

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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 January 2010 (14.01.2010)

(10) International Publication Number
WO 2010/003231 A1

PCT

(51) **International Patent Classification:**

C07K 5/11 (2006.01) C12Q 1/02 (2006.01)
A61K 38/01 (2006.01) G01N 33/53 (2006.01)
A61K 38/08 (2006.01) G01N 33/68 (2006.01)
A61P 35/00 (2006.01) CO1K 14/81 (2006.01)
C07K 5/10 (2006.01) C12N 9/64 (2006.01)
CO1K 1/06 (2006.01)

(21) **International Application Number:**

PCT/CA2009/000935

(22) **International Filing Date:**

6 July 2009 (06.07.2009)

(25) **Filing Language:**

English

(26) **Publication Language:**

English

(30) **Priority Data:**

61/079,820 11 July 2008 (11.07.2008) US

(71) **Applicant** (for all designated States except US):
SOCPPRA-SCIENCES ET GÉNIE S.E.C. [CA/CA];
Suite 100, 35, rue Radisson, Sherbrooke, Quebec J1L 1E2
(CA).

(72) **Inventors; and**

(75) **Inventors/Applicants** (for US only): **DAY, Robert** [CA/
CA]; 1615, rue Longchamp, Sherbrooke, Quebec J1H
1H9 (CA). **FUGÈRE, Martin** [US/US]; 4020, Centre
Street, San Diego, California 92103 (US). **NEUGE-
BAUER, Witold A.** [CA/CA]; 208, Provender Avenue,
Ottawa, Ontario K1K 4N7 (CA).

(74) **Agent: OGILVY RENAULT LLP/S.E.N.C.R.L., s.r.l.;**
Suite 2500, 1 Place Ville Marie, Montreal, Quebec H3B
IRI (CA).

(81) **Designated States** (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP,
KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI,
NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT,
TZ, UA, UG, US, UZ, VC, VN, 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, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted
a patent (Rule 4.1 I(H))

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(54) **Title:** MULTI-LEU PEPTIDES AND ANALOGUES THEREOF AS SELECTIVE PACE4 INHIBITORS AND EFFEC-
TIVE ANTIPROLIFERATIVE AGENTS

(57) **Abstract:** Disclosed herein are PACE4 inhibitors, compositions comprising PACE4 inhibitors and their uses thereof for low-
ering PACE4 activity, reducing cell proliferation, reducing tumor growth, reducing metastasis formation, preventing and/or treat-
ing cancer. Also provided are methods for lowering PACE4 activity, reducing the proliferation of a cell, reducing tumor growth
and/or treating and preventing cancer. Methods for screening PACE4 inhibitors and cell proliferation inhibitors are further provid-
ed.



WO 2010/003231 A1

MULTI-LEU PEPTIDES AND ANALOGUES THEREOF AS
SELECTIVE PACE4 INHIBITORS AND EFFECTIVE
ANTIPROLIFERATIVE AGENTS

TECHNICAL FIELD

5 [0001] The present invention relates to PACE4 inhibitors and their uses for limiting the proliferation of a cell.

BACKGROUND OF THE INVENTION

[0002] Cancer cells are characterized by multiple genetic alterations that confer physiological changes, leading to uncontrolled division and ability to
10 invade other tissues. These acquired capabilities, namely self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis are essential for malignant growth. Recent studies have associated the family of enzymes known as the proprotein convertases (PCs) to
15 cancer (Bassi et al., 2005, Mol. Carcinog., 44: 151-161; Khatib et al., 2002, Am. J. Pathol., 160: 1921-1935). PCs are serine proteases that optimally cleave substrates at **R-X-K/R-R** motif. These processing events, resulting in the activation of protein precursors, occur at multiple levels of cell secretory pathways, and even at the cell surface.

20 [0003] In mammalian cells, seven members of this family have been identified: furin, PACE4, PC1/3, PC2, PC4, PC5/6 and PC7, with differential expression in tissues, ranging from ubiquitous (eg. furin) to an endocrine restricted expression (PC1/3 and PC2).

