

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 November 2006 (30.11.2006)

PCT

(10) International Publication Number
WO 2006/126231 A1

(51) International Patent Classification:
C07K 14/435 (2006.01)

(21) International Application Number:
PCT/IT2006/000383

(22) International Filing Date: 22 May 2006 (22.05.2006)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
RM2005A000252 23 May 2005 (23.05.2005) IT

(71) Applicant (for all designated States except US): **AE-QUOTECH S.R.L.** [IT/IT]; Via Saragat 1, I-44100 Ferrara (IT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DI VIRGIO, Francesco** [IT/IT]; Aequotech S.r.l., Via Saragat 1, I-44100 Ferrara (IT). **FALZONI, Simonetta** [IT/IT]; Aequotech S.r.l., Via Saragat 1, I-44100 Ferrara (IT). **PELLEGATTI, Patrizia** [IT/IT]; Aequotech S.r.l., Via Saragat 1, I-44100 Ferrara (IT). **PINTON, Paolo** [IT/IT]; Aequotech S.r.l., Via Saragat 1, I-44100 Ferrara (IT). **RIZZUTO, Rosario** [IT/IT]; Aequotech S.r.l., Via Saragat 1, I-44100 Ferrara (IT).

(74) Agents: **GITTO, Serena** et al.; Ing. Barzano' & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

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

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.

(54) Title: CHIMERIC PROTEINS FOR MEASURING ATP CONCENTRATIONS IN PERICELLULAR SPACE AND RELATED SCREENING METHOD

(57) Abstract: The invention relates to luminescent chimeric proteins comprising a first N-terminal protein sequence, a second protein sequence and a third C-terminal protein sequence wherein: (i) said first and said third protein sequence are a leader sequence and an anchor sequence belonging to at least a receptor localized on a plasma membrane site; (ii) said second protein sequence encodes for the full-length or partial sequence of a photoprotein and is inserted in frame between said first and said third sequence (i); said chimeric protein being addressed to said plasma membrane site of the cell wherein it is expressed.



WO 2006/126231 A1

CHIMERIC PROTEINS FOR MEASURING ATP CONCENTRATIONS IN PERICELLULAR SPACE AND RELATED SCREENING METHOD

5 The present invention relates to chimeric proteins for the measurement of ATP concentrations into the pericellular space and related screening method.

 ATP is now accepted as an ubiquitous extracellular messenger (Burnstock, 2004; Di Virgilio et al., 2004; Wang et al., 2004). Responses elicited by this nucleotide, depending on the concentration and the given
10 P2R subtype expressed by the target cell, range from chemotaxis (Oshimi et al., 1999), to cell adhesion (Freyer et al., 1998), from cytokines release (Perregaux & Gabel, 1994), to neurotransmitter secretion (Illes & Norenberg, 1993), from activation of apoptosis (Zanovello et al., 1990), to stimulation of cell proliferation (Neary et al., 2003).

15 Further, several mediators (neurotransmitters, cytokines, hormones) work together to enhance or to reduce ATP release in the microenvironment directly close to cell plasma membrane wherein the factor carries out its paracrine or autocrine action, or like endogenous drug.

20 While it is generally agreed that many disease conditions (trauma, inflammation, ischemia) may lead to an increase in the extracellular ATP concentration as a consequence of mere cell lysis, the pathways that support non-lytic ATP release are less clear. Increasing attention is payed to those signals that alert the immune system during the early phases of
25 tissue damage or pathogen invasion (Matzinger, 2002; La Sala et al., 2003; Skoberne et al., 2004). Intracellular nucleotides are considered likely candidates to this role for their ubiquitous distribution, high intracellular concentration, negligible extracellular levels under quiescent conditions, presence of specific receptors and ability to modulate dendritic
30 cell differentiation. The additional feature described here, unveiling a non-lytic and self sustaining release mechanism, make ATP an even more appealing danger signal.

 The very low extracellular levels under quiescent conditions, the quick increases caused by many different stimuli, the fast degradation in
35 the extracellular space, and the presence of specific receptors make ATP an ideal extracellular messenger (Burnstock, 2004; Zimmermann, 2000). However, full appreciation of the role of ATP as an extracellular signal has been hampered by lack of proper probes for accurate measurement of the

extracellular concentration. Most measurements are performed by using the standard luciferin/luciferase assay in off- or on-line settings. All off-line techniques measure ATP in the cell-free supernatants, and therefore after ATP has diffused and equilibrated into the incubation medium. On-line measurements give a more accurate estimate of the actual ATP levels reached close to the site of release, but still involve manipulations that may seriously affect the measurements. The prototypic ATP probe is firefly luciferase, a bioluminescent ATP-dependent enzyme that can detect ATP in the pico-millimolar range. Luciferase is mostly used to assay the ATP concentration in cell-free supernatants after cell or tissue stimulation. This procedure, albeit technically very simple, involves manipulations that can cause cell rupture or unwanted stimulation (sampling, centrifugation, recovery of the supernatants). Furthermore, these off-line measurements do not allow detection of rapidly changing localized ATP transients close to the surface of the plasma membrane. Previous observations have clearly shown that ATP levels measured in the proximity of the plasma membrane surface can be up to 10-20 fold higher than those measured in the bulk solution by the soluble luciferase assay (Beigi et al., 1999).

On-line measurements give a more accurate estimate of the actual ATP levels reached close to the site of release, but still involve manipulations that may seriously affect the measurements, for instance, by producing positive false and/or negative false.

For example, a protein A-luciferase chimera was engineered by Dubyak et al., to detect local ATP release at the membrane level (Beigi et al., 1999). Use of this probe involves coating of the cell surface with IgG to allow binding of the luciferase chimera. The cell-attached probe yielded an ATP release from thrombin-stimulated platelets 10-15 fold higher than those recorded by soluble luciferase under similar experimental conditions. This method requires the use of specific antibodies for target cells that may alter the physiological properties. Further it is known that antibodies fixed on plasma membrane incur redistribution and endocytosis. This make very difficult to ensure stable levels of membrane luciferase.

Recently, a biosensor system based on cells and fragments thereof expressing ATP sensible P2X channels placed near to a source of ATP (Hayashi et al., 2004), that allow to measure ATP release as a change in the recorded current. This methodology makes use of "patch

clamp" electrophysiology technique, that is extremely complex (cell fragments or cells must be entire) and makes the method open to the presence of false positive or negative because of the existence of an intermediate step of channel opening. Thus, it results in a random and poorly repeatable measurement.

In the light of the above it is evident the need of a method for measuring of ATP levels in the pericellular space which is reliable, simple and repeatable and which allows more reliable measurements in comparison with the current methods.

The authors of the present invention have now created luminescent chimeric proteins able to localize on plasma membrane of the adopted cellular, to be used as probes for the measurement of actual ATP extracellular levels, highly selective as insensitive to all other ADP, UTP, UDP and GTP nucleotides.

Particularly, the method which employs these luminescent chimeric probes found by the authors has the following advantages: 1) chimeric probe is expressed as a plasma membrane protein, thus exposed to the actual environment wherein we aim to measure ATP; 2) chimeric probe can be engineered to be targeted to virtually any plasma membrane region, thus allowing the measurement of extracellular ATP at discrete plasma membrane sites; 3) genetic manipulation may allow to measure with this technique ATP levels *in vivo* in experimental models.

Such method for the measurement of ATP concentration in the periplasmalemmal space through the detection of a luminescent signal was carried out on cells expressing (es. HEK293 cells) the recombinant or native P2X₇ receptor (P2X₇R) transfected with the chimeric probes according to the invention to determine its function. Both cell types release large amounts of ATP (100 to 200 μ M) in response to P2X₇R activation. This novel approach unveils a hitherto unsuspected non lytic pathway for the release of amounts of ATP into the extracellular milieu in the low micromolar to millimolar level, thus easily measurable.

Indeed, the authors have shown that HEK293-pmeLUC transfectants did not appreciably release ATP in response to most stimuli applied. Expression of the P2X₇R on the contrary endows these cells with the ability to release large amounts of ATP in response to BzATP or ATP itself. The kinetic of BzATP-stimulated release is transient, reaching a peak within two min from the addition, and then rapidly declining to near

basal level (see Figure 3). This kinetic is surprising since it is well known that the P2X₇ is a non-desensitizing receptor, thus one would expect that so far the receptor stays open ATP should efflux. In fact opening of the P2X₇ pore allows to reach a periplasmalemmal ATP concentration in the hundred micromolar range, sufficient to activate even the low affinity P2X₇R. This observation supports the hypothesis that, once an initial event triggers ATP release to a level sufficient to activate P2X₇, neighbour cells expressing this receptor (mainly inflammatory cells) may function as amplification devices by sustaining a process of ATP-induced ATP release.

Hence these cellular systems transfected with the probes according to the invention may be used in screening method for the evaluation of drugs or molecules of interest that may cause a reduction or an increase of ATP levels in the periplasmalemmal space.

Furthermore these systems may be used as biosensors to detect the presence of toxic substances and/or environmental contaminants.

Thus, it is an object of the invention luminescent chimeric proteins comprising a first N-terminal protein sequence, a second protein sequence and a third C-terminal protein sequence wherein:

(i) said first and said third protein sequence are a leader sequence and an anchor sequence belonging to at least a receptor localized on a plasma membrane site;

(ii) said second protein sequence encodes for a photoprotein and is inserted in frame between said first and said third sequence (i); said chimeric protein being addressed to said plasma membrane site of the cell wherein it is expressed.

Preferably, the receptor localized on the plasma membrane site is selected from the group that consists in ionic-channel receptors, connexins, G protein coupled receptors, tyrosine-kinase activity receptors. More preferably, said photoprotein is selected from the group that consists in luciferase, aequorin, obelin.

According to a preferred embodiment of the present invention said first and said third protein sequence (i) are the leader sequence and the GPI anchor sequence of the folate receptor and said photoprotein (ii) is luciferase, preferably fire-fly luciferase. Most preferably the aminoacid sequence of the protein according to the invention, namely pMeLuc, is the following:

MAQRMTTQLLLLLVWVAVVGEAQTRIAEQKLISEEDLLQMEDAKNIKKGP
APFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVDITYAEYFEMSV
RLAEAMKRYGLNTNHRIVVCSNSLQFFMPVLGALFIGVAVAPANDIYNE
RELLNSMGISQPTVVVFSKKGLQKILNVQKKLPPIQKIIIMDSKTDYQGF
5 QSMYTFVTSHLPPGFNEYDFVPESFDRDKTIALIMNSSGSTGLPKGVALP
HRTACVRFSHARDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLICGFR
VLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFAKSTLIDKYDLSNLHE
IASGGAPLSKEVGEAVAKRFHLPGIRQGYGLTETTSAILITPEGDDKPGA
VGKVVPFPEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNPEATN
10 ALIDKDGWLHSGDIAYWDEDEHFFIVDRCLKSLIKYKGYQVAPAELESILLQ
HPNIFDAGVAGLPDDDAGELPAAVVVLEHGKTMTEKEIVDYVASQVTTA
KKLRGGVVFVDEVPKGLTGKLDARKIREILIKAKKGGKIAVAAAAMSGAGP
WAAWPFLLSLALMLLWLLS (SEQ ID No:1).

It is a further object of the present invention a nucleotidic
15 sequence encoding for one of the luminescent chimeric proteins
luminescenti as above defined. Particularly, among the nucleotidic
sequences encoding the above mentioned pmeLuc aminoacid sequence
is preferably used the following nucleotidic sequence:

ATGGCTCAGCGGATGACAACACAGCTGCTGCTCCTTCTAGTGTGGGT
20 GGCTGTAGTAGGGGAGGCTCAGACAAGGATTGCAGAACAAAACTAA
TAAGCGAGGAGGACCTGCTGCAGATGGAAGACGCCAAAAACATAAA
GAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGA
GAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAAC
AATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGA
25 GTACTTCGAAATGTCCGTTCCGTTGGCAGAAGCTATGAAACGATATG
GGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAACTCTCTTC
AATTCTTTATGCCGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTG
CGCCCGCGAACGACATTTATAATGAACGTGAATTGCTCAACAGTATG
GGCATTTCGCAGCCTACCGTGGTGTTCGTTTCCAAAAAGGGGTTGCA
30 AAAAATTTTGAACGTGCAAAAAAGCTCCCAATCATCAAAAAATTATT
ATCATGGATTCTAAAACGGATTACCAGGGATTTCAAGTCGATGTACACG
TTCGTCACATCTCATCTACCTCCCGGTTTTAATGAATACGATTTTGTG
CCAGAGTCCTTCGATAGGGACAAGACAATTGCACTGATCATGAACTC
CTCTGGATCTACTGGTCTGCCTAAAGGTGTCGCTCTGCCTCATAGAA
35 CTGCCTGCGTGAGATTCTCGCATGCCAGAGATCCTATTTTTGGCAAT
CAAATCATTCCGGATACTGCGATTTTAAGTGTTGTTCCATTCCATCAC
GGTTTTGGAATGTTTACTACACTCGGATATTTGATATGTGGATTTCA

GTCGTCTTAATGTATAGATTTGAAGAAGAGCTGTTTCTGAGGAGCCTT
CAGGATTACAAGATTCAAAGTGCGCTGCTGGTGCCAACCCTATTCTC
CTTCTTCGCCAAAAGCACTCTGATTGACAAATACGATTTATCTAATTTA
CACGAAATTGCTTCTGGTGGCGCTCCCCTCTCTAAGGAAGTCGGGGA
5 AGCGGTTGCCAAGAGGTTCCATCTGCCAGGTATCAGGCAAGGATATG
GGCTCACTGAGACTACATCAGCTATTCTGATTACACCCGAGGGGGAT
GATAAACCGGGCGCGGTTCGGTAAAGTTGTTCCATTTTTTTGAAGCGAA
GGTTGTGGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAAAGAG
GCGAACTGTGTGTGAGAGGTCCTATGATTATGTCCGGTTATGTAAAC
10 AATCCGGAAGCGACCAACGCCTTGATTGACAAGGATGGATGGCTACA
TTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATCG
TTGACCGCCTGAAGTCTCTGATTAAGTACAAAGGCTATCAGGTGGCT
CCCGCTGAATTGGAATCCATCTTGCTCCAACACCCCAACATCTTCGA
CGCAGGTGTCGCAGGTCTTCCCGACGATGACGCCGGTGAACCTCCC
15 GCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAAGA
GATCGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTGC
GCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGG
AAAACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGA
AGGGCGGAAAGATCGCCGTGGCTGCAGCCATGAGTGGGGCTGGGC
20 CCTGGGCAGCCTGGCCTTTCCTGCTTAGCCTGGCCCTAATGCTGCTG
TGGCTGCTCAGCTGA (SEQ ID No:2). This nucleotidic sequence is
preferably used into the VR1012 vector.

