiogenic effects.

To decipher the mechanisms of such interactions, the role of nucleotides in angiogenesis *cd39*-null (or *Entpd1*) mouse model, in which aberrant regulation of nucleotide P2 receptors has been observed, was investigated. CD39 (also referred to as nucleoside triphosphate diphosphohydrolase-1 (NTPDase1) was shown to be the major vascular endothelial membrane ectonucleotidases and to hydrolyse nucleoside triphosphates and diphosphates, ultimately to the nucleoside analogues; these products having mitogenic effects on endothelial cells *in vitro*. Because angiogenesis is critical to the progression of various diseases, for example, cancer, rheumatoid arthritis, and diabetic retinopathy, there exists a need for compounds capable of preventing or reducing angiogenesis in patients suffering from an angiogenesis-associated condition. NTPDase1/CD39 could now be used to reduce platelet aggregation, thrombogenicity as well as angiogenesis. However, the solubilization of the protein actually lead to a decrease in nucleoside phosphohydrolase activity. It would thus be highly desirable to be provided with a stable NTPDase for the treatment of prevention of diseases related to blood clotting or angiogenesis.

While some products related to ectohydroxynucleotides as described before exist in the art, there is still place for new molecules allowing platelet

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aggregation modulation and/or control in different physiological normal or pathological conditions.

DISCLOSURE OF THE INVENTION

- 5 One object of the present invention is to provide an isolated nucleoside triphosphate diphosphohydrolase (NTPDase) from a mammalian tissue characterized in that it comprises the amino acid sequence SEQ ID NO:1 or SEQ ID NO:3, a functional fragment thereof, or an amino acid sequence having at least eighty percent (70%) homology with said isolated and purified NTPDase.
- 10 Preferentially, the homology level at least of 80%.

Another object is to provide an isolated nucleic acid molecule consisting of a nucleotide sequence selected from the group consisting of :

- a nucleotide sequence encoding for a peptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:3;
- 15 a nucleotide sequence consisting of SEQ ID NO:2 or SEQ ID NO:4; and
- a nucleotide sequence complementary to at least 70% of a nucleotide sequence of a) or b).

The nucleotide sequences a), b) or c) encode for a peptide having ectonucleotidase activity.

- 20 Also, the nucleic acid expression vector may comprise a nucleic acid molecule as defined herein in operative association with a promoter sequence. The promoter can be an inducible or an ubiquitous promoter.

A further object of the invention is to provide a host cell containing the expression vector as defined herein. The host cell is preferably an eukaryotic cell.

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Another object of the invention is to provide an isolated peptide comprising the amino acid sequence SEQ ID NO:1, a functional fragment or an analog thereof, said isolated peptide having an ectonucleotidase activity.

Particularly, one object of the present invention is to provide an isolated
5 polynucleotide consisting of a nucleotide sequence set forth in SEQ ID NO:2.

In accordance with the present invention, there is provided a method for modulating at least one biological reaction selected from the group consisting of platelet aggregation, thrombosis, emboli, and angiogenesis, said method comprising depositing in blood or a medium comprising blood an amount sufficient of a
10 peptide encoded by a nucleic acid molecule or an isolated peptide as defined herein under condition allowing the modulation of platelet aggregation, thrombosis, or emboli.

Also, another object of the invention is to provide a method for modulating at least one biological reaction selected from the group consisting of
15 platelet aggregation, thrombosis, emboli, and angiogenesis, comprising administering to a patient an expression vector as defined herein.

Alternatively, there is provided a method for modulating at least one biological reaction selected from the group consisting of platelet aggregation, thrombosis, emboli, and angiogenesis, comprising administering to a patient a
20 partial expression vector without promoter and designed to incorporate a transcriptionnally active genomic region of a cell, said partial expression being transcribed under the control of a promoter endogenous to said genomic region.

The sequence complementary to the NTPDase8 molecule of the present invention can be obtained from mammalian tissue from which the NTPDase is
25 isolated, as for example from a human (SEQ ID NO:3), a porcine, a bovine, a primate, a caprine, an ovine, an equine, a murine, a canine, a grouse or a feline tissue.

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Another object of the present invention is to provide an isolated nucleotide sequence encoding the amino acid sequence of the isolated NTPDase described above or the complementary nucleotide sequence thereof, wherein said isolated nucleotide sequence is a gene, a complementary DNA (cDNA), a messenger RNA (mRNA), a complementary RNA (cRNA) or a fragment thereof.

A further object of the present invention is to provide a recombinant expression vector comprising a promoter sequence and a nucleotide sequence encoding the amino acid sequence of the isolated NTPDase described above.

Also, one object of the present invention is to provide a recombinant host cell, transformed or transfected with said recombinant expression vector comprising a nucleotide sequence encoding the amino acid sequence of the isolated NTPDase.

In one object of the present invention, there is provided a method for modulating platelet aggregation, thrombogenicity or angiogenesis which comprises an administration of a NTPDase.

A further object of the present invention is to provide a composition for modulating platelet aggregation, thrombogenicity or angiogenesis wherein said composition comprises the isolated NTPDase as active ingredient. The active ingredient can be found alone or in combination with an acceptable carrier.

In a further object of the present invention, there is provided a method for converting ATP into ADP and/or ADP into AMP comprising an administration of the NTPDase described herein.

Another object of the present invention is to provide A composition for converting ATP into ADP and/or ADP into AMP comprising the NTPDase described herein, together with a pharmaceutically acceptable carrier.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position

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of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

BRIEF DESCRIPTION OF DRAWINGS

Figs. 1A and 1B illustrate the hydrophobicity analysis and schematic representation of murine NTPDase8 ;

Fig. 2 illustrates the phylogenetic analysis of amino acid sequences of selected NTPDases and related proteins;

Fig. 3 illustrates a schematic representation of the genomic organization of mouse encoding gene *Entpd8*;

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Figs. 4A to 4D illustrate the biochemical characterization of mouse NTPDase8;

Figs. 5A to 5D illustrate the kinetics and profile of nucleotides hydrolysis by mouse NTPDase8;

5 Fig. 6 illustrates the effect of the isolated NTPDase8 on platelet aggregation.

MODES OF CARRYING OUT THE INVENTION

10 In accordance with the present invention, there is provided an isolated nucleoside triphosphate diphosphohydrolase (NTPDase) from a mammalian tissue characterized in that it comprises the amino acid sequence SEQ ID NO:1 and 3 , with corresponding encoding nucleotide sequences SEQ ID NO:2 and 4.

15 The present invention relates to methods for reducing the platelet activation or aggregation state in an individual, and methods for treating or preventing a vascular condition by administering to said individual a NTPDase8 under the peptide form or under its nucleic acid form.

Hence, the present invention relates to methods of reducing or inhibiting platelet activation by administering to an individual an effective amount of a NTPDase8, or analogues or derivatives thereof.

20 The platelet activation state refers to the occurrence of one or more of the following events: platelet aggregation, platelet adhesion, platelet agglutination, platelet release reactions (e.g., osteonectin, platelet factor 4 or beta-thrombomodulin), expression of platelet external receptors (e.g., GPIIb/IIIa or P-selectin), or platelet interaction with other blood components (e.g., collagen or
25 fibrinogen) and cells (e.g., leukocytes). Administration of a NTPDase8 decreases, reduces or inhibits one or more of these occurrences, thereby reducing the platelet activation state.