[0004] The association of PCs with cancer was firstly done by comparative
25 studies of normal and cancerous cells showing higher expression of PCs in small cell lung cancer (Clark et al., 1993, Peptides, 14: 1021-1028), non-small cell lung carcinoma (Mbikay et al., 1997, Cancer, 75: 1509-1514), breast (Cheng et al., 1997, Int. J. Cancer, 71: 966-971), colon (Tzimas et al., 2005, BMC Cancer, 5: 149), and head and neck (Bassi et al., Mol. Carcinog., 31: 224-

- 2 -

232) tumors cells. A correlation between expression of some PCs, namely furin and PACE4, and tumor cell aggressiveness has been established for different cell types. It as been demonstrated that the overexpression of PACE4 in non-malignant keratinocyte cell lines renders these cells malignant. Non-selective inhibitors that target several PCs together (such as furin, PACE4 and PC5/6 together) have been described (Bassi et al., 2005, Cancer Res., 65: 7310-7319; Mahloogi et al., 2002, Carcinogenesis, 23: 565-572; Bassi et al., 2000, Mol. Carcinog., 28: 63-69; Hubbard et al., 1997, Cancer Res., 57: 5226-5231).

[0005] Moreover, it has been proposed that PC activity regulates epithelial cell differentiation in a prostate cancer cell line. One possible mechanism underlying these observations could be on the basis of the precursors activation by overexpressed PCs. Thus, it is hypothesized that aberrant processing events provide cancer cells a higher capacity to (i) remodel the extracellular; (H) to interact with their host micro-environment to favor tumor cell adhesion and; (Hi) to modulate their proliferation and differentiation. Alternatively, PC's overexpression is required to sustain these pathophysiological functions to maintain cancer cells immortality

[0006] The situation becomes more complex as the expression/activity of PCs are modulated differently in various cancer cells or cancer models. If one wishes to understand the specific contribution of each PC in tumorigenesis, the necessity for potent, specific and cell effective inhibitors, either pharmacologic or molecular, for each member of this enzyme family is crucial. Until now, these pharmacological tools are limited and lack specificity for single PCs.

[0007] It would be highly desirable to be provided with selective PCs inhibitors. It would also be highly desirable to be provided with selective PCs inhibitors that are effective in treating cancer. More specifically, it would be highly desirable to be provided with selective PCs inhibitors that have antiproliferative effects.

SUMMARY OF THE INVENTION

[0008] In accordance with the present invention there is now provided PACE4 inhibitors and their uses for limiting the proliferation of a cell.

[0009] According to one aspect of the present invention, there is provided a
5 PACE4 inhibitor comprising a peptide sequence consisting of the following formula:



wherein

-Arg₁ is an arginine, arginine derivative, arginine mimetic or a transition
10 state analogue;

-Xaa₂ and Xaa₃ are any amino acids or stereoisomers thereof; and

-Y is absent or comprises the formula Z-Xaa₈-Xaa₇-Xaa₆-Xaa₅, wherein

Xaa₅, Xaa₆, Xaa₇ and Xaa₈ have an hydrophobicity score between
about 4.5 to -0.4 based on a Kyte-Doolittle hydrophobicity plot, or
15 Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are independently selected from the
group consisting of Lys, His and Arg;

Z is absent or comprises an N-terminal acyl group linked to the N-
terminal of the peptide sequence;

with the proviso that Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are not aromatic or negatively
20 charged amino acids.

[0010] Particularly, Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are positively charged amino
acids or stereoisomers thereof. More preferably, Xaa₃ is Val. Preferably, Xaa₂
and Xaa₃ are independently selected from of Gly and Ala. More preferably, Xaa₂
is Lys or Arg or an analogue thereof.

25 [0011] In a particular embodiment, Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are Leu.

[0012] In an embodiment, Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are aliphatic
hydrophobic amino acids, such as Leu, Iso or Val.

- A -

[0013] In another embodiment, Xaa₇ and Xaas are small amino acids.

[0014] In a particular embodiment, the N terminus of the inhibitor is acylated (e.g. acetylated). Further, the N terminus acylation is with fatty omega amino acids or with steroid derivatives.

5 [0015] The fatty omega amino acids can be C2 to C18, preferably C2 to C11, more preferably the fatty omega amino acids are selected from the group consisting of 11-amino undecanoyl, 8-amino octanoyl and the steroid derivatives are cholyl.

[0016] In another embodiment, the inhibitor comprises at least one of the
10 following amino acid sequences: SEQ ID NO: 2, 3, 4, 5, 6 and 7.

[0017] According to another aspect of the present invention, there is provided a composition comprising the PACE4 inhibitor as defined herein and a carrier.

[0018] In another embodiment, the composition further comprises at least
15 one anti-cancer drug.