It is an object of the present invention an expression vector
comprising the nucleotidic sequence as above defined, preferably
25 pcDNA3 or VR1012.

The present invention provides a primary cell culture transfected
with the above mentioned expression vector or with the nucleotidic
sequence according to the invention, such as fibroblasts isolated from skin
or microglial cells isolated from cerebral tissue of newborn mice.

30 It is further object of the present invention a cell line transfected
with the above defined expression vector or with the nucleotidic sequence
according to the invention. More preferably, said cell line is characterized
by the native or recombinant expression of a receptor of interest preferably
selected between CD14, P2Y (several subtypes) and P2X (several
35 subtypes), preferably P2X₇. Preferably, the cell line is selected between
HEK 293, HeLa, ACN, N9, N13, PC12, J774, A549. These two latter cell
lines constitutively expressing both P2X₇, and CD14 receptor represent a

particularly advantageous cellular system to be employed. Particularly, the macrophage cell line may be considered particularly advantageous in the systems for the analysis of substances toxicity due to macrophage ability to phagocytize toxic substances. Parallely the pulmonary epithelial cells
5 may be considered particularly advantageous in the systems for the analysis of environmental contaminants because of their specific location at level of respiratory airways.

The present invention concerns the use of luminescent chimeric proteins according to the invention, as probes for the measurement of
10 extracellular ATP levels into *in vitro* or *in vivo* systems.

It is a further object of the invention the use of cell lines as above defined for the measurement of extracellular ATP levels in *in vitro* systems or alternatively as experimental model in screening method and/or analysis method of compounds of interest able to modulate
15 extracellular ATP levels.

Finally, it is an object of the present invention a screening method of compounds of interest (e.g. anti-inflammatory drugs, immunomodulators, vasodilators) able to modulate extracellular ATP levels comprising the following steps:

- 20 a) contacting the cell line as above defined wherein the photoprotein is luciferase with said compound of interest in the presence of O_2 , Mg^{2+} and of the luciferin substrate;
- b) detecting cps or luminescence percent over basal value or the value of a preceding stimulation with an agonist as a control and determining the
25 activity as an agonist or antagonist in relation to cps increase or reduction over basal value or the value of a preceding stimulation with an agonist as a control, respectively.

According to a preferred embodiment the present invention relates to a method for the analysis of the presence of toxic substances and/or environmental contaminants able to induce the increase of
30 extracellular ATP levels as an early index of cell suffering in a measure proportional to the toxicity of the substance and to the exposure time, comprising the following steps:

- a) contacting a cell line sensible to the toxic substance(s) to be assayed
35 as above defined when the photoprotein is luciferase with said compound of interest in the presence of O_2 , Mg^{2+} and of the luciferin substrate;
- b) detecting the presence or the absence of a toxic substance in relation to

the cps or luminescence percent increase or reduction over the basal value.

Preferably, when the analysis method is for the detection of LPS and/or ozone the cell line of step a) is selected between pulmonary epithelial cells, and macrophage cells of mammalian origin, preferably of human origin. Preferably, said human pulmonary epithelial cells are A549 cells. Preferably, said human macrophage cells are J774 cells.

According to a preferred embodiment of the analysis method of the invention said toxic substance to be assayed is LPS and/or ozone. In a particularly preferred embodiment of the invention if ozone and/or LPS are assayed the above mentioned A549 and/or J774 cell lines are employed.

The present invention refers to a biosensor comprising a cell line a cell line characterized in that it is transfected with the above defined expression vector or with the nucleotidic sequence according to the invention. Said line may be further characterized by the native or recombinant expression of a receptor (e.g. P2X₇). Preferably, said cell line is selected from the group that consists in HEK 293, HeLa, ACN, N9, N13, PC12, J774, A549.

The invention refers to the use of the above mentioned biosensor for the measurement of extracellular ATP levels in *in vitro* systems, for example for the analysis of environmental toxicity or for the screening of compounds of interest (e.g. anti-inflammatory drugs, immunomodulators, vasodilators) able to alter extracellular ATP levels.

According to an alternative embodiment of biosensor, the present invention further concerns a biosensor comprising at least one of the luminescent chimeric proteins contemplated by the present invention. Finally, the invention concerns the use of the above mentioned biosensor for the measurement of extracellular ATP levels in *in vitro* systems for example for the analysis of environmental toxicity or for the screening of compounds of interest (e.g. anti-inflammatory drugs, immunomodulators, vasodilators) able to alter extracellular ATP levels.

The present invention will be now described for illustrative but non-limiting purposes, according to its preferred embodiments, with particular reference to the figures of the enclosed drawings, in which:

figure 1 shows the structure and localization of pmeLUC construct; panel A shows the structure comprising the full length coding sequence of luciferase inserted in frame between the N-terminal, leader

sequence (26 aa) and the C-terminal GPI anchor sequence (28 aa) of the folate receptor, panel B shows a schematic rendering of the plasma membrane localization of pmeLUC; panels C and D reproduce immunofluorescence and FACS analysis of HEK293 cells transfected with pmeLUC (HEK293-pmeLUC) or with the empty vector (HEK293-mock), respectively;

figure 2 shows the response of HEK293-pmeLUC cells to extracellular nucleotides in panel A, and ATP calibration curve in panel B; HEK293-pmeLUC cell monolayers were placed in the luminometer chamber, and were then perfused with solutions containing increasing concentrations of nucleotides; basal cps before addition of the nucleotides ranged between 2500 and 3200; luminescence increase is shown as percent increase over basal; in panel B luminescence increase is correlated to the ATP concentration to build a calibration curve;

figure 3 shows ATP release through the P2X₇ receptor; panels A and B show HEK293 cells co-transfected with hP2X₇ and pmeLUC (HEK293-hP2X₇/pmeLUC) placed in the luminometer chamber and perfused with a BzATP-containing solution, with or without prior treatment with 300 μ M oATP for 2 hours; HEK293-pmeLUC cells are used as a control; luminescence increase was expressed as cps in panel A and luminescence percent increase over basal in panel B; in panel C total pmeLUC protein expressed in HEK293-pmeLUC, HEK293-hP2X₇/pmeLUC and HEK293-rP2X₇/pmeLUC cells is determined by western blotting wherein wtHEK293 cells are shown as a control; in panel (D) plasma membrane-expressed pmeLUC is measured by FACS analysis in these three cell populations; panel E shows ATP release from human neuroblastoma ACN cells incubated in the absence or presence of 300 μ M oATP;

figure 4 shows extracellular ATP release from P2X₇-expressing cells. HEK293 cells transfected with the rat (panel A) or human (panel B) P2X₇R and control cells were placed in the luminometer chamber and perfused with increasing ATP concentrations;

figure 5 shows the increase of ATP release triggered by membrane stretching due to the expression of the P2X₇R; in panel A HEK293-hP2X₇/pmeLUC and HEK293-pmeLUC cells were placed in the luminometer chamber and perfused with isotonic buffer followed by a hypotonic solution, followed again by isotonic buffer; in panel B HEK293-

hP2X₇/pmeLUC and HEK293-pmeLUC cells were perfused with isotonic buffer followed by a hypertonic solution;

figure 6 A and 6 B show two diagram showing the measurement of ATP release from J774 cells expressing pmeLUC following exposure to different substances (panel A: treatment with different ATP concentrations ranging from 5 μ M to 1 mM; panel B: treatment with LPS);

figure 7 A and 7 B show two diagram showing the measurement of ATP release from A459 cells expressing pmeLUC following exposure to different substances (panel A: treatment with different ATP concentrations ranging from 5 μ M to 1 mM; panel B: treatment with LPS 1 μ g/ml).

EXAMPLE 1: Preparation of the luminescent chimeric probe pMeLUC for ATP measurements and HEK293-P2X₇ cells transfected with said probe

MATERIALS AND METHODS

Benzoyl ATP (BzATP), oxidized ATP (oATP), DMEM, DMEM-F12, MEM non-essential amino acid solution 100x were from Sigma-Aldrich (St. Louis, MO, USA). ATP, ADP, UTP, UDP and GTP were purchased from Boehringer-Roche Diagnostics (Mannheim, Germany). Luciferin used for ATP measurements with pmeLUC was from DUCHEFA Biochemie (Amsterdam, The Netherlands). Luciferin-luciferase solutions for ATP measurements with the Firezyme luminometer were from Promega (Madison, WI, USA). Dithiothreitol (DTT) was purchased from Merck (Damstadt, Germany). All experiments were performed in a saline solution containing: 135 mM NaCl, 5 mM KCl, 0.5 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5.5 mM glucose and 20 mM Hepes, pH 7.4 at 37°C.

PmeLUC engineering

Plasma membrane luciferase pmeLUC was obtained as follows: luciferase cDNA was amplified from a modified pGL3 plasmid (kind gift of Dr Guy Rutter, University of Bristol, UK) using the following primers:

5' - CCC TGC AGA TGG AAG ACG CCA AAA ACA TAA AGA AAG G 5' -
 CCC TGC AGA TGG AAG ACG CCA AAA ACA TAA AGA AAG G - 3'
 (SEQ ID No:3) (corresponding to the sequence encoding amino acids 1–9 of luciferase; *Pst*I site underlined) and

5' – GCT GCA GCC ACG GCG ATC TTT CCG CCC TTC TTG G – 3'
 (SEQ ID No:4) (including amino acids 542–549 of luciferase cDNA without the stop codon; *Pst*I site underlined).

The PCR product was transferred to pBSK⁺ vector (Stratagene), digested with the enzyme *Pst*I and inserted in the right frame between a

*Pst*I fragment encoding the complete N-terminal leader sequence of the human folate receptor (26 aa) fused with myc tag (10 aa) and a *Pst*I fragment of the GPI anchor protein (28 aa), to generate the construct shown in Figure 1A. Particularly, the nucleotidic sequence used was the following:

5
10
15
20
25
30
35

```
ATGGCTCAGCGGATGACAACACAGCTGCTGCTCCTTCTAGTGTGGG
TGGCTGTAGTAGGGGAGGCTCAGACAAGGATTGCAGAACAAAACT
AATAAGCGAGGAGGACCTGCTGCAGATGGAAGACGCCAAAAACATA
AAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTG
GAGAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGG
AACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACG
CTGAGTACTTCGAAATGTCCGTTTCGGTTGGCAGAAGCTATGAAACGA
TATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACCTC
TCTTCAATTCTTTATGCCGGTGTTGGGCGCGTTATTTATCGGAGTTG
CAGTTGCGCCCGCGAACGACATTTATAATGAACGTGAATTGCTCAAC
AGTATGGGCATTTTCGCAGCCTACCGTGGTGTTCGTTTCCAAAAAGG
GGTTGCAAAAAATTTGAACGTGCAAAAAAAGCTCCCAATCATCCAA
AAAATTATTATCATGGATTCTAAAACGGATTACCAGGGATTTAGTCG
ATGTACACGTTTCGTACATCTCATCTACCTCCCGGTTTTAATGAATAC
GATTTTGTGCCAGAGTCCTTCGATAGGGACAAGACAATTGCACTGAT
CATGAACTCCTCTGGATCTACTGGTCTGCCTAAAGGTGTCGCTCTGC
CTCATAGAACTGCCTGCGTGAGATTCTCGCATGCCAGAGATCCTATT
TTTGCAATCAAATCATTCCGGATACTGCGATTTTAAGTGTTGTTCCA
TTCCATCACGGTTTTTGAATGTTTACTACACTCGGATATTTGATATGT
GGATTTTCGAGTCGTCTTAATGTATAGATTTGAAGAAGAGCTGTTTCT
GAGGAGCCTTCAGGATTACAAGATTCAAAGTGCGCTGCTGGTGCCA
ACCCTATTCTCCTTCTTCGCCAAAAGCACTCTGATTGACAAATACGAT
TTATCTAATTTACACGAAATTGCTTCTGGTGGCGCTCCCCTCTCTAA
GGAAGTCGGGGAAGCGGTTGCCAAGAGGTTCCATCTGCCAGGTATC
AGGCAAGGATATGGGCTCACTGAGACTACATCAGCTATTCTGATTAC
ACCCGAGGGGGATGATAAACCGGGCGCGGTTCGGTAAAGTTGTTCC
ATTTTTTGAAGCGAAGGTTGTGGATCTGGATACCGGGAAAACGCTG
GGCGTTAATCAAAGAGGCGAACTGTGTGTGAGAGGTCCTATGATTAT
GTCCGGTTATGTAAACAATCCGGAAGCGACCAACGCCTTGATTGAC
AAGGATGGATGGCTACATTCTGGAGACATAGCTTACTGGGACGAAG
ACGAACACTTCTTCATCGTTGACCGCCTGAAGTCTCTGATTAAGTAC
AAAGGCTATCAGGTGGCTCCCGCTGAATTGGAATCCATCTTGCTCCA
```

ACACCCCAACATCTTCGACGCAGGTGTCGCAGGTCTTCCCGACGAT
 GACGCCGGTGAACCTCCCGCCGCCGTTGTTGTTTTGGAGCACGGAA
 AGACGATGACGGAAAAAGAGATCGTGGATTACGTCGCCAGTCAAGT
 AACAAACCGCGAAAAAGTTGCGCGGAGGAGTTGTGTTTGTGGACGAA
 5 GTACCGAAAGGTCTTACCGGAAAACTCGACGCAAGAAAAATCAGAG
 AGATCCTCATAAAGGCCAAGAAGGGCGGAAAGATCGCCGTGGCTGC
AGCCATGAGTGGGGCTGGGCCCTGGGCAGCCTGGCCTTTCCTGCT
TAGCCTGGCCCTAATGCTGCTGTGGCTGCTCAGCTGA (SEQ ID
 No:5).