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Several platelet activation markers exist which can be measured to assess the platelet activation state. Platelet activation can be assessed using platelet activation markers that are currently assessed in the art, as well as those that will later be discovered. Examples of platelet activation markers are: CD9, GPIb, GPIIb, CDIa-IIa, P-selectin, PECAM-1, GPIIb/IIIa, vitronectin, integrins and adhesive molecules. A reduction in the platelet activation state also refers to a decrease in or an absence of one or more platelet activation markers. One or more platelet activation markers is decreased by at least about 10% (e.g., 20% to 100%), as compared to the level just prior to administration. Hence, one can measure the presence, absence or level of one or more platelet activation markers, and compare the result against a control. For example, one can obtain a suitable sample and compare the level of one or more platelet activation markers from previous time points (e.g., prior to administration of the NTPDase8 or during the onset of a vascular event, disease or disorder). The level of one or more platelet activation markers decreases after administration of the NTPDase8, as compared to the level during the onset of the vascular event. One can also measure platelet activation markers in an individual prior to the onset of a vascular event (e.g., in a resting state or during a check-up), and determine the individuals baseline. Accordingly, administration of a NTPDase8 after the onset of a vascular disease decreases the levels of one or more platelet activation markers, as compared to those levels occurring during the onset of the vascular disease.

The level of platelet activation markers assessed can also be compared to a standard or control obtained from normal individuals. In one example, levels of platelet activation markers can be assessed in a population of healthy individuals or individuals who have not had a vascular event, disease or disorder. Such levels are referred to as a "negative control." Conversely, platelet activation marker levels can also be obtained from a pool of individuals who are undergoing a vascular event, disease or disorder, e.g., a "positive control." After administration of a NTPDase8, the level of one or more platelet activation markers decreases; the platelet

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activation marker level gets closer to the level of the negative control, and farther from the positive control. The level of a platelet activation marker decreases as compared to the level the platelet activation marker during the onset of the vascular event, disease or disorder. Hence, the methods include reducing the platelet
5 activation state, or inhibiting platelet activation with administration of a NTPDase8, wherein one or more platelet activation markers is reduced or decreased, as compared to those levels during the occurrence of the vascular event, disease or disorder, or immediately prior to the administration of a NTPDase8.

In another embodiment, the present invention relates to a method for
10 preventing the onset of a vascular event, disease or disorder. An effective amount of at least one NTPDase8 can be administered to prevent platelet activation state from increasing, or lessen platelet activation state that would otherwise become active without NTPDase8 administration. For example, an individual who is a risk for a vascular event, disease or condition can take a NTPDase8 on a daily basis (or
15 every other day), to prevent the platelet activation state from increasing as compared to a control or baseline. Baseline levels of the platelet activation state can be obtained prior to and/or during the course of administration of a NTPDase8. The platelet activation state, as measured by platelet markers can stay the same, or can even decrease. Similarly, the platelet activation marker level can be compared to a
20 negative or positive control, wherein upon administration of NTPDase8, the levels are closer to the negative control, than the positive control. However measured, the platelet activation state is prevented from increasing, thereby preventing the occurrence of a vascular event, disease or disorder.

The present invention also relates to reducing or inhibiting platelet
25 activation by contacting the platelets with a NTPDase8 or derivatives or fragments thereof. This embodiment of the invention can be carried out *in vivo* or *in vitro*. The method reduces the level of one or more platelet activation markers, as compared to the level prior to contact of the NTPDase8 with the platelets.

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The present invention pertains to methods for preventing or treating an individual at risk for a vascular event, disease or disorder. Platelet activation is the cause or a significant contributor of several vascular diseases. Prevention of a vascular event, disease or disorder (e.g., vascular condition) refers to delaying or suppressing the onset of the vascular condition, or one or more of its symptoms. To treat an individual at risk for a vascular condition means to alleviate or ameliorate one or more of its symptoms. An individual at risk for a vascular condition refers to an individual with a history of vascular disease, an individual experiencing symptoms or risk factors (e.g., gender, weight) associated with or caused by the vascular condition, an individual undergoing a vascular procedure, or an individual who has tested positive for a vascular condition using a diagnostic test (e.g., electrocardiogram, cardiac catheterization, stress test, ultrasound poppler techniques). A vascular condition is a event, disease or disorder that involves a thrombosis or a narrowing of a blood vessel. Vascular events, diseases or disorders include cardiovascular diseases (e.g., coronary heart disease, myocardial infarction, angina or a disease in which a narrowing of a blood vessel occurs in at least one major artery), cerebrovascular diseases (e.g., stroke or transient ischemic attacks), vascular procedures (e.g., thrombotic re-occlusion subsequent to a coronary intervention procedure, heart or vascular surgery) or any other thrombotic event (e.g., pulmonary embolism, deep vein thrombosis or peripheral vascular thrombosis). Vascular conditions also include Syndrome X, which is a disease that is associated with unidentified chest pain.

The NTPDases 8 used in the present invention can be administered with or without a carrier. The terms "pharmaceutically acceptable carrier" or a "carrier" refer to any generally acceptable excipient or drug delivery composition that is relatively inert and non-toxic. Exemplary carriers include sterile water, salt solutions (such as Ringer's solution), alcohols, gelatin, talc, viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, calcium carbonate, carbohydrates (such as lactose, sucrose, dextrose, mannose, albumin, starch,

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cellulose, silica gel, polyethylene glycol (PEG), dried skim milk, rice flour, magnesium stearate, and the like. Suitable formulations and additional carriers are described in Remington's Pharmaceutical Sciences, (17^{sup}.th Ed., Mack Pub. Co., Easton, Pa.). Such preparations can be sterilized and, if desired, mixed with
5 auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, preservatives and/or aromatic substances and the like which do not deleteriously react with the active compounds. Typical preservatives can include, potassium sorbate, sodium metabisulfite, methyl paraben, propyl paraben, thimerosal, etc. The compositions
10 can also be combined where desired with other active substances, e.g., enzyme inhibitors, to reduce metabolic degradation. A carrier (e.g., a pharmaceutically acceptable carrier) is preferred, but not necessary to administer the compound.

The NTPDase8 can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The method of
15 administration can dictate how the composition will be formulated. For example, the composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

20 The NTPDase8 used in the invention can be administered intravenously, parenterally, intramuscular, subcutaneously, orally, nasally, topically, by inhalation, by implant, by injection, or by suppository. The composition can be administered in a single dose or in more than one dose over a period of time to confer the desired effect.

25 The actual effective amounts of compound or drug can vary according to the specific composition being utilized, the mode of administration and the age, weight and condition of the patient. For example, as used herein, an effective amount of the drug is an amount which reduces the platelet activation state.

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Dosages for a particular individual patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

For enteral or mucosal application (including via oral and nasal mucosa), particularly suitable are tablets, liquids, drops, suppositories or capsules. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Liposomes, microspheres, and microcapsules are available and can be used.