[0019] Preferably, the composition is adapted for delivery by at least one of the following route selected from the group consisting of oral, mucosal, intranasal, intraocular, intratracheal, intrabronchial, intrapleural, intraperitoneal, intracranial, intramuscular, intravenous, intraarterial, intralymphatic,
20 subcutaneous, intratumoral, gastric, enteral, colonic, rectal, urethral and intravesical route.

[0020] According to still another aspect of the present invention, there is provided a method of lowering PACE4 activity in a cell, comprising contacting the PACE4 inhibitor or the composition as defined herein with the cell, thereby
25 lowering PACE4 activity in the cell.

[0021] According to yet another aspect of the present invention, there is provided a method of reducing the proliferation of a cell in a subject, comprising

- 5 -

administering the PACE4 inhibitor or the composition as defined herein to the subject, thereby reducing the proliferation of the cell in the subject.

[0022] According to a further aspect of the present invention, there is provided a method of reducing tumor growth in a subject, comprising
5 administering the PACE4 inhibitor or the composition as defined herein to the subject, thereby reducing tumor growth in the subject.

[0023] According to yet a further aspect of the present invention, there is provided a method for the prophylaxis or treatment of a cancer in a subject, comprising administering to said subject a therapeutically effective amount of
10 the PACE4 inhibitor or the composition as defined herein, thereby preventing or treating the cancer in the subject.

[0024] Preferably, the cell is in a subject. More preferably, the cell is a cancer cell. More preferably, the cell has increased PACE4 activity.

[0025] According to still a further aspect of the present invention, there is provided the use of the PACE4 inhibitor or the composition as defined herein in
15 the manufacture of a medicament for preventing or treating cancer in a subject.

[0026] According to another aspect of the present invention, there is provided the use of the PACE4 inhibitor or the composition as defined herein for preventing or treating cancer in a subject.

20 [0027] More specifically, the cancer is a prostate cancer or a metastasis thereof.

[0028] According to yet another aspect of the present invention, there is provided the use of the PACE4 inhibitor or the composition as defined herein for lowering PACE4 activity in a cell, for reducing proliferation of a cell in a subject,
25 and for reducing tumor growth in a subject.

[0029] In a particular embodiment, the inhibitor or the composition reduces cell proliferation, tumor growth or metastasis formation.

- 6 -

[0030] In another embodiment, there is provided a method of screening for a PACE4 inhibitor comprising the steps of contacting an agent with a PACE4 protein; assessing the activity of the PACE4 protein, wherein a reduction of the activity of the PACE4 protein compared to the basal activity of the PACE4 protein that has not been in contact with the agent is indicative that the agent is an inhibitor of PACE4.

[0031] According to another aspect of the present invention, there is provided a method of identifying a cell proliferation inhibitor, comprising the steps of contacting an agent with a PACE4 protein in a cell; assessing the activity of the PACE4 protein, wherein a reduction of the activity of the PACE4 protein compared to the basal activity of the PACE4 protein that has been in contact with the agent is indicative that the agent is an inhibitor of PACE4 inhibiting cell proliferation.

[0032] In a further embodiment, the method further comprises the step of comparing the proliferation rate of the cell to a control cell not contacted with the agent.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] Having thus generally described the nature of the invention, reference will now be made to the accompanying drawings, showing by way of illustration, a preferred embodiment thereof, and in which:

[0034] Fig. 1 illustrates the overexpression of PACE4 mRNA in prostate cancer as measured by (A) quantitative PCR and (B) *in situ* hybridization of normal prostate tissue shows PACE4 mRNA localized to epithelial cells lining the ducts, while in (C) tumor tissues PACE4 expression is widespread, disorganized and localized into the stroma. The (*) indicate that values are mean \pm SEM; *P < 0.05.

[0035] Figure 2 illustrates expression of PACE4-SOFA- δ Rz vector transfected into the DU145 cell line, a highly invasive, androgen-independent prostate epithelial tumor cell line, (A) by Northern blot analysis on total RNA

- 7 -

extracts performed from wild-type DU145 cell line (DU145), on DU145 cells transfected with ptRNAVal-PACE4-SOFA- δ Rz (4-2) and, on DU145 cells transfected with ptRNAVal-PACE4-SOFA- δ Rz and co-transfected with PACE4 cDNA expression vector (4-2+PACE4). In (B), a densitometric analysis using 5 18S ribosomal RNA as loading control to quantify the mRNA levels in the cell line illustrated in (A) is shown. The Northern blot analysis of mRNA levels of two others endogenous expressed PCs is shown and confirms the specificity of the PACE4-SOFA- δ Rz cleavage, wherein levels of (C) furin and (D) PC7 mRNAs remained mostly unchanged in the 4-2 cells, confirming the reduction of PACE4 10 expression without significantly affecting the expression of other endogenous PCs. The (*) indicates that values are mean \pm SEM (n=3); *P < 0.05.