10 Legend: leader sequence is shown in italics; myc epitope is indicated in
 bold; fire-fly luciferase sequence is indicated in capitals; GPI sequence of
 folate receptor is underlined.

The whole final construct was excised by a NotI/XhoI or XbaI
 digestion, and cloned into the expression vectors pcDNA3 or VR1012,
 15 respectively. The clone was checked by sequence analysis carried out on
 service at the Bio Molecular Research sequencing core of the CRIBI-
 University of Padova.

Cell transfection

HEK293 cells were cultured in DMEM-F12 (Sigma-Aldrich).
 20 Media were supplemented with 10% heat-inactivated FBS, 100 U/ml
 penicillin and 100 µg/ml streptomycin (Invitrogen corporation, San Giuliano
 Milanese, Italy). ACN neuroblastoma cells were cultured in DMEM
 supplemented with MEM non-essential amino acid solution 100x (Sigma-
 Aldrich). HEK293 cells were transfected with the calcium phosphate
 25 method. Cells transiently expressing the pmeLUC construct were assayed
 36 hours after transfection. Clones stably expressing pmeLUC or the
 P2X₇R were generated by culture of the transfected cells in the presence
 of G418 (0.8 mg/ml, added 48 hours after transfection) for 3 weeks. Stable
 P2X₇-or pmeLUC-expressing clones were kept in the continuous presence
 30 of 0.2 mg/ml of G418 sulphate (Geneticin) (Calbiochem, La Jolla, CA). ACN
 cells were transfected with pmeLUC by lipofectamine (Invitrogen) and
 tested 24 hours after transfection. Briefly, cells were incubated in 250 µl
 serum-free transfection medium (OPTIMEM) in the presence of
 lipofectamine-plus-DNA (0.4 µg per well). After 3 hours incubation 1 ml of
 35 DMEM plus 10% FBS supplemented with MEM non-essential amino acid
 solution 100 X (Sigma-Aldrich) was added. Cells were assayed 24 hours
 after transfection. In order to allow a high level of plasma membrane

expression of the transfected constructs, cells were incubated overnight in the presence of 1 mM DTT (Mezghrani *et al.*, 2001). Furthermore, they were also kept at room temperature (21°C) for two hours prior to transfer into the thermostated luminometer chamber. These treatments that did not perturb luciferase activity or P2X₇ function maximized pmeLUC surface expression by improving transport to the plasma membrane and slowing down recycling.

Immunofluorescence

HEK293 cells, seeded onto 24 mm coverslips, were fixed with 4% formaldehyde in PBS solution for 30 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature, rinsed three times with PBS, and incubated for 30 minutes with 0.2% gelatine in PBS to block non specific binding-sites. Immunostaining was carried out for 1 hour at 37 °C with a commercial monoclonal antibody against the c-myc epitope tag (Santa Cruz Biotechnology Inc., CA, USA) at a 1:100 dilution in 0.2% gelatine in PBS. Immunodetection was carried out using Texas-Red conjugated goat anti-mouse IgG (Santa Cruz) used at 1:50 dilution in 0.2% gelatine in PBS. After immunostaining, cells were imaged with a Zeiss LSM 510 Confocal Laser Scanning Microscope.

ATP measurement

ATP was measured in the custom-made luminometer described by Rizzuto and co-workers (Brini *et al.*, 1999; Jouaville *et al.*, 1999). For experiments, cells were plated onto 13 mm coverslips and were placed in a 37°C thermostatted chamber (diameter 15 mm, height 2 mm) and perfused with a saline solution supplemented with luciferin at a concentration of 5 µM. The chamber was held in a photomultiplier kept in a dark refrigerated (4°C) box. Light emission was detected by a Thorn EMI photon counting board installed in an IBM-compatible computer. The board allowed storing of the data in the computer memory for further analysis. During the experiments the thermostatted chamber was continuously perfused with buffer by means of a Gilson peristaltic pump. Alternatively, ATP was measured in the supernatants using soluble luciferase in a Firezyme luminometer as previously described (Baricordi *et al.*, 1999; Solini *et al.*, 2004; Zanovello *et al.*, 1990).

FACS analysis

Non-permeabilized HEK293 cells stably transfected with pmeLUC or with the empty vector were labeled with the murine monoclonal

antibody (Santa Cruz) directed against the pmeLUC c-myc tag at a 1:100 dilution in PBS for 1 hour at 4°C. At the end of this incubation, cells were incubated with FITC-conjugated anti-mouse antibody at a 1:50 dilution in PBS for 1 hour at 4°C. Fluorescence emission was analysed with an argon laser cytofluorometer FACS Scan Vantage (Beckton Dickinson, Franklin Lakes, NJ, USA).

RESULTS

The structure of the novel ATP probe (pmeLUC) is shown in Figure 1A. This chimeric protein, thanks to the folate receptor leader sequence, is targeted to the plasma membrane and detects ATP in the extracellular space close to the cell surface (Figure 1B). The aminoacid sequence of pmeLuc is preferably:

MAQRMTTQLLLLLVWVAVVGEAQTRIAEQKLISEEDLLQMEDAKNIKKGP
APFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVDITYAEYFEMSV
RLAEAMKRYGLNTNHRIVVCSNSLQFFMPVLGALFIGVAVAPANDIYNE
RELLNSMGISQPTVVFVSKKGLQKILNVQKKLPQQKIIMDSKTDYQGF
QSMYTFVTSHLPPGFNEYDFVPESFDRDKTIALIMNSSGSTGLPKGVALP
HRTACVRFSHARDPIFGNQIIPDTAILSVPFHHGFGMFTTLGYLICGFR
VVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFAKSTLIDKYDLSNLHE
IASGGAPLSKEVGEAVAKRFHLP GIRQGYGLTETTSAILITPEGDDKPGA
VGKVVPFFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNPEATN
ALIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLIKYKGYQVAPAELESILLQ
HPNIFDAGVAGLPDDDAGELPAAVVLEHGKTMTEKEIVDYVASQVTTA
KKLRGGVVFVDEV PKGLTGKLDARKIREILIKAKKGGKIAVAAAMSGAGP
WAAWPFLLSLALMLLWLLS (SEQ ID No:1).

Immunofluorescence and FACS analysis of cells transfected with this construct confirm that pmeLUC is expressed on the plasma membrane (Figures 1C and D). Cells expressing pmeLUC have a basal level of luminescence emission that depends on the amount of expressed luciferase, however in the stable HEK293-pmeLUC clones generated and used by the authors of the invention in the experiments, basal luminescence emission was comprised within a fairly narrow cps (counts per second) range (i.e. from a lower emission of about 2500 cps, to a higher of about 3200 cps).

To minimize variations due to minor changes in pmeLUC expression luminescence can be expressed as percent increase over basal, as shown in Figure 2A, where HEK293-pmeLUC cells are

challenged with different nucleotides in order to test affinity and selectivity of the probe. Affinity of pmeLUC for ATP is rather low, with a threshold of about 10 μ M, however, subsequent ATP additions elicit further increases in light emission that allow building a calibration curve (Figure 2B).
5 Importantly, pmeLUC is insensitive to all other nucleotides tested (ADP, UTP, UDP and GTP) (Figure 2A). To check for ability of pmeLUC to monitor ATP release triggered by receptor-directed stimuli, the HEK293-pmeLUC cells were challenged with various agonists of G protein-coupled receptors (e.g. carbachol, histamine, bradykinin), obtaining negligible ATP
10 release (not shown).

Early experiments carried out not only by the authors of the present invention had shown that supernatants from cells expressing the P2X₇ receptor contained a high level of ATP, which was not due to an accelerated rate of cell lysis (Baricordi et al., 1999; Adinolfi et al., 2003;
15 Solini et al., 2004). This suggested that the P2X₇R might be one of the pathways mediating ATP translocation across the plasma membrane. To test such hypothesis several HEK293 clones were generated stably transfected with the human or rat P2X₇R (HEK293-hP2X₇ or HEK293-rP2X₇, respectively). These clones were then transfected with pmeLUC
20 (HEK293-P2X₇/pmeLUC). As shown in Figure 3A, the HEK293-hP2X₇/pmeLUC cells exhibit a several fold higher level of basal fluorescence compared to HEK293-pmeLUC (12250 $\pm$ 1500 versus 2300 $\pm$ 850 cps, respectively, n = 8). Addition of BzATP, a potent P2X₇ agonist, triggers a large luminescence increase in the HEK293-hP2X₇/pmeLUC or HEK293-rP2X₇/pmeLUC (only HEK293-hP2X₇/pmeLUC shown), but not in the HEK293-pmeLUC cells. The
25 luminescence increase triggered by BzATP is fully blocked by pre-treatment with oxidized ATP (oATP), a powerful and irreversible blocker of the P2X₇R (Murgia et al., 1993).

30 To rule out a possible inhibitory effect of oATP on the luciferase itself, a calibration curve was performed in the presence and absence of this inhibitor, showing that the ATP-dependent luminescence increase is not affected (not shown but see also Figure 4). Calibration of BzATP triggered luminescence increase yields a peak ATP concentration of about
35 250 μ M.

Differences in basal levels of luminescence emission are neutralized by expressing luminescence as percentage increase over

basal (Figure 3B), however, the higher basal luminescence of HEK293-hP2X₇/pmeLUC might reflect a real increased level of pericellular ATP compared to HEK293-pmeLUC, rather than a higher expression of pmeLUC. In this case, reporting luminescence as percent increase over basal would mask such a difference in the extracellular ATP concentration. To clarify this issue, total pmeLUC protein was measured by western blotting (Figure 3C). Blots show that, although there is some variability in protein expression in the different stable transfectants, HEK293-hP2X₇/pmeLUC or HEK293-rP2X₇/pmeLUC have a lower content of luciferase compared to HEKpmeLUC, and this cannot account for the higher basal luminescence emission in the P2X₇-transfected clones. To measure quantitatively surface pmeLUC expression, different clones were analyzed by FACS. As shown in Figure 3D, pmeLUC expression profiles of HEK293-hP2X₇/pmeLUC, HEK293-rP2X₇/pmeLUC and HEK293-pmeLUC closely overlaps. Therefore, in keeping with previous findings (Baricordi et al., 1999; Solini et al., 2004), these data suggest that cells expressing the P2X₇R maintain a higher ATP concentration. Reduction of basal luminescence by oATP pretreatment (5800 ± 1300 cps, $n = 8$) also supports this interpretation. Finally, the effect of BzATP on the human ACN neuroblastoma, a cell line expressing the native P2X₇R, was assayed (Raffaghello L., Pistoia V., Di Virgilio F., unpublished observations). As shown in Figure 3E, also in this case BzATP induces a large ATP release which is fully blocked by oATP. Basal luminescence total levels in this cell line were 3500 ± 350 cps and 1500 ± 260 cps ($n = 5$), before and after treatment with oATP, respectively, further supporting the finding that cells expressing a functional P2X₇R maintain a higher ATP concentration in the pericellular space.

As an independent proof that HEK293-P2X₇ cells release a larger amount of ATP than mock-transfected HEK293 (HEK293-mock), extracellular ATP was measured in the supernatants using soluble luciferase. Quiescent HEK293-mock cells maintained an extracellular ATP concentration of 80 ± 20 nM ($n = 12$), compared to 220 ± 34 nM ($n = 10$) for HEK-hP2X₇. Addition of BzATP had no effect on the HEK293-mock, but increased extracellular ATP levels to about 400 ± 55 nM ($n = 10$) in the HEK293-hP2X₇ cells supernatants.