Pulmonary administration can be accomplished, for example, using any of various delivery devices known in the art such as an inhaler.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. In particular, carriers for parenteral administration include aqueous solutions of dextrose, saline, pure water, ethanol, glycerol, propylene glycol, peanut oil, sesame oil, polyoxyethylene-polyoxypropylene block polymers, and the like. Ampoules are convenient unit dosages.

The administration of the NTPDase8 and the vascular treating compound can occur simultaneously or sequentially in time. The vascular treating compound can be administered before, after or at the same time as the NTPDase8. Thus, the term "co-administration" is used herein to mean that the NTPDase8 and the vascular treating compound will be administered at times to achieve a reduction of the platelet activation state. The methods of the present invention are not limited to the sequence in which the NTPDASE8 and vascular treating compound are administered, so long as the vascular treating compound is administered close enough in time to produce the desired effect of reducing the platelet activation state.

Finally, in addition to being small, potent and specific, the preferred cyclic peptide platelet aggregation inhibitor should not produce substantial

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untoward in vivo side effects such as an increased cutaneous bleeding time, reduction in platelet count, or decreased peripheral blood flow in a mammal treated with the inhibitor. Thus, for example, the time it takes for bleeding to stop, on an incision made in a mammal treated with a platelet aggregation inhibitor, dosed to
5 inhibit about 90-100% of platelet aggregation, should not be more than about twice that of the mammal prior to treatment. Similarly, peripheral blood flow and other hemodynamic factors should not be adversely affected by treatment with doses sufficient to achieve 90-100% inhibition of platelet aggregation.

The present invention will be more readily understood by referring to the
10 following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Effect of calcium and magnesium on ATPase and ADPase activity 15 of murine NTPDase8

Materials and Methods

Agarose, aprotinin, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N-N-N'-N'-tetraacetic acid (EGTA), 2-(4-
20 morpholino)-ethane sulfonic acid (MES), nucleotides, phenylmethanesulfonyl fluoride (PMSF), sodium acetate, tetrabutylammonium hydrogen sulphate (TBA) and tris(Hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich (Oakville, On, Canada). Glycine was provided by Fisher Scientific Ltd (Nepean, On, Canada). All cell culture media were obtained from Invitrogen (Burlington,
25 On, Canada) and the 24-well plates from VWR Canlab (Mont-Royal, Qc, Canada).

RT-PCR cloning

Total RNA was isolated from mouse liver with Trizol™ reagent (Invitrogen). cDNA was synthesized with SuperScript II (Invitrogen) from 500 ng

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of total RNA with oligo (dT)₁₈ as the primer, according to the manufacturer's instructions (Invitrogen). For amplification, one fifth of the reverse transcription (RT) reaction volume was used as template in a final volume of 50 µl containing 0.4 µM primer, 200 µM dNTP and 0.7 U Tgo DNA polymeraseTM (Roche, Laval, Qc, Canada). The two following sets of primers were designed based on the 5' and 3' ends of incomplete mouse EST sequences (GenBank accession numbers BB610017 and AV026718, respectively): 1) forward 5'GGA-GAC-GGG-GTG-TGG-AGG-AC3' (SEQ ID NO:5), reverse 5'GGG-GTT-CAT-AAG-GGC-AGG-CA3' (SEQ ID NO:6); 2) forward 5'GTA-GGT-GGA-GAC-GGG-GTG-TG3'(SEQ ID NO:7), reverse 5'GGG-TTC-ATA-AGG-GCA-GGC-AT3'(SEQ ID NO:8). Amplification was started with 2 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 2 min annealing at 60°C, 2 min primer extension at 72°C and ended with a 7 min incubation at 72°C. The PCR product of approximately 1.8 kb was purified on agarose gel using the QIAEX IITM gel extraction kit (Qiagen, Mississauga, On, Canada) and ligated to pcDNA3.1/V5-His (Invitrogen). Plasmid DNA was purified with the QIAprep Spin Miniprep kitTM (Qiagen) and the orientation of the insert determined by restriction enzyme mapping. Two independent clones (one for each set of primers) with the orientation allowing the expression of the protein were amplified and sequenced. One clone was completely sequenced in both directions and the second clone in one orientation to confirm the sequence obtained from the first clone. Both sequences were identical.

COS-7 cell transfection and protein preparation

COS-7 cells were transfected in 10 cm plates using LipofectamineTM (Invitrogen), as previously described (Kaczmarek et al, 1996, JBC, 271:33116-33122). Briefly, cells were incubated for 5 h at 37°C in DMEM in absence of foetal bovine serum (FBS) with 6 µg of plasmid DNA and 24 µl of LipofectamineTM reagent. The reaction was stopped by adding an equal volume of DMEM containing 20% FBS and cell were harvested 40-44 h later. For protein

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preparation, transfected cells were washed three times with Tris-saline buffer at 4°C, harvested by scraping in 95 mM NaCl, 0.1 mM PMSF and 45 mM Tris, pH 7.5, and washed twice by 300 × g centrifugation for 10 min at 4°C. Cells were resuspended in the harvesting buffer containing 10 µg/ml aprotinin and sonicated.

5 Nucleus and cellular debris were discarded by another centrifugation as described above and the supernatants stored at -80°C until used. Protein concentration was estimated by the Bradford microplate assay using bovine serum albumin (BSA) as a standard of reference (Bradford, 1976, *Analyt. Biochem.*, **72**:248-254).

NTPDase activity measurement

10 Enzyme activity in the protein fractions was determined as previously described (Sévigny et al, 1997, *Biochimica Biophysica Acta* **1334**:73-88) Briefly, enzyme activity was measured at 37°C in 0.5 ml of the following incubation medium: 5 mM CaCl₂ either with 100 mM Tris, pH 7.4 or with 100 mM MES, pH 6.4 or as indicated. Enzyme preparation was added to the incubation mixture and

15 pre-incubated at 37°C for 3 min. Reaction was started by the addition of 0.5 mM substrate (ATP, ADP, UTP, UDP or AMP) and stopped after 20 min by the addition of 0.125 ml of the malachite green reagent (Baykov and Evtushenko, 1988, *Analytical Biochemistry* **171**:266-270). Activity on intact cells was carried out in similar conditions in 24 wells plates, also 40-44 h after transfection, with the

20 addition of 145 mM NaCl to the incubation medium. The reaction was stopped by taking 0.2 ml of the reaction mixture to a new tube containing 50 µl of malachite reagent. The inorganic phosphate (P_i) released from the hydrolysis of exogenous nucleotides was measured according to Baykov *et al.* (Baykov and Evtushenko, 1988, *Analytical Biochemistry* **171**:266-270)

25 Optimum pH was determined in the presence of 5 mM CaCl₂ with the following buffers: 100 mM acetate (pH 4.0-5.5), 100 mM MES (pH 5.5-7.0), 100 mM Tris (pH 7.0-9.0) or 100 mM glycine pH (9.0-11.0). In the indicated experiments CaCl₂ was substituted by MgCl₂. To remove traces of divalent cations

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1 mM EDTA plus 1 mM EGTA was added to the medium. One unit of enzyme activity corresponds to the release of 1 μ mole P_i /min at 37°C (LeBel et al., 1980, J. Biol. Chem. **255**:1227-1233). All experiments were performed in triplicate with the appropriate controls.