[0036] Figure 3 illustrates that PACE4 knockdown slows DU145 proliferation *in vitro* since in (A) the total cell number of the stable cell lines showed a significant reduction of proliferation for the 4-2 cells (200,000 \pm 14,000 cells; 15 white histogram) when compared to untransfected DU145 (375,000 \pm 40,000 cells; black histogram) or 4-2+PACE4 cells (gray histogram), 96 hours after the initial plating. Also shown (B) is an *in vitro* clonogenic assay on the same cell lines to detect the proportion of cells that retained the capacity to grow into a colony confirming the lower proliferation of DU145 with lowered PACE4 20 expression (4-2). The (*) indicates that values are mean \pm SEM (n=9 for DU145 and 4-2+PACE4 and n=7 for 4-2 cells); *P < 0.05.

[0037] Figure 4 illustrates in (A) that PACE4 inhibition prevents tumor growth in xenograft tumor model. Results are shown as mean tumor volume (mm³) as the reduction of PACE4 mRNA levels reduced dramatically the ability of 4-2 25 cells (Δ) to induce tumor growth, while untransfected DU145 cells (π) were able to develop into well-defined tumor masses. Histological analysis in (B) shows the well define tumor mass when DU145 cells (panels A and B) are implanted, which is not seen with the 4-2 cells (panels C and D). Panels B and D of figure 4B represent a 400X magnification of panels A and C respectively.

30 [0038] Figures 5A-G illustrates the effects of adding from 0- to 6 leucine residues (multileucine or ML) to the N-terminal of the **RXKR** consensus

- 8 -

sequence (with X chosen to be a Val) on the inhibition of (A) PACE4, (B) PC5/6, (C) PC7, (D) Furin, (E) PC2, (F) PC1/3 and (G) PC4.

[0039] Figure 6 illustrates the inhibition of the **LLLLRVKR** (SEQ ID NO: 5) peptide on (A) PC1/3 (454 nM), (B) PC2 (18769 nM), (C) Furin (114 nM), (D) PACE4 (5.5 nM), (E) PC5/6 (245 nM), (F) PC7 (54 nM) and (G) PC4 (205 nM) as plotted as an histogram wherein the y axis is a log scale of the inhibitory constants measured in nM.

[0040] Figure 7 illustrates flow cytometry results of the ability of the ML peptide to penetrate into DU145 cells wherein cells were treated with the cholyl-ML peptide linked to FITC demonstrating that there is a clear shift of the cells indicating that the cholyl-ML FITC peptide has penetrated the cells with (A) or without (B) being treated with trypsin to insure that the observed shift was not due to the cholyl-ML FITC peptide unabsorbed on the cell surface.

[0041] Figure 8 illustrates the proliferation index in function of the concentration of acetyl-ML peptide added for various cell types. For comparison, a vehicle treatment (control) was also administered. Small cell carcinoma cell line H345 (white histogram), glioblastoma U251 cell line (black histogram), prostatic cell line DU145 (shaded histogram) and sarcofibroma HT1080 cell line (gray histogram) were all treated with increasing amounts of the acetyl-ML peptide.

[0042] Figure 9 illustrates the proliferation index in function of the concentration of peptide added for DU145 cells. For comparison, a vehicle treatment (control) was also administered. 8-amino octanoyl-ML peptide (black histogram), 11-amino undecanoyl-ML peptide (white histogram) or cholyl-ML peptide (gray histogram) were all administered to DU145 cells. The (*) indicates that values are mean \pm SEM; *P < 0.05.

[0043] Figure 10 illustrates (A) areas covered by colonies as a % of DU145 cell lines treated with a vehicle (control), 10 or 100 μ M of acetyl-ML (black histogram), 8-amino octanoyl-ML (white histogram), 11-amino undecanoyl-ML (gray histogram) and cholyl-ML (shaded histogram). Photographic

- 9 -

representations of a dish showing colonies of DU145 cells (B) treated with the vehicle or (C) 100 μ M of 8-amino octanoyl-ML is illustrated. The (*) indicates that values are mean \pm SEM (n=2 to 4); *P < 0.05.