Like BzATP, ATP itself should trigger ATP release in the HEK293-P2X₇/pmeLUC cells, since, albeit at high concentrations, ATP is

the only known physiological activator of P2X₇ (Di Virgilio et al., 2001; North, 2002). Then, ATP addition to HEK293-P2X₇/pmeLUC cells should trigger an extra increase in luminescence compared to HEK293-pmeLUC cells. The extra increase in luminescence should be due to ATP release via the P2X₇R. Figure 4 shows that this is the case, whether the transfected receptor is the human or rat P2X₇R. Interestingly, in the HEK293 cells transfected with the rat receptor (Figure 4A) the extra-increase in luminescence emission (expressed as percent increase over basal) is detectable already at the lowest ATP concentrations used (10-50 μ M), while in cells transfected with the human ortholog (Figure 4B) a luminescence increase over control cells is detectable only at ATP concentrations higher than 100 μ M. This is in keeping with the known lower affinity for ATP of the human receptor (Rassendren et al., 1997; Suprenant et al., 1996). If the extra luminescence observed in the HEK293-P2X₇/pmeLUC cells is due to ATP release via P2X₇, then it should be abolished by pre-treatment with oATP. This prediction is fulfilled, as in the oATP-treated cells the luminescence increase matches exactly that of HEK293-pmeLUC (Figures 4A and B).

EXAMPLE 2: Increase of ATP release P2X₇R mediated

One of the most potent stimuli for ATP secretion is plasma membrane stretching. To test ability of pmeLUC to detect stretch-induced ATP release HEK293-P2X₇/pmeLUC and HEK293-pmeLUC cells were exposed to a change in tonicity of the perfusion buffer. Cell monolayers were initially perfused with the usual isotonic solution used in all the experiments, and then switched to a hypotonic buffer, obtained by diluting the standard saline buffer with distilled water (1 to 4) (final tonicity 78 mOsm/L). The tonicity shift causes a clearly detectable release of ATP from both clones (Figure 5A). However, ATP release is several fold higher in the P2X₇-transfected cells. These cells, as shown in the previous experiments, also maintain a higher basal pericellular ATP level with respect to HEK293-pmeLUC (14200 $\pm$ 2300 versus 3150 $\pm$ 970 cps, n = 7). Interestingly, a peak of ATP release is triggered both by a shift from isotonicity to hypotonicity and from hypotonicity to isotonicity, as well as from a shift from isotonicity to hypertonicity (Figure 5B). In this case HEK293-hP2X₇/pmeLUC and HEK293-pmeLUC cells were perfused with isotonic buffer followed by a hypertonic solution obtained by dissolving 25 ml of sucrose into 75 ml of standard saline solution (final osmolarity 560

mOsm/L).

These findings suggest that, although membrane stretch-induced ATP release can occur independently of the P2X₇R, it is strongly dependent on the expression of this receptor.

5 EXAMPLE 3: Measurement of ATP release induced by toxic agents in J774 macrophage cells and A549 pulmonary epithelial cells expressing pmeLUC probe

MATERIALS AND METHODS

Chimeric probe pmeLUC construction

10 Chimeric probe pmeLUC described in Example 1 was used to transfect J774 macrophage cells and A549 pulmonary epithelial cells.

Cell line engineering

15 In the example shown it was not necessary to previously make the cell line employed expressing any receptor trigger cell response, since this such a cell line constitutively expresses the molecular complex necessary for ATP release. However, it is possible, depending on study necessities, to make the cells expressing a specific receptor for the molecule/drug to be tested to the aim of ensuring an effective binding level, thus simplifying the functional analysis of transduction systems.

20 *Cellular transfection*

 Cells cultured in flasks for cell culture (75 cm²) were transfected with a vector containing the produced chimeric probe, by transfection techniques more suitable for the cell line under examination. As experimental model established macrophage cells, named J774, and
25 established pulmonary epithelial cells, named A459, were used after being transfected with pmeLUC probe using TransFectin (Biorad) that ensure the major percent of positive cells for these cell lines.

Harvesting of cells expressing the chimeric probe

30 36 hours after transfection cells were removed from the bottom of flasks for trypsinization. Cell suspension was transferred in a Falcon tube (from 15 to 50 ml) and centrifuged at 1200 rpm at 20°C and finally cell precipitate was re-suspended in DMEM medium supplemented with DTT (1 mM).

Seeding on multi-well plates

50 µl of suspension containing transfected cells (corresponding to about 50.000 cells) were seeded in each well of the plate. Cells were incubated overnight.

5 *Response detection*

The day after seeding cells were incubated with a saline solution (KRB: Krebs-Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na₃PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5,5 mM glucose, 20 mM HEPES, pH 7.4, 37°C) supplemented with luciferin (5 µM) and were directly contacted with a photomultiplier measuring photon emission by luciferase.

10 RESULTS

This test was carried out to assay the presence of toxic substances inducing ATP release in live cells.

Figures 6 (J774 cells) and 7 (A549 cells) show diagrams wherein it is shown the measurement of ATP release following exposure to several substances.

The example was carried out on two parallel "batch" of cells, J774 and A549:

20 A) J774 cells/A549 cells expressing pmeLUC probe treated with several ATP concentrations (from 5 µM to 1 mM) (control).

B) J774 cells/A549 cells expressing pmeLUC probe treated with a known toxic agent, bacterial endotoxin (LPS) acting on the constitutive CD14 receptor of both lines (1 µg/ml) (thin dark line).

25 In absence of outer ATP photon emission has very low intensity. Indeed, in such conditions extracellular ATP concentration, where is localized pmeLUC probe is very low (0,1 µM).

30 As it is notable from diagrams represented in figures 6A and 7A the addition of increasing outer ATP concentrations induces a photon emission by the pmeLUC probe proportional to ATP concentration applied to demonstrate the sensitivity of such biosensors for the detection of extracellular ATP.

Figures 6B and 7B show ATP release, by J774 cells and A549 cells in response to a toxic agent such as LPS.

35 It is evident from the diagram that the treatment with toxic substances activated mechanisms capable to induce ATP release at the pericellular level inside cells under examination that is detected by pmeLUC probe.

Such a result shows applicability of such system as cellular biosensor suitable to detect and identify substances causing ATP release such as environmental toxic substances such as LPS and ozone.

BIBLIOGRAPHY

- 5 - Adinolfi, E., Kim, M., Young, M.T., Di Virgilio, F., and Surprenant, A. (2003). *J. Biol. Chem.* 278, 37344-37351.
- Anderson, C.M., Bergher, J.P., and Swanson, R.A. (2004). *Journal of Neurochemistry* 88, 246-256.
- Baricordi, O.R., Melchiorri, L., Adinolfi, E., Falzoni, S., Chiozzi, P., Buell, G., and Di Virgilio, F. (1999). *J. Biol. Chem.* 274, 33206-33208.
- 10 - Beigi, R., Kobatake, E., Aizawa, M., and Dubyak, G.R. (1999). *Am J Physiol Cell Physiol* 276, C267-C278.
- Brini, M., Pinton, P., King, M.P., Davidson, M., Schon, E.A., and Rizzuto, R. (1999). *Nat. Med.* 5, 951-954.
- 15 - Burnstock, G. (2004). *Curr. Top. Med. Chem.* 4, 793-803.
- Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J.M., Morelli, A., Torboli, M., Bolognesi, G., and Baricordi, O.R. (2001). *Blood* 97, 587-600.
- 20 - Di Virgilio, F., Baricordi, O.R., Romagnoli, R., and Baraldi, P.G. (2004). *Curr Drug Targets*, 5, 85-99.
- Duan, S., Anderson, C.M., Keung, E.C., Chen, Y., Chen, Y., and Swanson, R.A. (2003). 23, 1320-1328.
- Ferrari, D., Chiozzi, P., Falzoni, S., Hanau, S., and Di Virgilio, F. (1997). *J. Exp. Med.* 185, 579-582.
- 25 - Freyer, D.R., Boxer, L.A., Axtell, R.A., and Todd, R.F. III. (1988). *J. Immunol.* 141, 580-586.
- Hayashi, S., Hazama, A., Dutta, A.K., Sabirov, R.Z., and Okada, Y. (2004). *Sci. STKE.* 258, 114.
- 30 - Illes, P., and Norenberg, W. (1993). *Trends Pharmacol. Sci.* 14, 50-54.
- Jouaville, L.S., Pinton, P., Bastianutto, C., Rutter, G.A., and Rizzuto, R. (1999). *Proc. Natl. Acad. Sci. USA* 96, 13807-13812.
- La Sala, A., Ferrari, D., Di Virgilio, F., Idzko, M., Norgauer, J., and Girolomoni, G. (2003). *J. Leukoc. Biol.* 73, 339-343.
- 35 - Matzinger, P. (2002). *Science* 296, 301-305.

- Mezghrani, A., Fassio, A., Benham, A., Simmen, T., Braakman, I., and Sitia, R. (2001). *EMBO J.* 20, 6288-6296.
- Murgia, M., Hanau, S., Pizzo, P., Rippa, M., and Di Virgilio, F. (1993). *J. Biol. Chem.* 268, 8199-8203.
- 5 - Neary, J.T., Kang, Y., Willoughby, K.A., and Ellis, E.F. (2003). *J. Neurosci.* 23, 2348-2356.
- Newman, E.A. (2001). *J. Neurosci.* 21, 2215-2223.
- North, R.A. (2002). *Physiol. Rev.* 82, 1013-1067.
- Oshimi, Y., Miyazaki, S., and Oda, S. (1999). *Immunology* 10 98, 220-227.
- Perregaux, D., and Gabel, C.A. (1994). *J. Biol. Chem.* 269, 15195-15203.
- Rassendren, F., Buell, G.N., Virginio, C., Collo, G., North, R.A., and Surprenant, A. (1997). *J. Biol. Chem.* 272, 5482-5486.
- 15 - Skoberne, M., Beignon, A.S., and Bhardwaj, N. (2004). *Trends. Mol. Med.* 10, 251-257.
- Solini, A., Chiozzi, P., Morelli, A., Adinolfi, E., Rizzo, R., Baricordi, O.R., and Di Virgilio, F. (2004). *Arterioscler. Thromb. Vasc. Biol.* 24, 1240-1245.
- 20 - Surprenant, A., Rassendren, F., Kawashima, E., North, R.A., and Buell, G. (1996). *Science* 272, 735-738.
- Wang, X., Arcuino, G., Takano, T., Lin, J., Peng, W.G., Wan, P., Li, P., Xu, Q., Liu, Q.S., Goldman, S.A., and Nedergaard, M. (2004). *Nat. Med.* 10, 821-827.
- 25 - Zanollo, P., Bronte, V., Rosato, A., Pizzo, P., and Di Virgilio, F. (1990). *J. Immunol.* 145, 1545-1550.
- Zimmermann, H. (2000). *Naunyn Schmiedeberg's Arch. Pharmacol.* 362, 299-309.

CLAIMS

1. Luminescent chimeric proteins comprising a first N-terminal protein sequence, a second protein sequence and a third C-terminal protein sequence wherein:
- 5 (i) said first and said third protein sequence are a leader sequence and an anchor sequence belonging to at least a receptor localized on a plasma membrane site;
- (ii) said second protein sequence encodes for the full-length or partial sequence of a photoprotein and is inserted in frame between said first and
- 10 said third sequence (i); said chimeric protein being addressed to said plasma membrane site of the cell wherein it is expressed.
2. Proteins according to claim 1, wherein said receptor localized on the plasma membrane site is selected from the group that consists in ionic-channel receptors, connexins, G protein coupled receptors, tyrosine-
- 15 kinase activity receptors.
3. Proteins according to claims 1 or 2, wherein said photoprotein is selected from the group that consists in luciferase, aequorin, obelin.
4. Proteins according to claims 1-3, wherein said first and said third protein sequence (i) are the leader sequence and the GPI anchor
- 20 sequence of the folate receptor and said photoprotein (ii) is luciferase.
5. Proteins according to claim 4, wherein luciferase is fire-fly luciferase.
6. Protein according to claim 5 having the following aminoacidic sequence:
- 25 MAQRMTTQLLLLLVWVAVVGEAQTRIAEQKLISEEDLLQMEDAKNIKKGP
APFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVDITYAEYFEMSV
RLAEAMKRYGLNTNHRIVVCSNSLQFFMPVLGALFIGVAVAPANDIYNE
RELLNSMGISQPTVVFVSKKGLQKILNVQKKLPPIQKIIIMDSKTDYQGF
QSMYTFVTSHLPPGFNEYDFVPESFDRDKTIALIMNSSGSTGLPKGVALP
30 HRTACVRFSHARDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLICGFR
VVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFAKSTLIDKYDLSNLHE
IASGGAPLSKEVGEAVAKRFHLPGIRQGYGLTETTSAILITPEGDDKPGA
VGKVVPFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNPEATN
ALIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLIKYKGYQVAPAELESILLQ
35 HPNIFDAGVAGLPDDDAGELPAVVVLEHGKTMTEKEIVDYVASQVTTA
KKLRGGVVVDEVKGLTGKLDARKIREILIKAKKGGKIAVAAAMSGAGP
WAAWPFLLSLALMLLWLLS(SEQ ID No:1).