5 Separation and quantification of nucleotides by HPLC

For HPLC analysis, activity assays were performed in 5 mM $CaCl_2$ and 100 mM MES, pH 6.4 as described above with the following modifications. Aliquots of 20 μ L were taken at different time points from the enzymatic reaction and activity stopped by the addition of an equal volume of ice-cold 1 M perchloric acid. The samples were centrifuged for 5 min at $1000 \times g$ at 4°C. Supernatants
10 were neutralized with 1 M KOH (4°C), centrifuged for a subsequent 5 min at $1000 \times g$ and then lipids were removed by liquid-liquid extraction with n-heptane (5:1, v/v). An aliquot of 20 μ L from the lipid extracted sample was applied to a column connected to a HPLC system.

15 Adenine nucleotides (ATP, ADP and AMP) were separated on a 15 cm x 4.6 mm, 3 μ m SUPELCOSILTM LC-18-T column (Supelco, Bellefonte, PA, USA) with a mobile phase composed of 25 mM TBA, 5 mM EDTA, 100 mM KH_2PO_4/K_2HPO_4 , pH 7.0 and 2% methanol (v/v), at a flow-rate of 1 ml/min. Uridine nucleotides (UTP, UDP and UMP) were resolved using SUPELCOSILTM
20 LC-18-T column (25 cm x 4.6 mm, 5 μ m, Supelco) as described above with the difference that the mobile phase did not contain methanol. Combined adenine and uridine nucleotide samples were analyzed with the latter column with a mobile phase composed of 16.7 mM TBA, 3.3 mM EDTA, 66.7 mM KH_2PO_4/K_2HPO_4 , pH 7.0. The flow rate was 0.5 ml/min for the first 20 min and 1 ml/min up to 90 min.
25 The nucleotides were detected by UV absorption at 260 nm and identified by comparison of the retention time and UV spectrum with the appropriate standards.

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Results

Cloning and characterization of mouse *Entpd8* cDNA

The sequence includes a protein of 497 amino acid residue (SEQ ID NO:1) with a predicted molecular mass of 54 650 Da and a calculated isoelectric point of 5.94, and a respectively encoding nucleotide sequence (SEQ ID NO:2) having an open reading frame of 1491 nucleotides which is translated into. The deduced amino acid sequence contains eight potential N-glycosylation sites, the five apyrase conserved regions and various potential phosphorylation sites including one for both Protein kinase C and Casein kinase II on serine 4.

Hydrophobicity analysis of mouse NTPDase8 predicts two transmembrane domains in the polypeptide chain, one near the N terminus (amino acids 9-30) and one near the C terminus (amino acids 469-490; Fig. 1A). A schematic representation of the protein is shown on Fig. 1B. Distance estimation of the amino acid sequences of various NTPDases and related proteins was performed with GeneBee software (<http://www.genebee.msu.su/>). The phylogenetic tree obtained is shown in Fig. 2. The percentage of amino-acid identity of these proteins with mouse NTPDase8 was determined by pair wise alignment using ALIGNp (http://www.infobiogen.fr/services/analyseseq/cgi-bin/alignp_in.pl). The mouse NTPDase8, chicken ecto-ATPDase, human CD39L1, mouse CD39L1, rat ecto-ATPase, chicken ecto-ATPase, human CD39, mouse CD39, human HB6, human CD39L4, human CD39L2, rat NTPDase6, potato apyrase, pea apyrase, human UDPase, human LALP1, and toxoplasma gondii apyrase, were compared. Murine NTPDase8 had highest homology with chicken ecto-ATPDase with 52.7% amino acid identity.

Genomic characterization of *Entpd 8*

The full cDNA against the mouse genomic sequence reveals that mouse *Entpd8* covers approximately 5410 bases and is organized into 10 exons and 9 introns, all in agreement with the GT/AG rule. The coding sequence, including

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exon 2 to 10, Was cloned from mouse genomic DNA, and its sequence confirmed the intron/exon junctions found in the entry. Fig. 3 and Table 1 summarize the genomic structure of mouse NTPDase8 gene.

5

Table 1.
Summary of the genomic structure of mouse Entpd8.

Exon	Splice acceptor	Length of exon (bases)	Exon position in NT_039205.1	Splice donor	Length of intron (bases)	Nucleotide position of important sequences in current exon
1	ccaggtcag/TTCCAAAGT	246	2557096-2557341	CCAGTGCAG/gtgagcgac	1254	Promoter -260 to -179
2	cccttgccag/CTCAGAGCC	142 (from Met 126)	2558596-2558737	GACACCAAG/gtttgcaca	85	Met 17 - 19
3	tatccctag/TTTGGGATT	118	2558823-2558940	AGATAGAAG/gtcagtgga	777	ACR1 16 - 39
4	gtgtttcag/GACCTGGAA	151	2559718-2559868	GCTGCTCAG/gtgacacag	141	ACR2 126 - 143
5	tctgcccag/CCAGAAGAA	160	2560010-2560169	CTCCTGAAG/gtcagggg	118	ACR3 98 - 112
6	tgtggtcag/TATTCCTCT	237	2560288-2560524	GACAGGTG/gtatgtgaa	90	ACR4 55 - 78
7	tctctgcag/AGCAGCCAG	264	2560615-2560878	CAGTTCAT/gtaagcatg	172	
8	cttcccag/GCATTTTTC	111	2561051-2561161	TGGAAACTG/gtgggtgac	354	
9	gaacccaag/GTGAAGTC	135	2561516-2561650	CAGAAGCAG/gtgactgcc	100	
10	gtctccag/GCAGGTGAC	755 (up to TAG 189)	2561751-2562488	TAG	Introns + exons 5410	ACR5 22 - 33 TAG 190 - 192 polyA 540 bp after TAG

Sizes and junctions of the exons and introns of mouse Entpd8(= NTPDase8) are presented according to the sequence of accession No. NT_039205.1. Intron sequences are given in small characters and exons in capitals.

10

Biochemical characterization of NTPDase8

The biochemical characteristics of this novel NTPDase were determined with crude protein extracts, in COS-7 cells transiently transfected with an expression vector (pcDNA3.1/V5-His) containing the *Entpd8* cDNA. Firstly, we determined the time course of the reaction by stopping the reaction at different time points from 5 to 60 min. The result on Fig. 4A shows that the reaction was linear for the first 30 min with both ATP and ADP as substrates. All of the following assays were, therefore, carried out for 12 to 20 min. The effect of pH on ATPase and ADPase activity was, then, evaluated from pH 4.0 to 11.0. Fig. 4B shows that murine NTPDase8 is highly active between pH 5.5-7.0 with optimal activity around

20

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pH 6.4 for ADPase activity. The enzyme showed a marked preference for Ca^{2+} over Mg^{2+} for the nucleotides tested (Fig. 4C). In the presence of 1 mM EDTA and 1 mM EGTA, that remove traces of divalent cations, no activity could be detected with any of the substrates tested (Fig. 4C). The substrate specificity of NTPDase8 in the presence of Ca^{2+} is illustrated on Fig. 4D. Although all triphospho- and diphosphonucleosides tested were hydrolyzed, NTPDase8 preferred triphosphonucleosides with a ratio in the order of ~2:1 (ATP/ADP = 1.6 ± 0.3 and UTP/UDP = 2.1 ± 0.3). Like other NTPDases, this enzyme did not hydrolyze AMP (Fig. 4D). As a control, the biochemical activity measured in untransfected COS-7 cell extracts was less than 7% of the activity detected in transfected cells with a maximum of 0.05 U/mg of protein at pH 8.0 with ATP as substrate.