[0044] Figure 11 illustrates (A) the *in vivo* volume of tumors inhibition by a vehicle (grey) or choly-ML peptide (black) of DU145 cells implanted sc at two sites on the backs of Nu/Nu mice, which lack an immune system. Representative control (1) and treated mice (2) are shown on the (B) panel, while panels (C) and (D) show the histology of the control (C) and treated tumors (D). The (*) indicates that values are mean \pm SEM (n=5).

10 [0045] Figure 12 illustrates flow cytometry results showing the apoptosis induction using annexin-V-FITC/propidium iodide staining wherein dot plots show the presence of extracellular phosphatidylserine and the permeability for propidium iodide (PI) of DU145 (A and B) and 4-2 cells (C and D) untreated (A and C) or incubated (B and D) for 48h with 66 μ M cisplatin, and wherein for 15 each plot, the horizontal lines separate annexin-V positive from negative cells; the vertical lines separate PI-positive and -negative cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0046] The present application provides selective PACE4 inhibitors which have antiproliferative effects.

20 [0047] The relationship between the expression/activity of PCs and cancer has become stronger within the last few years. Since cancer cell lines generally express varying cocktail of PCs, it always remains unclear whether one PC is more important or whether the cell simply establish multiple PC overexpression to assure redundancy. It is disclosed herein that specific inhibition of a PC (e.g. 25 PACE4 expression) in cancer (e.g. such as the cell line DU145) causes a reduction in cell proliferation and clonogenic capacity both *in vitro* and *in vivo* (e.g. as shown in Figs. 3 and 4).

[0048] Therefore, the unavailability of potent and specific PC inhibitors represents a problem for the determination of the specific functions of

- 10 -

overexpressed PCs in cancer cells. While the hypothesis of PCs importance in cancer has much credibility, studies with specific PC inhibitors are crucial, since each cancer cells overexpress multiple PCs. This variable PC expression pattern suggests that each PC can contributes differently to the apparition and the maintenance of given cancers and their specific functions have to be defined within each cancer cell.

[0049] Overexpression of PACE4 in different clinical stages prostate cancer tissues (Fig. 1) is disclosed herein. This result demonstrate the PACE4 specific contribution to prostate cancer, since other co-expressed PC (including furin and PC7) did not show significant variation in their expression levels.

[0050] To test the impact of PACE4 in overall tumor progression, the well-established model cell line, the DU145 epithelial-like cell line, derived from a human metastatic carcinoma of the prostate was used. These androgen non-responsive cells are tumorigenic in nude mice forming adenocarcinoma (grade II) consistent with prostatic primary tumors.

[0051] Targeted inhibition studies in tumoral cell lines with endogenously high expression levels of PCs are useful to understand the specific contribution of these enzymes into the generation of cancer related proteins, although functional redundancy might be observed for some substrates.

[0052] A stable DU145 cell line in which the expression of PACE4 would be silenced or significantly reduced was also established. A delta ribozyme (δ Rz) technology was used to accomplish this, the new δ Rz generation harboring a biosensor module that activates the molecule only in the presence of the appropriate RNA target substrat. A specific on/off adapter (SOFA module) gives a higher specificity of the δ Rz toward its target, but also a higher cleavage capacity.

[0053] The expression vector used in this study produced a chimeric RNA transcript constituted of a tRNA^{Val} motif and the PACE4-SOFA- δ Rz. This molecule had the same cleavage capacity than the PACE4-SOFA- δ Rz itself by

performing as observed in an *in vitro* cleavage assay performed transfecting DU145 cells.

[0054] After hygromycin selection, a very low number of stable cells were analyzed, since transfected DU145 cells grew very slowly. This is the consequence of lowered PACE4 level, thus arguing for the important role of this PC for DU145 cells proliferation. This link between PACE4 and cell proliferation could explain why no clones with a lower expression levels was obtained.

[0055] Considering the high specificity potential of PACE4-SOFA- δ Rz, the cell line with the lowest levels of PACE4 mRNA levels was chosen for further studies (see Fig. 2A). Northern blots performed for two other endogenous expressed PCs showed that this effect is specific to PACE4 (Figs. 2C and 2D). The stable cell line was transfected with the SOFA- δ Rz expression vector and named 4-2, while the 4-2 cell line was stably transfected with the PACE4 expression vector and named 4-2+PACE4.

[0056] The consequences of lowered levels of PACE4 were well illustrated by the reduced cell proliferation rate and the incapacity of these cells to form subcutaneous tumors in nude mice (Figs. 3 and 4). The restoration of PACE4 expression levels in this cell line allowed a partial recovery of the *in vitro* proliferation rate, demonstrating that PACE4 is a key player for tumoral growth and its levels have to be high to achieve this function.