7. Nucleotidic sequence encoding for one of the protein according to anyone of claims 1 to 6.

8. Nucleotidic sequence according to claim 7 when dependent on claim 6, having the following nucleotidic sequence:

5 ATGGCTCAGCGGATGACAACACAGCTGCTGCTCCTTCTAGTGTGGGT
GGCTGTAGTAGGGGAGGCTCAGACAAGGATTGCAGAACAAAACTAA
TAAGCGAGGAGGACCTGCTGCAGATGGAAGACGCCAAAAACATAAA
GAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGA
GAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAAC
10 AATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGA
GTACTTCGAAATGTCCGTTCCGTTGGCAGAAGCTATGAAACGATATG
GGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACCTCTCTTC
AATTCTTTATGCCGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTG
CGCCCGCGAACGACATTTATAATGAACGTGAATTGCTCAACAGTATG
15 GGCATTTGCGAGCCTACCGTGGTGTTCGTTTCCAAAAAGGGGTTGCA
AAAAATTTTGAACGTGCAAAAAAGCTCCCAATCATCCAAAAAATTATT
ATCATGGATTCTAAAACGGATTACCAGGGATTTTCAGTCGATGTACACG
TTCGTCACATCTCATCTACCTCCCGGTTTTAATGAATACGATTTTGTG
CCAGAGTCCTTCGATAGGGACAAGACAATTGCACTGATCATGAACTC
20 CTCTGGATCTACTGGTCTGCCTAAAGGTGTCGCTCTGCCTCATAGAA
CTGCCTGCGTGAGATTCTCGCATGCCAGAGATCCTATTTTTGGCAAT
CAAATCATTCCGGATACTGCGATTTTAAGTGTTGTTCCATTCCATCAC
GGTTTTGGAATGTTTACTACACTCGGATATTTGATATGTGGATTTCGA
GTCGTCTTAATGTATAGATTTGAAGAAGAGCTGTTTCTGAGGAGCCTT
25 CAGGATTACAAGATTCAAAGTGCGCTGCTGGTGCCAACCCTATTCTC
CTTCTTCGCCAAAAGCACTCTGATTGACAAATACGATTTATCTAATTTA
CACGAAATTGCTTCTGGTGGCGCTCCCCTCTCTAAGGAAGTCGGGGA
AGCGGTTGCCAAGAGGTTCCATCTGCCAGGTATCAGGCAAGGATATG
GGCTCACTGAGACTACATCAGCTATTCTGATTACACCCGAGGGGGAT
30 GATAAACC GGCGCGGTTCGGTAAAGTTGTTCCATTTTTTGAAGCGAA
GGTTGTGGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAAAGAG
GCGAACTGTGTGTGAGAGGTCCTATGATTATGTCCGGTTATGTAAAC
AATCCGGAAGCGACCAACGCCTTGATTGACAAGGATGGATGGCTACA
TTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATCG
35 TTGACCGCCTGAAGTCTCTGATTAAGTACAAAGGCTATCAGGTGGCT
CCCGCTGAATTGGAATCCATCTTGCTCCAACACCCCAACATCTTCGA
CGCAGGTGTCGCAGGTCTTCCCGACGATGACGCCGGTGAACCTCCC

GCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAAGA
GATCGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTGC
GCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGG
AAACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGA
5 AGGGCGGAAAGATCGCCGTGGCTGCAGCCATGAGTGGGGCTGGGC
CCTGGGCAGCCTGGCCTTTCCTGCTTAGCCTGGCCCTAATGCTGCTG
TGGCTGCTCAGCTGA (SEQ ID No:2).

9. Expression vector comprising the nucleotidic sequence as defined according to anyone of the claims 7 or 8.
10. Vector according to claim 9, wherein said vector is selected between pcDNA3, VR 1012.
11. Primary cell culture transfected with the expression vector according to anyone of the claims 9-10 or with the nucleotidic sequence according to anyone of the claims 7 or 8.
12. Cell line transfected with the expression vector according to anyone of the claims 9-10 or with the nucleotidic sequence according to anyone of the claims 7 or 8.
13. Cell line according to claim 12, characterized by the native or recombinant expression of a receptor of interest whose activation trigger an increase of extracellular ATP levels.
14. Cell line according to claim 13, wherein said receptor of interest is selected between CD14, P2Y, P2X.
15. Cell line according to claim 14, wherein said receptor P2X is P2X₇.
16. Cell line according to anyone of the claims 12-15, selected from the group that consists in HEK 293, HeLa, ACN, N9, N13, PC12, J774, A549.
17. Use of luminescent chimeric proteins according to anyone of the claims 1 to 6, as probes for the measurement of extracellular ATP levels in *in vitro* systems.
18. Use of luminescent chimeric proteins according to anyone of the claims 1 to 6, as probes for the measurement of extracellular ATP levels in *in vivo* systems.
19. Use of cell lines according to anyone of the claims from 12 to 16 for the measurement of extracellular ATP levels in *in vitro* systems.
20. Use of cell lines according to anyone of the claims from 12 to 16 as experimental model in screening method and/or in analysis method of compounds of interest able to modulate extracellular ATP levels.

21. Screening method of compounds of interest able to modulate extracellular ATP levels comprising the following steps:

- 5 a) contacting the cell line as defined according to anyone of the claims 12-16, when dependent on anyone of claims 4-6, with said compound of interest in the presence of O_2 , Mg^{2+} and of the luciferin substrate;
- b) detecting cps or luminescence percent over basal value or the value of a preceding stimulation with an agonist as a control and determining the activity as an agonist or antagonist in relation to cps increase or reduction over basal value or the value of a preceding stimulation with an agonist as a control, respectively.
- 10

22. Method for the analysis of the presence of toxic substances and/or environmental contaminants able to induce the increase of extracellular ATP levels comprising the following steps:

- 15 a) contacting at least a cell line sensible to the toxic substance(s) to be assayed as defined according to anyone of the claims 12-16, when dependent on anyone of claims 4-6, with a sample to be tested in the presence of O_2 , Mg^{2+} and of the luciferin substrate;
- b) detecting the presence or the absence of a toxic substance for cells in relation to the cps or luminescence percent increase or reduction over the basal value.
- 20

23. Method for the analysis according to claim 22, wherein the cell line of step a) is selected between pulmonary epithelial cells and macrophage cells of mammalian origin, preferably of human origin.

24. Method for the analysis according to claim 23, wherein said human pulmonary epithelial cells are A459 cells.

25

25. Method for the analysis according to claim 23, wherein said human macrophage cells are J774 cells.

26. Method for the analysis according to anyone of the claims 22-25 wherein said toxic substance to be tested is LPS and/or ozone.

30 27. Biosensor comprising a cell line characterized in that it is transfected with an expression vector according to anyone of the claims 9-10 or with the nucleotidic sequence according to anyone of the claims 7 or 8.

28. Biosensor according to claim 27, wherein said cell line is as defined according to anyone of the claims 13-15.

35

29. Biosensor according to claim 27 or 28, wherein said cell line is selected from the group consisting in HEK 293, HeLa, ACN, N9, N13,

PC12, J774, A549.

30. Use of biosensor according to anyone of the claims 27-29, for the measurement of extracellular levels of ATP in *in vitro* systems.

5 31. Biosensor comprising at least one of the luminescent chimeric protein as defined according to anyone of the claims from 1 to 6.