All four nucleotides tested were hydrolyzed by NTPDase8 consistently with Michaelis-Menten model of kinetics (Fig. 5A, not shown for ADP, UTP and UDP). The apparent K_m constants were calculated by plotting the data according to Woolf-Augustinsson-Hofstee method (Fig. 5A) and are summarized in Table 2. Triphosphonucleosides had lower apparent K_m compared to their respective diphosphonucleoside by over three folds while the apparent $V_{\max}$ were comparable for the four substrates tested. Based on these observations it could be predicted that ATP and UTP hydrolysis by NTPDase8 would result in an accumulation of the corresponding diphospho-derivative. As these intermediate products can also activate subset of P2 receptors, we followed their apparition by HPLC analysis over a time period of two hours. Incubation of ATP or UTP with protein extracts from NTPDase8 expressing COS-7 or with intact transfected cells, led to the formation of high levels of the corresponding diphosphonucleoside during the early phase of the reaction (Figs. 5B, C). In these experiments, ADP and UDP were further hydrolyzed to the corresponding monophosphonucleoside, only when ATP and/or UTP concentration had significantly decreased. After 120 min, ADP was completely hydrolyzed to AMP (Fig. 5B) while UDP was still detectable at a concentration of 140 μM (Fig. 5C). When both ATP and UTP were added

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together, adenine nucleotides were slightly preferentially hydrolyzed giving rise to an accumulation of UDP for an important period of time (Fig. 5D). These data were also in agreement with the lower K_m values for adenine nucleotides compared to uridine nucleotides.

5

Table 2
Kinetic constants of NTPDase8.

Substrate	$K_{m, app}$ (μ M)	$V_{max, app}$ (U/mg of protein)
ATP	13 ± 6	0.82 ± 0.02
ADP	41 ± 6	0.95 ± 0.08
UTP	47 ± 1	1.13 ± 0.02
UDP	171 ± 15	1.08 ± 0.06

10

$K_{m, app}$ and $V_{max, app}$ were estimated using Woolf-Augustinsson-Hofstee plot. Results are expressed as the mean $\pm$ SEM. The curves drawn from these data gave a r^2 of 0.99, 0.98, 0.99 and 0.96 for ATP, ADP, UTP and UDP respectively.

It is noteworthy that comparable experiments were conducted as in Fig. 4D and Figs. 5B-D with confluent intact COS-7 cells transiently transfected with NTPDase8. Similar substrate specificity and pattern of hydrolysis were observed as detected by either P_i determination or HPLC analysis of the products. In addition, the tight association of NTPDase8 to a membrane fraction was confirmed by an ultracentrifugation of the protein extracts at $100\,000 \times g$ for 1 hour. Indeed, over 90% of the total activity collected was found in the pellet fraction. These experiments demonstrated that mouse NTPDase8 is an ectoenzyme tightly bound to the plasma membrane.

20

EXAMPLE II

Effect of murine NTPDase8 on platelet aggregation

5 **Transient Transfection**

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Cells were seeded at 5×10^5 cells per 30-mm well and transfected 20-24 h later with Lipofectamine™ (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, the cells
10 were exposed to 1µg of DNA (pCDNA3 or pCDNA3-CD39) and 4µl of Lipofectamine in Dulbecco's modified Eagle's medium without FCS for 5h, followed by the addition of an equal volume of Dulbecco's modified Eagle's medium containing 20% FCS. Twenty-four h after transfection, the culture medium was changed (Dulbecco's modified Eagle's medium/10% FCS), and ~70 h post-
15 transfection, COS-7 cells were used for analyses. Control COS-7 cells used for transfection were negative for CD39 as analyzed by Western blotting and FACS with anti-CD39 mAb; ATPDase biochemical activity was negligible.

Cell membrane preparation and platelet aggregation assays were performed as described before.

20 **Results**

PRP prepared from human donors was tested for platelet activation in the presence of exogenous ADP and protein extracts from COS cell transfected with pcDNA3.1/V5-His encoding mouse NTPDase8 (mNTPDase8) or mouse (mNTPDase1) (Fig. 6). Optic density at 600nm was recorded over a period of 8
25 minutes. Before the addition of 4uM ADP, PRP was preincubated for 25 seconds with 250ug mNTPDase8 or 3ug mNTPDase1 protein extracts, 250ug untransfected COS cells or without protein extract (agonist: 4uM ADP). Note that 4uM ADP induced high levels of platelet aggregation in control samples. This could be

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reversed by the addition of mNTPDase8 (53% inhibition) and mNTPDase1 (100% inhibition).

EXAMPLE III

Human NTPDase8

Human NTPDase8 was isolated and analyzed as described in the previous EXAMPLES. It shows having amino acid sequence SEQ ID NO:3 and nucleotide sequence SEQ ID NO:4. Its activity has been found to be similar to the activity of the mouse equivalent described herein.

The activity of the protein extracts of human NTPDase8 was carried out as described previously herein and the results are shown in Table 3.

Table 3

Activity on protein extracts from human NTPDase8 transient transfected COS-7 cells.

Substrate (number of independent experiments each in triplicate)	Activity (nmols P _i /min/mg)
ATP (4)	264 ± 7
ADP (4)	55 ± 15
AMP (3)	2 ± 0.6
UTP (3)	271 ± 35
UDP (3)	38 ± 5

Molecular mass: 53.9 kDa; Isoelectric point: 5.42; Homology with mouse NTPDase8: 81.3% amino acid identity (63.4% at the nucleotide level, coding sequence only)

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or

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customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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CLAIMS:

1. An isolated nucleic acid molecule consisting of a nucleotide sequence selected from the group consisting of :
 - a) a nucleotide sequence encoding for a peptide comprising the amino acid sequence of SEQ ID NO:1 or 3 or an analog or an active fragment thereof;
 - b) a nucleotide sequence consisting of SEQ ID NO:2 or 4; and
 - c) a nucleotide sequence complementary to at least 70% of a nucleotide sequence of a) or b).
2. The nucleic acid molecule of claim 1, wherein said nucleotide a), b) or c) encodes for a peptide having ectonucleotidase activity.
3. A nucleic acid expression vector comprising a nucleic acid molecule as defined in claim 1 in operative association with a promoter sequence.
4. The expression vector of claim 3, wherein said promoter is an inducible or an ubiquitous promoter.
5. A host cell containing the expression vector of claim 3.
6. The host cell of claim 5 being an eukaryotic cell.

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7. An isolated peptide comprising the amino acid sequence SEQ ID NO:1 or SEQ ID NO:3, a functional fragment or an analog thereof, said isolated peptide having an ectonucleotidase activity.
8. An isolated polynucleotide consisting of a nucleotide sequence set forth in SEQ ID NO:2 or SEQ ID NO:4).
9. A method for modulating at least one biological reaction selected from the group consisting of platelet aggregation, thrombosis, emboli, and angiogenesis, said method comprising depositing in blood or a medium comprising blood in a sufficient amount of a peptide encoded by a nucleic acid molecule as defined in claim 1 or an isolated peptide as defined in claim 8 in condition allowing the modulation of platelet aggregation, thrombosis, or emboli.
10. A method for modulating at least one biological reaction selected from the group consisting of platelet aggregation, thrombosis, emboli, and angiogenesis, comprising administering to a patient an expression vector as defined in claim 3.
11. A method for modulating at least one biological reaction selected from the group consisting of platelet aggregation, thrombosis, emboli, and angiogenesis, comprising administering to a patient a partial expression vector without promoter and designed to incorporate a transcriptionnally active genomic region of a cell, said partial expression vector being transcribed under the control of a promoter endogenous to said genomic region.

1/6

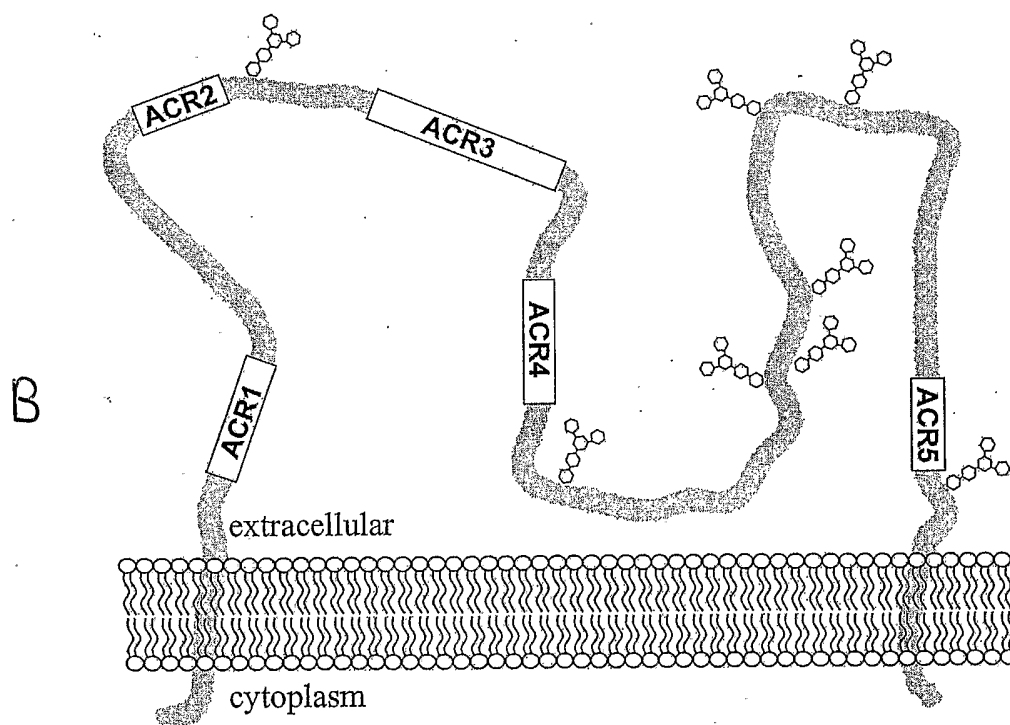
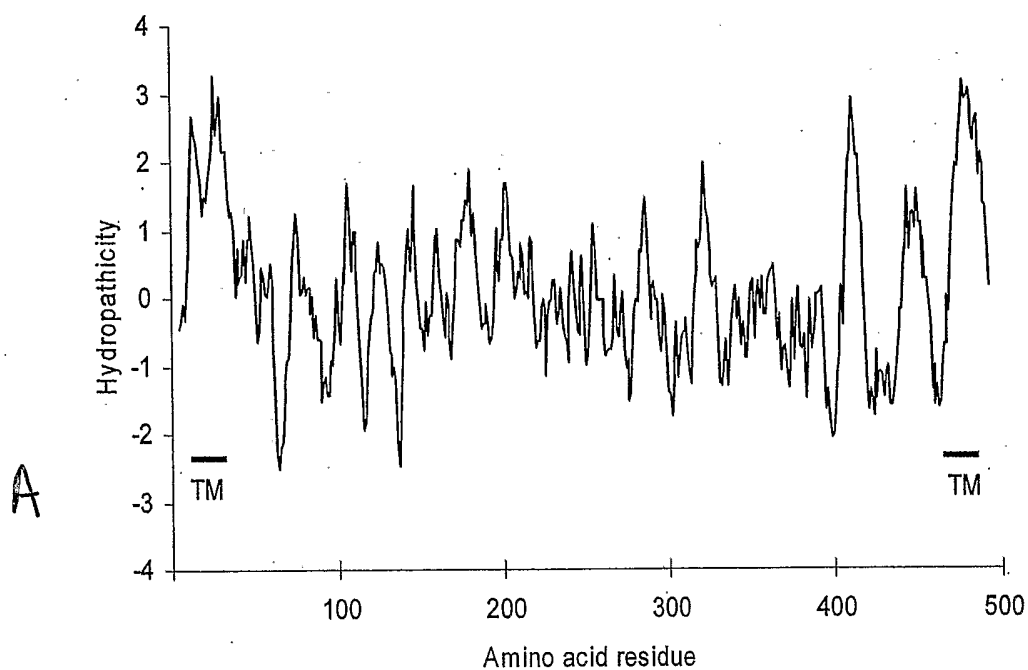


Fig. 1

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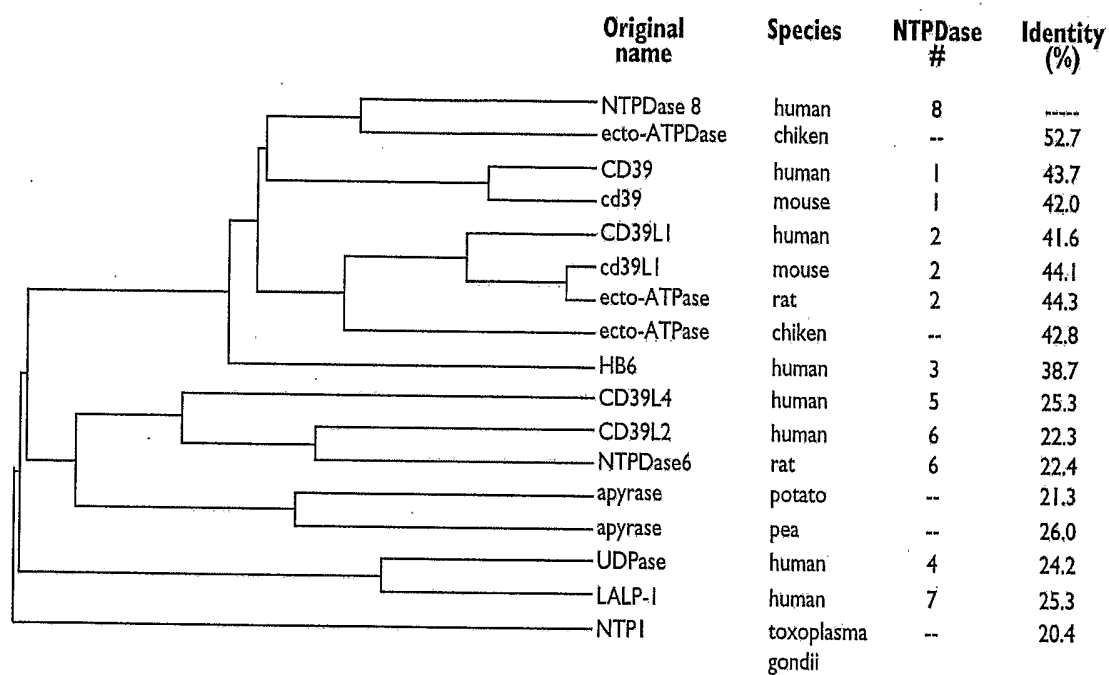