32. Use of biosensor according to claim 31, for the measurement of extracellular levels of ATP in *in vitro* systems.

1/9

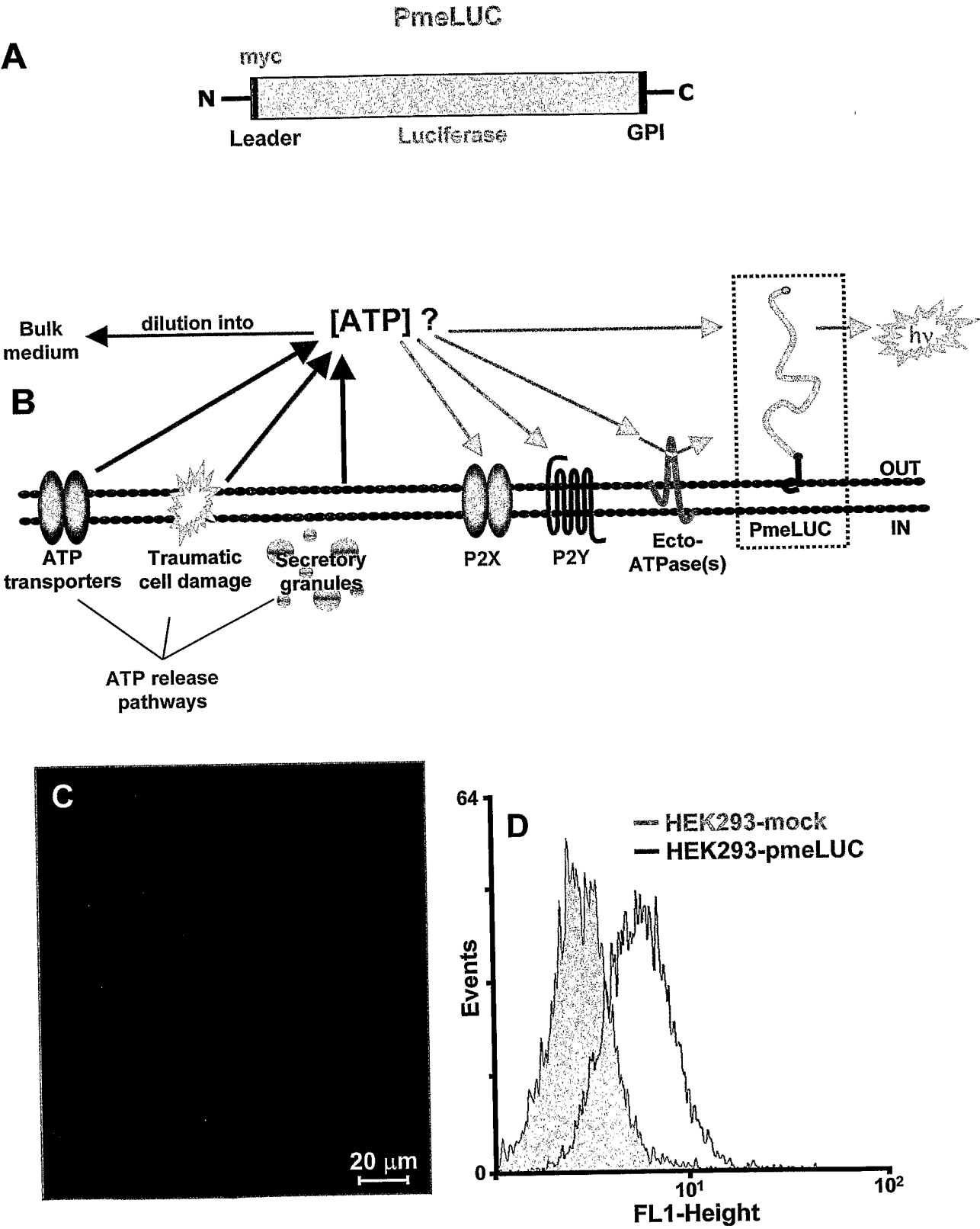


Fig. 1

2/9

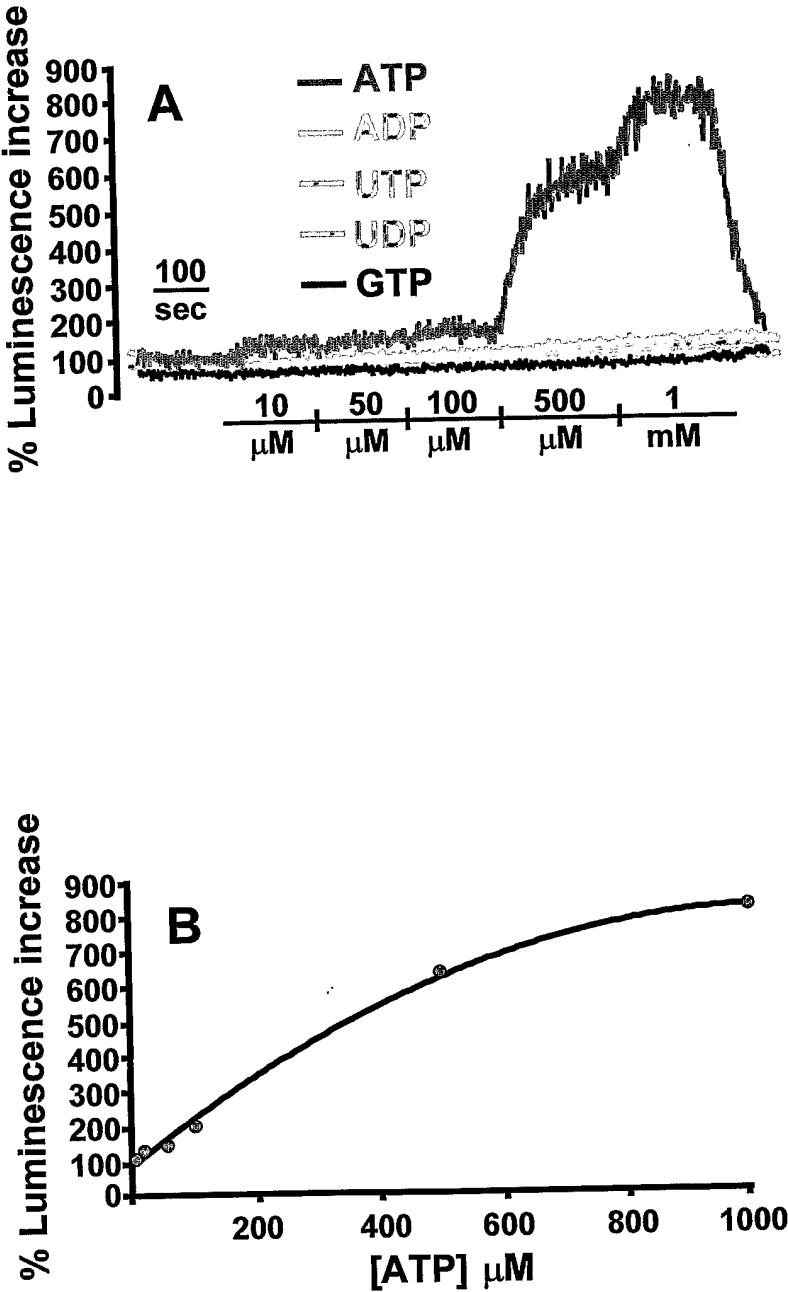


Fig. 2

3/9

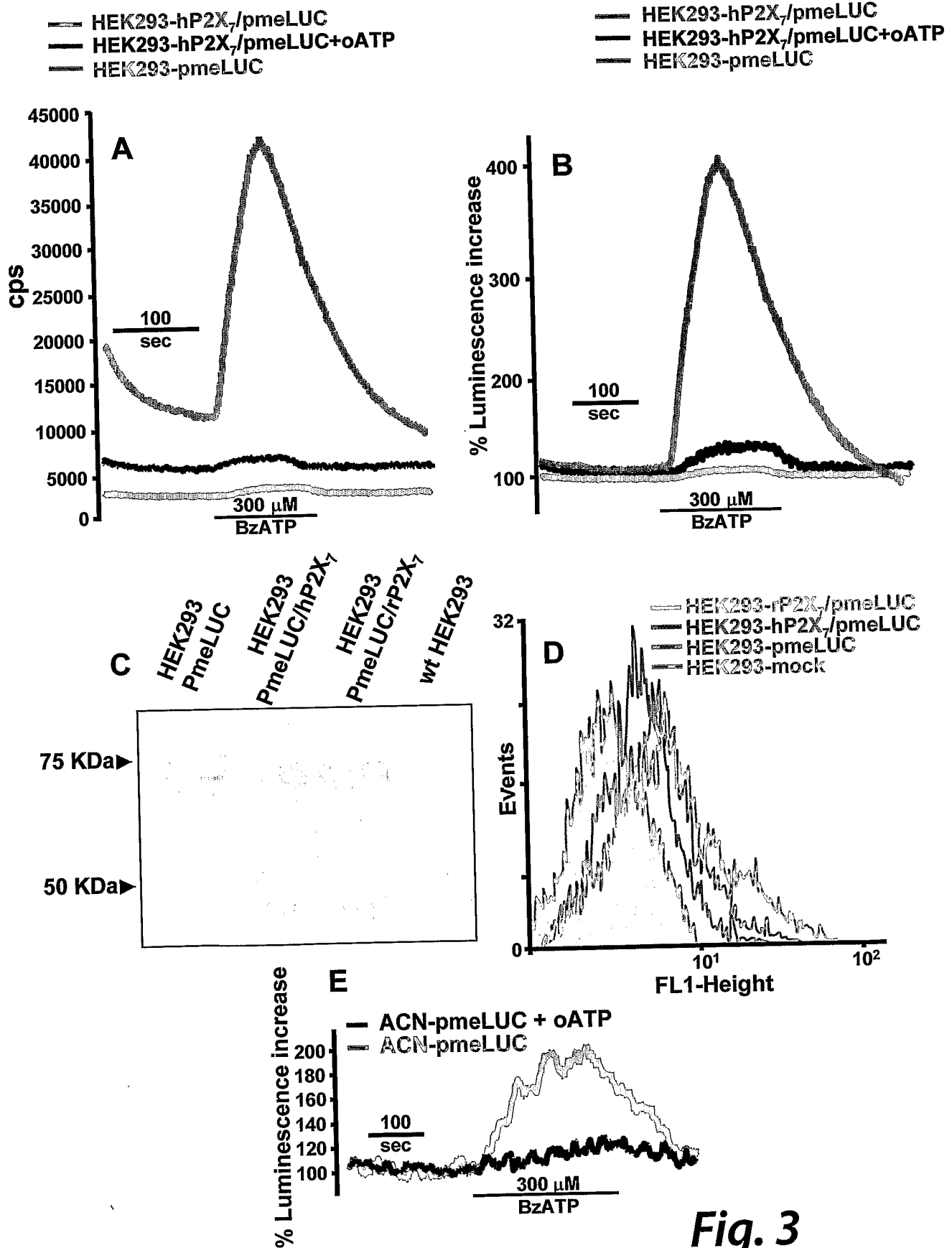
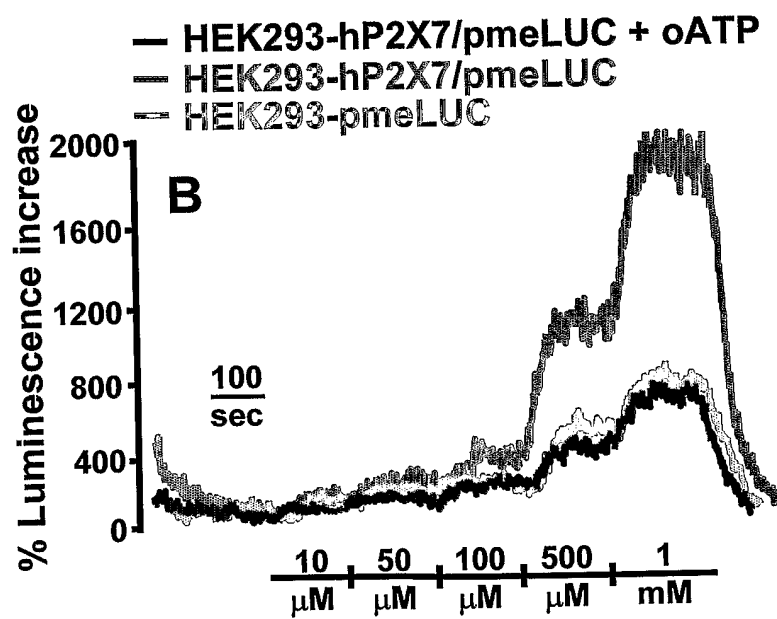
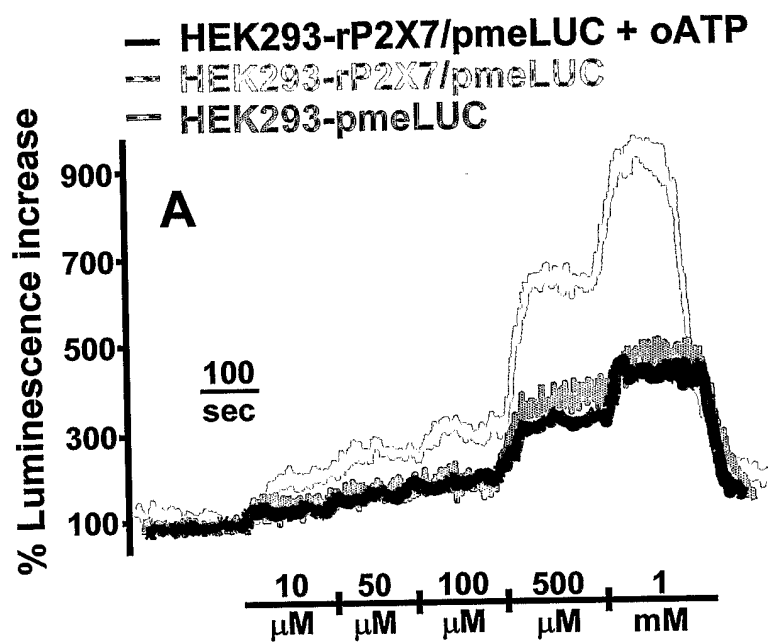