Fig. 2

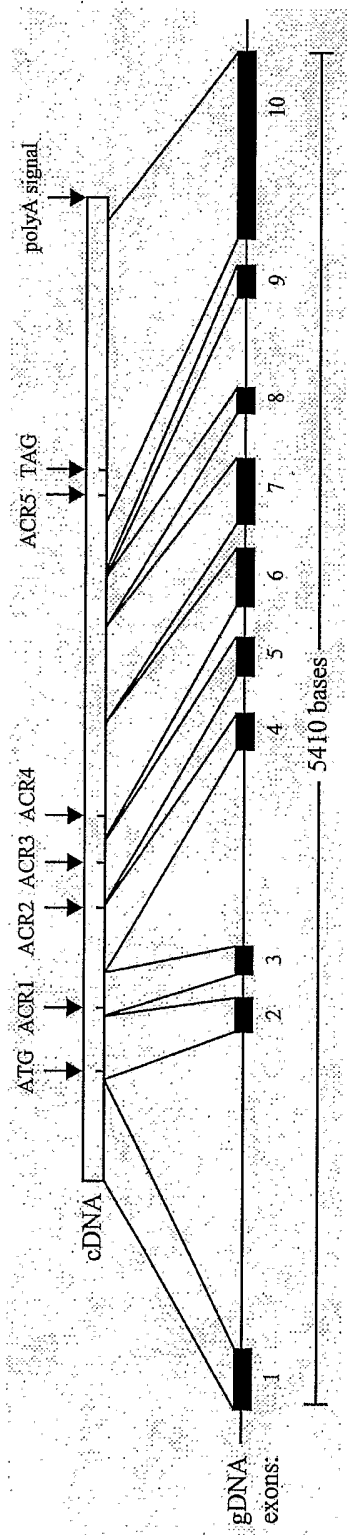


Fig. 3

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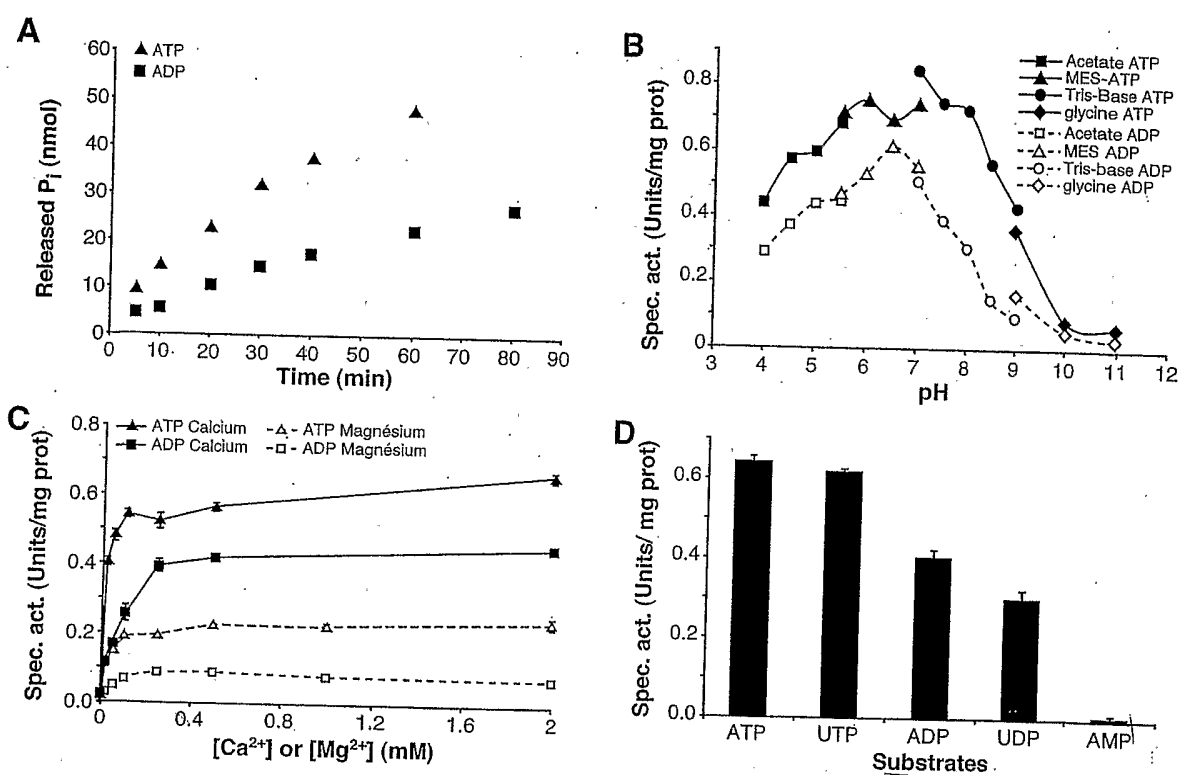


Fig. 4

5/6

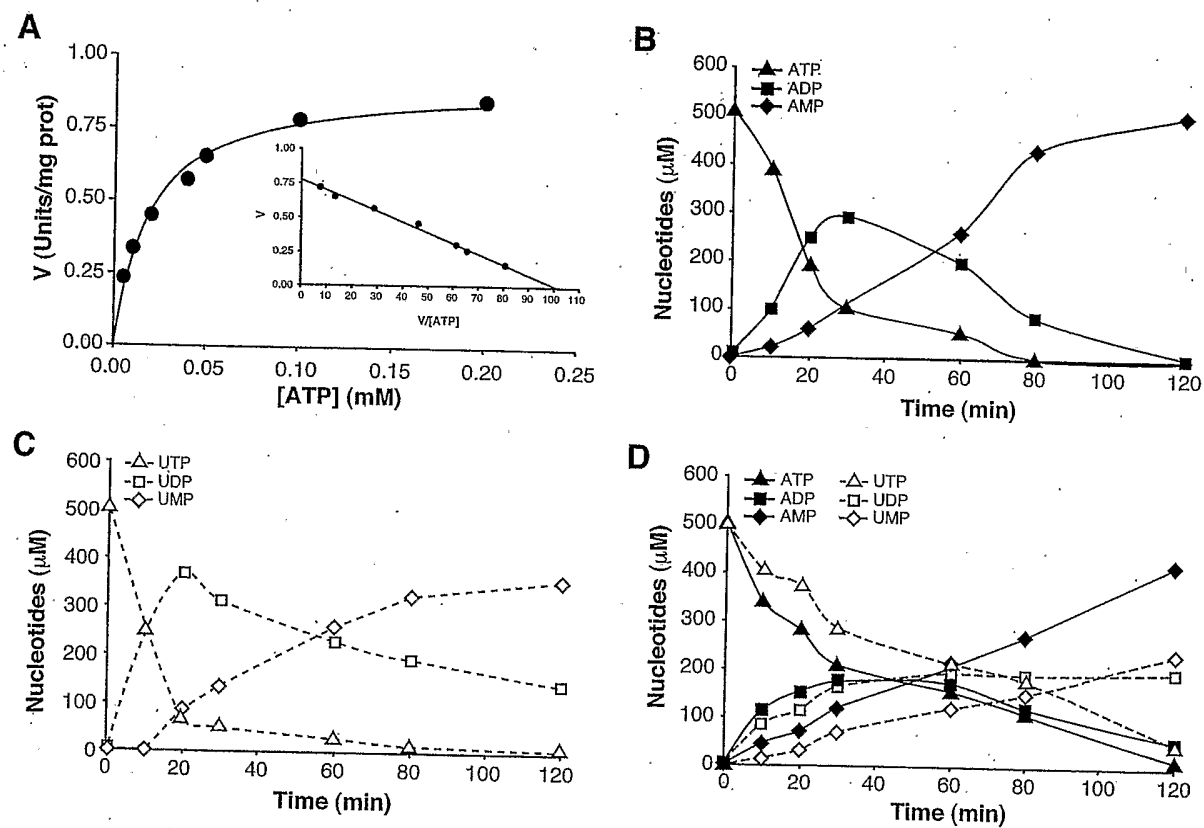


Fig. 5

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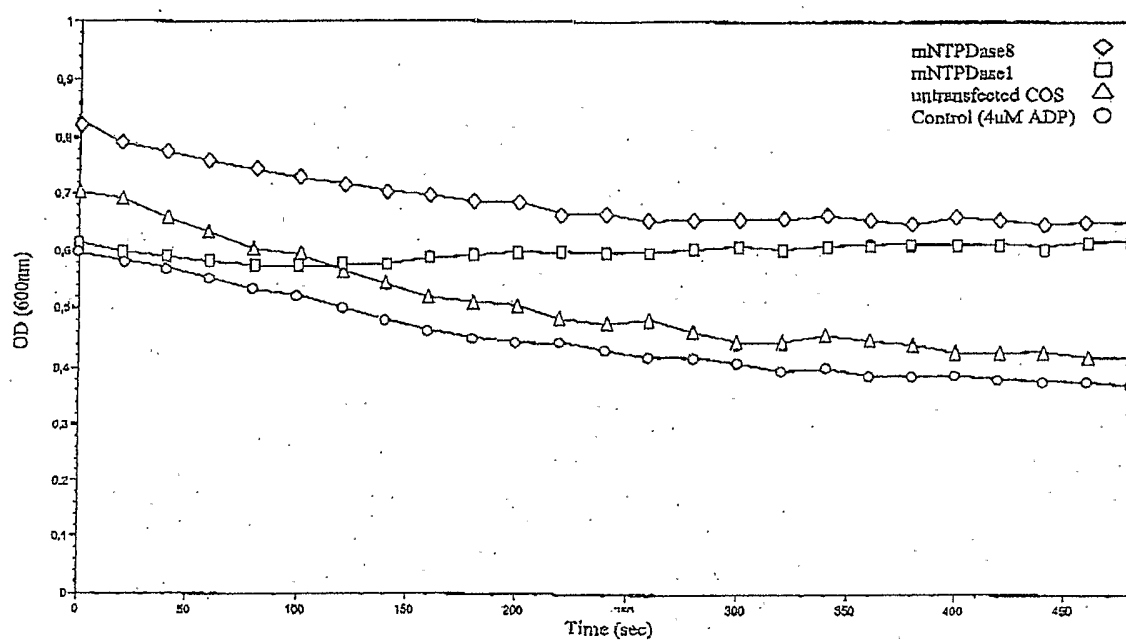


Fig. 6

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Ile Gln Pro Pro Glu Glu Met Leu Val Gly Ala Leu Asp Met Gly Gly
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 03/01397

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/14 C12N15/55 C12N15/85 C12N5/10 A61K38/46
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, BIOSIS, EMBASE, MEDLINE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 46380 A (INCYTE PHARMA INC ;CORLEY NEIL C (US); YUE HENRY (US); BANDMAN OLG) 16 September 1999 (1999-09-16) page 22, line 18 -page 25, line 27 page 32, line 15 -page 35, line 1 Sequence listing: SEQ ID Nos 3 and 8 --- -/--	1-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

14 January 2004

Date of mailing of the international search report

13/02/2004

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Donath, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 03/01397

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! Database accession no. BC031143 XP002266864 the whole document -& STRAUSBERG R L ET AL: "Generation and initial analysis of more than 15,000 full -length human and mouse cDNA sequences" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 99, no. 26, 24 December 2002 (2002-12-24), pages 16899-16903, XP002245220 ISSN: 0027-8424 the whole document	1-5,7
X	DATABASE EMBL 'Online! Database accession no. AK008590 XP002266865 the whole document -& KAWAI J ET AL: "Functional annotation of a full-length mouse cDNA collection" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 409, no. 6821, 8 February 2001 (2001-02-08), pages 685-690, XP002207921 ISSN: 0028-0836 the whole document	1-5,7
X	WO 02 071062 A (TAKASHIMA AKIRA ;UNIV TEXAS (US); KUMAMOTO TADASHI (US); MIZUMOTO) 12 September 2002 (2002-09-12) page 20, line 14 -page 22, line 18 page 41, line 1 -page 56, line 29	9-11
A	CHADWICK B P ET AL: "The CD39-like Gene Family: Identification of Three New Human Members (CD39L2, CD39L3, and CD39L4), Their Murine Homologues, and a Member of the Gene Family from Drosophila melanogaster" GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 50, no. 3, 15 June 1998 (1998-06-15), pages 357-367, XP004449112 ISSN: 0888-7543 the whole document	1-11

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 03/01397

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LECLERC M C ET AL: "Identification, characterization, and immunolocalization of a nucleoside triphosphate diphosphohydrolase in pig liver." ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS. UNITED STATES 15 MAY 2000, vol. 377, no. 2, 15 May 2000 (2000-05-15), pages 372-378, XP002266862 ISSN: 0003-9861 the whole document ----	1-8
A	IMAI MASATO ET AL: "Suppression of ATP diphosphohydrolase/CD39 in human vascular endothelial cells" BIOCHEMISTRY, vol. 38, no. 41, 12 October 1999 (1999-10-12), pages 13473-13479, XP002266863 ISSN: 0006-2960 the whole document -----	9-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 03/01397

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 9 - 11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 03/01397

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9946380	A	16-09-1999	US 2002086379 A1	04-07-2002
			AU 3072999 A	27-09-1999
			CA 2322771 A1	16-09-1999
			EP 1062338 A2	27-12-2000
			JP 2002505880 T	26-02-2002
			WO 9946380 A2	16-09-1999
WO 02071062	A	12-09-2002	US 2003087247 A1	08-05-2003
			WO 02071062 A2	12-09-2002