Fig. 3

4/9

*Fig. 4*

5/9

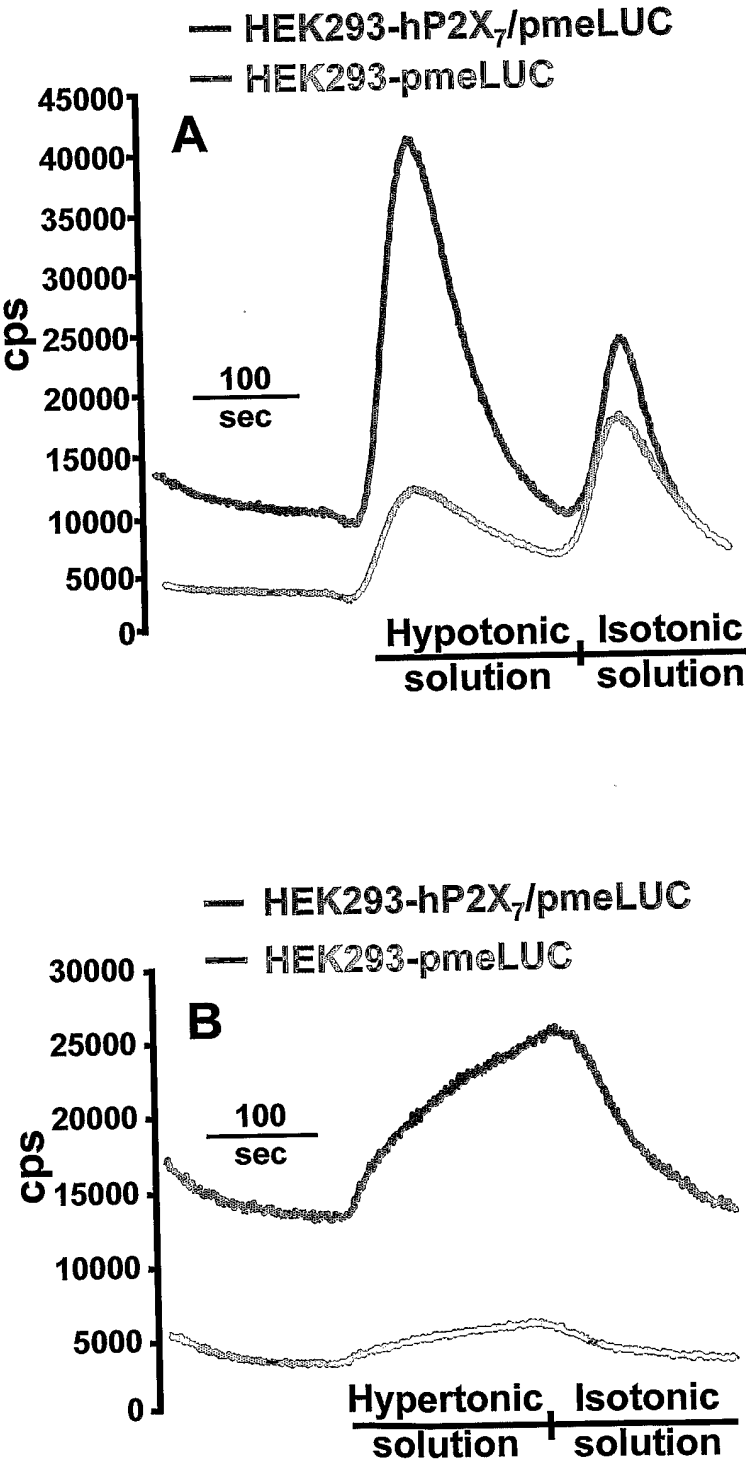


Fig. 5

6/9

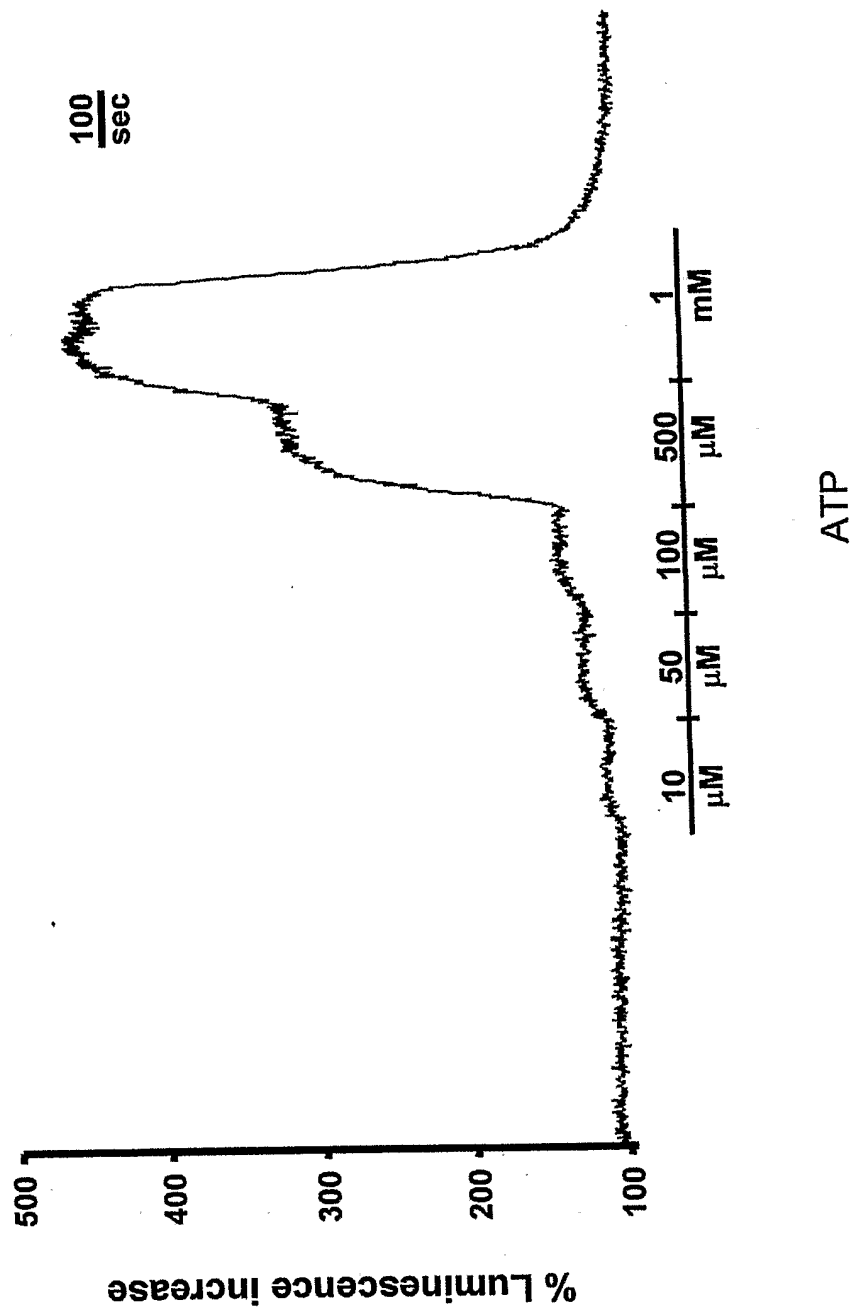


Fig. 6A

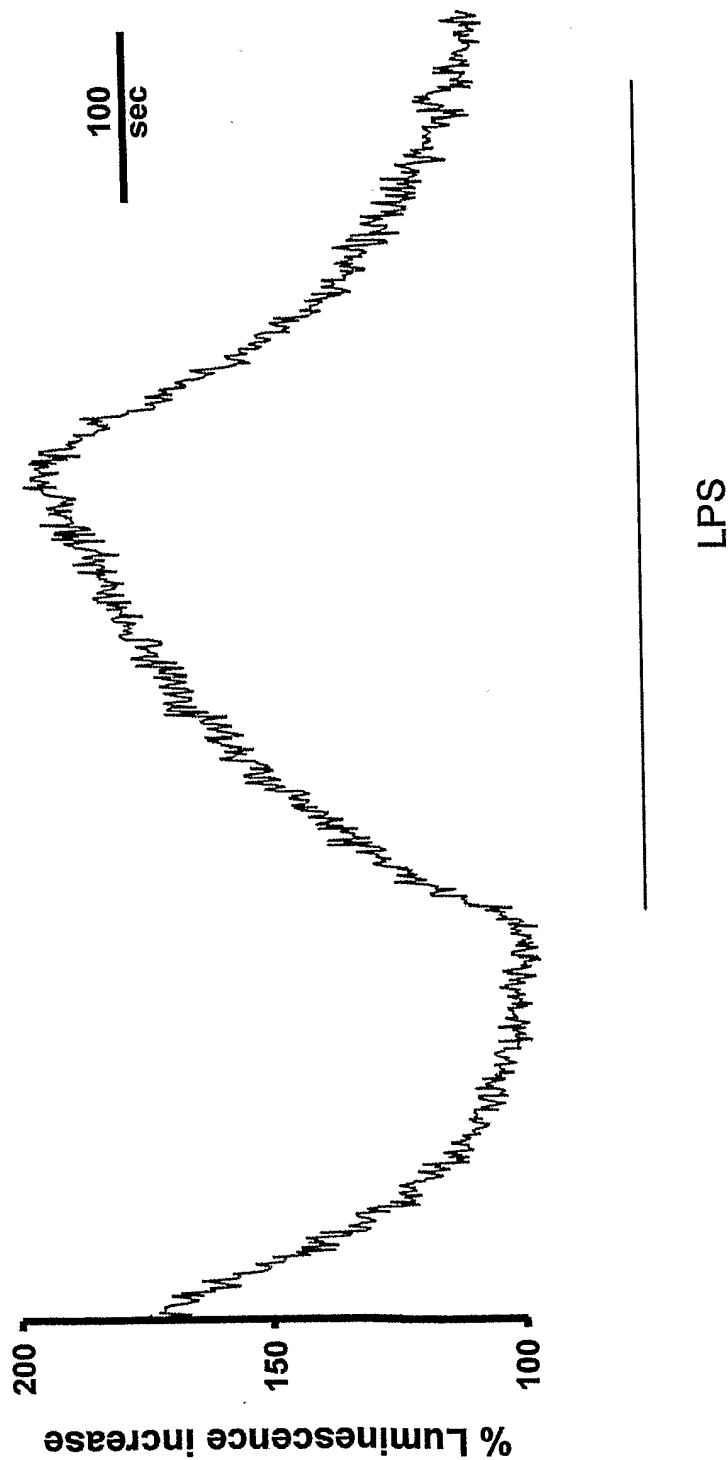
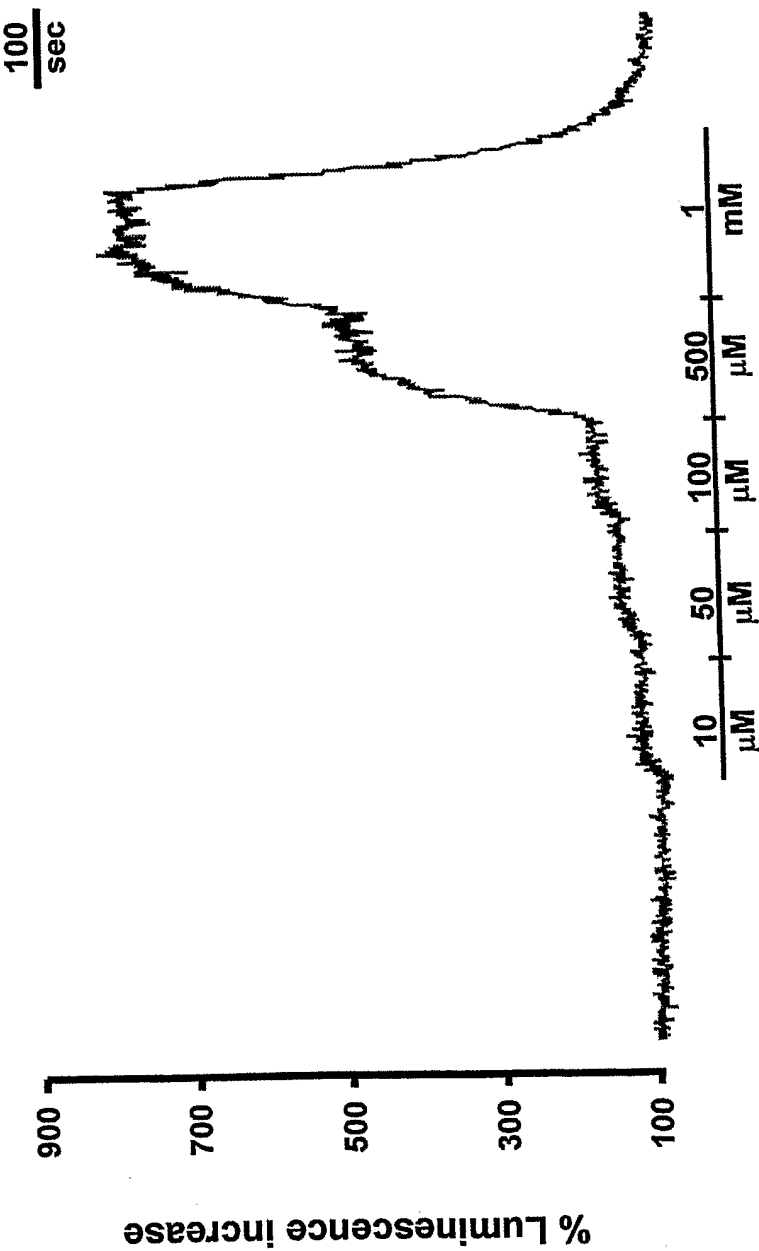
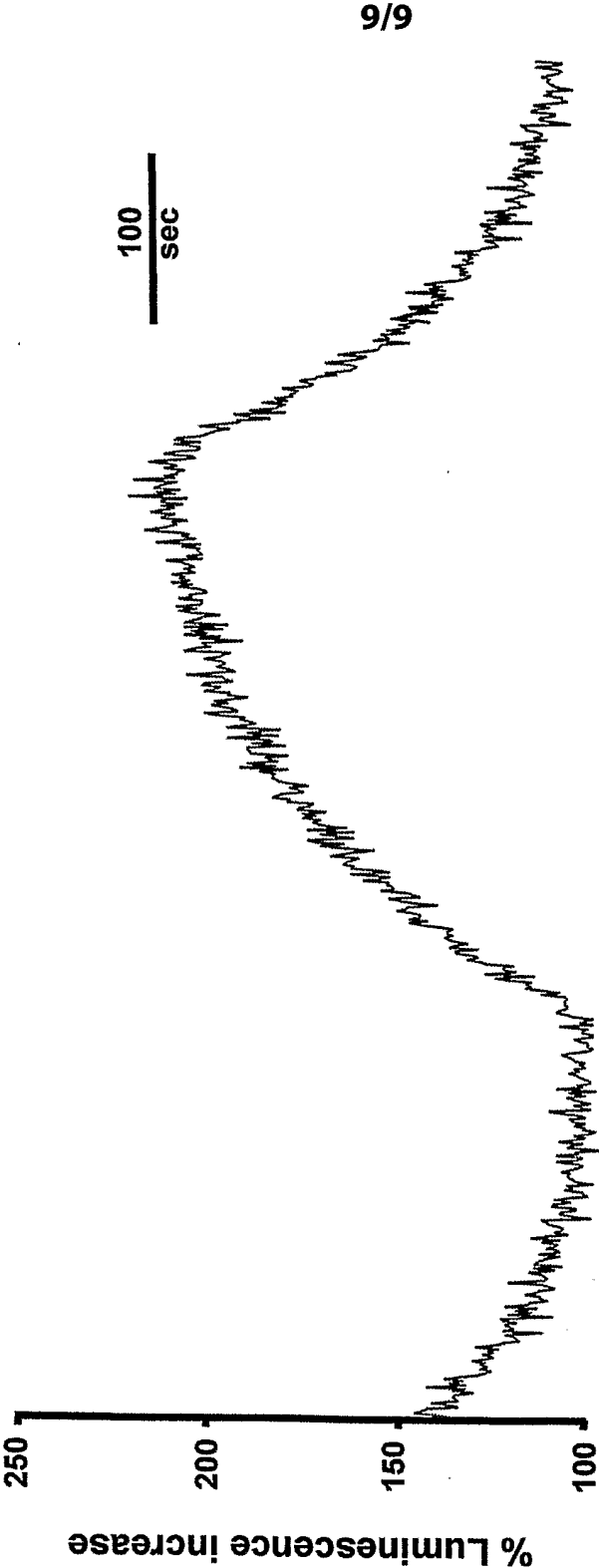


Fig. 6B



ATP

Fig. 7A



LPS

Fig. 7B

AEQUOTECH.ST25
SEQUENCE LISTING

<110> AEQUOTECH s.r.l.

<120> Chimeric proteins for measuring ATP concentrations in the pericellular space and relevant screening method

<130> PCT26059

<150> RM2005A000252

<151> 2005-05-23

<160> 5

<170> PatentIn version 3.2

<210> 1

<211> 617

<212> PRT

<213> Artificial

<220>

<223> Chimeric pMeLuc probe

<400> 1

Met Ala Gln Arg Met Thr Thr Gln Leu Leu Leu Leu Val Trp Val
1 5 10 15

Ala Val Val Gly Glu Ala Gln Thr Arg Ile Ala Glu Gln Lys Leu Ile
20 25 30

Ser Glu Glu Asp Leu Leu Gln Met Glu Asp Ala Lys Asn Ile Lys Lys
35 40 45

Gly Pro Ala Pro Phe Tyr Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln
50 55 60

Leu His Lys Ala Met Lys Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala
65 70 75 80

Phe Thr Asp Ala His Ile Glu Val Asp Ile Thr Tyr Ala Glu Tyr Phe
85 90 95

Glu Met Ser Val Arg Leu Ala Glu Ala Met Lys Arg Tyr Gly Leu Asn
100 105 110

Thr Asn His Arg Ile Val Val Cys Ser Glu Asn Ser Leu Gln Phe Phe
115 120 125

Met Pro Val Leu Gly Ala Leu Phe Ile Gly Val Ala Val Ala Pro Ala
130 135 140

Asn Asp Ile Tyr Asn Glu Arg Glu Leu Leu Asn Ser Met Gly Ile Ser
145 150 155 160

Gln Pro Thr Val Val Phe Val Ser Lys Lys Gly Leu Gln Lys Ile Leu
165 170 175

Pagina 1

AEQUOTECH.ST25

Asn Val Gln Lys Lys Leu Pro Ile Ile Gln Lys Ile Ile Ile Met Asp
 180 185 190
 Ser Lys Thr Asp Tyr Gln Gly Phe Gln Ser Met Tyr Thr Phe Val Thr
 195 200 205
 Ser His Leu Pro Pro Gly Phe Asn Glu Tyr Asp Phe Val Pro Glu Ser
 210 215 220
 Phe Asp Arg Asp Lys Thr Ile Ala Leu Ile Met Asn Ser Ser Gly Ser
 225 230 235 240
 Thr Gly Leu Pro Lys Gly Val Ala Leu Pro His Arg Thr Ala Cys Val
 245 250 255
 Arg Phe Ser His Ala Arg Asp Pro Ile Phe Gly Asn Gln Ile Ile Pro
 260 265 270
 Asp Thr Ala Ile Leu Ser Val Val Pro Phe His His Gly Phe Gly Met
 275 280 285
 Phe Thr Thr Leu Gly Tyr Leu Ile Cys Gly Phe Arg Val Val Leu Met
 290 295 300
 Tyr Arg Phe Glu Glu Glu Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys
 305 310 315 320
 Ile Gln Ser Ala Leu Leu Val Pro Thr Leu Phe Ser Phe Phe Ala Lys
 325 330 335
 Ser Thr Leu Ile Asp Lys Tyr Asp Leu Ser Asn Leu His Glu Ile Ala
 340 345 350
 Ser Gly Gly Ala Pro Leu Ser Lys Glu Val Gly Glu Ala Val Ala Lys
 355 360 365
 Arg Phe His Leu Pro Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr
 370 375 380
 Thr Ser Ala Ile Leu Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala
 385 390 395 400
 Val Gly Lys Val Val Pro Phe Phe Glu Ala Lys Val Val Asp Leu Asp
 405 410 415
 Thr Gly Lys Thr Leu Gly Val Asn Gln Arg Gly Glu Leu Cys Val Arg
 420 425 430
 Gly Pro Met Ile Met Ser Gly Tyr Val Asn Asn Pro Glu Ala Thr Asn
 435 440 445

AEQUOTECH.ST25

Ala Leu Ile Asp Lys Asp Gly Trp Leu His Ser Gly Asp Ile Ala Tyr
 450 455 460

Trp Asp Glu Asp Glu His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu
 465 470 475 480

Ile Lys Tyr Lys Gly Tyr Gln Val Ala Pro Ala Glu Leu Glu Ser Ile
 485 490 495

Leu Leu Gln His Pro Asn Ile Phe Asp Ala Gly Val Ala Gly Leu Pro
 500 505 510

Asp Asp Asp Ala Gly Glu Leu Pro Ala Ala Val Val Val Leu Glu His
 515 520 525

Gly Lys Thr Met Thr Glu Lys Glu Ile Val Asp Tyr Val Ala Ser Gln
 530 535 540

Val Thr Thr Ala Lys Lys Leu Arg Gly Gly Val Val Phe Val Asp Glu
 545 550 555 560

Val Pro Lys Gly Leu Thr Gly Lys Leu Asp Ala Arg Lys Ile Arg Glu
 565 570 575

Ile Leu Ile Lys Ala Lys Lys Gly Gly Lys Ile Ala Val Ala Ala Ala
 580 585 590

Met Ser Gly Ala Gly Pro Trp Ala Ala Trp Pro Phe Leu Leu Ser Leu
 595 600 605

Ala Leu Met Leu Leu Trp Leu Leu Ser
 610 615

<210> 2
 <211> 1854
 <212> DNA
 <213> Artificial

<220>
 <223> pMeLuc nucleotidic sequence

<400> 2
 atggctcagc ggatgacaac acagctgctg ctccttctag tgtgggtggc tgtagtaggg 60
 gaggctcaga caaggattgc agaacaaaaa ctaataagcg aggaggacct gctgcagatg 120
 gaagacgccca aaaacataaa gaaaggcccg gcgccattct atccgctgga agatggaacc 180
 gctggagagc aactgcataa ggctatgaag agatacgccc tggttcctgg aacaattgct 240
 ttacagatg cacatatcga ggtggacatc acttacgctg agtacttcga aatgtccgtt 300
 cggttggcag aagctatgaa acgatatggg ctgaatacaa atcacagaat cgtcgtatgc 360
 agtgaaaact ctcttcaatt ctttatgccg gtgttgggcg cggtatttat cggagttgca 420

AEQUOTECH.ST25

gttgcgccccg cgaacgacat ttataatgaa cgtgaattgc tcaacagtat gggcatttcg 480
 cagcctaccg tgggtgttcgt ttccaaaaag ggggttgcaaa aaattttgaa cgtgcaaaaa 540
 aagctcccaa tcatccaaaa aattattatc atggattcta aaacggatta ccagggattt 600
 cagtcgatgt acacgttcgt cacatctcat ctacctcccg gttttaatga atacgatttt 660
 gtgccagagt ctttcgatag ggacaagaca attgcactga tcatgaactc ctctggatct 720
 actggtctgc cttaaagggtgt cgctctgcct catagaactg cctgcgtgag attctcgcatt 780
 gccagagatc ctatttttgg caatcaaatac attccggata ctgcgatttt aagtgttggt 840
 ccattccatc acggttttgg aatgtttact acactcggat atttgatatg tggatttcga 900
 gtcgtcttaa tgtatagatt tgaagaagag ctgtttctga ggagccttca ggattacaag 960
 attcaaagtgc cgctgctggt gccaaccccta ttctccttct tcgccaaaag cactctgatt 1020
 gacaaatacg atttatctaa ttacacgaa attgcttctg gtggcgctcc cctctctaag 1080
 gaagtcgggg aagcggttgc caagagggtc catctgccag gtatcaggca aggatatggg 1140
 ctactgaga ctacatcagc tattctgatt acacccgagg gggatgataa accgggagcg 1200
 gtcggtaaag ttgttccatt ttttgaagcg aagggttggtg atctggatac cgggaaaacg 1260
 ctgggaggtta atcaaagagg cgaactgtgt gtgagagggtc ctatgattat gtccgggttat 1320
 gtaaacaatc cggaagcgac caacgccttg attgacaagg atggatggct acattctgga 1380
 gacatagctt actgggacga agacgaacac ttcttcatcg ttgaccgcct gaagtctctg 1440
 attaagtaca aaggctatca ggtggctccc gctgaattgg aatccatctt gctccaacac 1500
 cccaacatct tcgacgcagg tgtcgcagggt cttcccgacg atgacgccgg tgaacttccc 1560
 gccgccgttg ttgttttggg gcacggaaaag acgatgacgg aaaaagagat cgtggattac 1620
 gtcgccagtc aagtaacaac cgcgaaaaag ttgcgcggag gagttgtgtt tgtggacgaa 1680
 gtaccgaaaag gtcttaccgg aaaactcgac gcaagaaaaa tcagagagat ctcataaag 1740
 gccagaagg gcggaaagat cgccgtgggt gcagccatga gtggggctgg gccctgggca 1800
 gcctggcctt tctgtcttag cctggcccta atgctgctgt ggctgctcag ctga 1854

<210> 3
 <211> 37
 <212> DNA
 <213> Artificial

<220>
 <223> Primer corresponding to aminoacids 1-9 of luciferase

<400> 3
 ccctgcagat ggaagacgcc aaaaacataa agaaagg

37

<210> 4
 <211> 34
 <212> DNA
 <213> Artificial

<220>

AEQUOTECH.ST25

<223> Primer corresponding to aminoacids 542-549 of luciferase cDNA

<400> 4
gctgcagcca cggcgatctt tccgcccttc ttgg 34

<210> 5
<211> 1854
<212> DNA
<213> Artificial

<220>
<223> pMeLuc nucleotidic sequence in the expression construct

<400> 5
atggctcagc ggatgacaac acagctgctg ctcccttctag tgtgggtggc tgtagtaggg 60
gaggctcaga caaggattgc agaacaaaaa ctaataagcg aggaggacct gctgcagatg 120
gaagacgcca aaaacataaa gaaaggcccc gcgccattct atccgctgga agatggaacc 180
gctggagagc aactgcataa ggctatgaag agatacgccc tggttcctgg aacaattgct 240
tttacagatg cacatatcga ggtggacatc acttacgctg agtacttcga aatgtccgtt 300
cggttggcag aagctatgaa acgatatggg ctgaatacaa atcacagaat cgtcgtatgc 360
agtgaaaact ctcttcaatt ctttatgccg gtgttgggcg cgttatttat cggagttgca 420
gttgcgcccc cgaacgacat ttataatgaa cgtgaattgc tcaacagtat gggcatttcg 480
cagcctaccg tgggtgttcgt ttccaaaaag gggttgcaaa aaattttgaa cgtgcaaaaa 540
aagctcccaa tcatccaaaa aattattatc atggattcta aaacggatta ccagggattt 600
cagtcgatgt acacgttcgt cacatctcat ctacctccg gttttaatga atacgatttt 660
gtgccagagt ctttcgatag ggacaagaca attgcaactga tcatgaactc ctctggatct 720
actggctctgc ctaaagggtgt cgctctgcct catagaactg cctgcgtgag attctcgcag 780
gccagagatc ctatTTTTTg caatcaaadc attccggata ctgagatttt aagtgttggt 840
ccattccatc acggttttgg aatgtttact acactcggat atttgatatg tggatttcga 900
gtcgtcttaa tgtatagatt tgaagaagag ctgtttctga ggagccttca ggattacaag 960
attcaaagtg cgctgctggt gccaaacctt ttctccttct tcgccaaaag cactctgatt 1020
gacaaatacg atttatctaa ttacacgaa attgcttctg gtggcgctcc cctctctaag 1080
gaagtcgggg aagcggttgc caagaggttc catctgccag gtatcaggca aggatatggg 1140
ctcactgaga ctacatcagc tattctgatt acacccgagg gggatgataa accgggagcg 1200
gtcgggtaaag ttgttccatt ttttgaagcg aaggttgtgg atctggatac cgggaaaacg 1260
ctgggcgtta atcaaagagg cgaactgtgt gtgagaggtc ctatgattat gtccggttat 1320
gtaaacaatc cggaagcgac caacgccttg attgacaagg atggatggct acattctgga 1380
gacatagctt actgggacga agacgaacac ttcttcatcg ttgaccgcct gaagtctctg 1440
attaagtaca aaggctatca ggtggctccc gctgaattgg aatccatctt gctccaacac 1500
cccaacatct tcgacgcagg tgcgcagggt cttcccgacg atgacgccgg tgaacttccc 1560
gccgccgttg ttgttttggg gcacggaaag acgatgacgg aaaaagagat cgtggattac 1620

AEQUOTECH.ST25

gtcgccagtc aagtaacaac cgcgaaaaag ttgcgcggag gagttgtgtt tgtggacgaa	1680
gtaccgaaag gtcttaccgg aaaactcgac gcaagaaaaa tcagagagat cctcataaag	1740
gccaagaagg gcggaaagat cgccgtggct gcagccatga gtggggctgg gccctgggca	1800
gcctggcctt tcctgcttag cctggcccta atgctgctgt ggctgctcag ctga	1854

INTERNATIONAL SEARCH REPORT

International application No
PCT/IT2006/000383

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435

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)
C07K

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, BIOSIS, WPI Data, Sequence Search, PAJ, EMBASE, INSPEC, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 246 340 A (LUNDIN ET AL) 20 January 1981 (1981-01-20) abstract; claims 1-16 -----	6-32
P,X	PELLEGATTI PATRIZIA ET AL: "A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion" MOLECULAR BIOLOGY OF THE CELL, vol. 16, no. 8, August 2005 (2005-08), pages 3659-3665, XP002399311 ISSN: 1059-1524 the whole document -----	6-32

☐ Further documents are listed in the continuation of Box C.

☒ See patent family 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

19 September 2006

Date of mailing of the international search report

12/10/2006

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Stachowiak, Olaf

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-5

Present claims 1-5 relate to an extremely large number of possible chimeric proteins. Support and disclosure in the sense of Article 6 and 5 PCT is to be found however for only a very small proportion of the compounds claimed, i.e., for the plasmid pMeLUC encoding a chimeric luciferase fusion protein (sequences disclosed in SEQ ID nos. 1 and 2), see specification, pp. 10/17, examples 1 and 2) comprising a leader and a GPI anchor sequence of the folate receptor, and luciferase (cf. specification, p. 4, last para.).

However, the very broad language used in present claims 1-5 which refers to a 'photoprotein', as well as a 'a full-length or partial sequence' thereof, and in addition recites a 'receptor localized on a plasma membrane site' encompasses an unlimited number of different compounds and mutants thereof. As a consequence, the skilled person is not able to determine the scope of the above-mentioned claims, which contravenes Art 6 PCT. Furthermore, searching these claims over the entire claimed scope is considered impossible due to the extremely large number of chimeric proteins encompassed by the scope of the claims.

The non-compliance with the substantive provisions is to such an extent, that with respect to claims 1-5 no search has been conducted.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IT2006/000383

Box II Observations where certain claims were found unsearchable (Continuation of item 2 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:
2. ☒ Claims Nos.: 1-5
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:
see FURTHER INFORMATION sheet PCT/ISA/210
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 III Observations where unity of invention is lacking (Continuation of item 3 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/IT2006/000383

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4246340	A	20-01-1981	BE 876662 A1 17-09-1979
			CA 1116060 A1 12-01-1982
			DE 2921975 A1 06-12-1979
			FR 2427603 A1 28-12-1979
			GB 2022247 A 12-12-1979
			JP 55013893 A 31-01-1980
			NL 7904308 A 04-12-1979
			SE 428379 B 27-06-1983
			SE 7806296 A 01-12-1979