[0057] The cell lines characterized in this study constitute important tools for the identification of cellular proteins processed by PACE4. The results obtained with conditioned media showed that PACE4 is important for the generation of secreted proliferation factors; but also showed that these cells had a lower capacity to react when exposed to conditioned media issued from untransfected cells.

[0058] One of the keys to the development of potent and selective PC inhibitors is an understanding of the substrate-binding pocket. The deepest region of the substrate-binding pocket accommodates the consensus motif **RXKR** (i.e. P₄-P₃-P₂-Pi) and is nearly identically in all PCs. Potency and

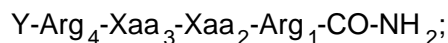
- 12 -

selectivity are determined by a less deeper region that interacts with P₈-P₇-P₆-P₅ of the inhibitor peptide (see Henrich et al., 2005, J. Mol. Biol., 345: 211-227; Fugere and Day, 2005, Trends Pharmacol. ScL, 26: 294-301 ; Henrich et al., 2003, Nat. Struct. Biol., 10: 520-526).

5 **[0059]** Endogenous inhibitors are often a good starting point in the development of pharmacological compounds. For example, proSAAS and the 7B2 C-terminal peptide are two endogenous inhibitors identified that inhibit PC1/3 and PC2, respectively. PC pro-domains are autoprocessed *in cis* by their cognate PC, but remain bound to the active site through their C-terminal PC-
10 recognition sequence until the complex reaches the compartment of zymogen activation. Thus, pro-domains are dual-function molecules, being the first substrate and first inhibitor encountered by PCs in cells.

[0060] The deepest region of the substrate-binding pocket accommodates the consensus motif **RXKR** (P₄-P₃-P₂-P₁) nearly identical in all PCs. Using an
15 incremental peptide assay (IPA), the core warhead sequence, **RVKR** (SEQ ID NO: 1), was extended one amino acid at a time.

[0061] In a first aspect, it is provided a PACE4 inhibitor comprising a peptide sequence consisting of the following formula:



20 wherein

-Arg_i is an arginine, arginine derivative, arginine mimetic or a transition state analogue;

-Xaa₂ and Xaa₃ are any amino acids or stereoisomers thereof; and

-Y is absent or comprises the formula Z-Xaa₈-Xaa₇-Xaa₆-Xaa₅, wherein

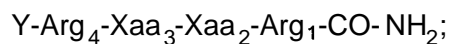
25 Xaa₅, Xaa₆, Xaa₇ and Xaa₈ have an hydrophobicity score between about 4.5 to -0.4 based on a Kyte-Doolittle hydrophobicity plot, or

Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are Lys, His or Arg;

Z is absent or comprises an N-terminal acyl group linked to the N-terminal of the peptide sequence;

with the proviso that Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are not aromatic or negatively charged amino acids.

- 5 [0062] The PACE4 inhibitor described herein can comprise a peptide sequence having amino acids that can be any non-natural amino acids, such as for example 2-aminoadipic acid, 3-aminoadipic acid, alanine, 3-aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, piperidinic acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2,4-diaminobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, 6-N-methyllysine, N-methylvaline, norvaline, norleucine or ornithine.
- 10
- 15 [0063] In another aspect, it is provided a PACE4 inhibitor consisting of a peptide sequence consisting of the following formula:



wherein

- Arg_i is an arginine, arginine derivative, arginine mimetic or a transition state analogue;
- 20 -Xaa₂ and Xaa₃ are any amino acids or stereoisomers thereof; and
- Y is absent or comprises the formula Z-Xaa₈-Xaa₇-Xaa₆-Xaa₅, wherein
- Xaa₅, Xaa₆, Xaa₇ and Xaa₈ being positively charged amino acids or stereoisomers thereof;
- 25 Xaa₅, Xaa₆, Xaa₇ and Xaa₈ have an hydrophobicity score between about 4.5 to -0.4 based on a Kyte-Doolittle hydrophobicity plot, or Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are Lys, His or Arg;

- 14 -

Z is absent or comprises an N-terminal acyl group linked to the N-terminal of the peptide sequence;

with the proviso that Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are not aromatic or negatively charged amino acids.

- 5 **[0064]** In another aspect, it is provided a PACE4 inhibitor consists essentially of a peptide sequence consisting of the following formula:



wherein

- 10 -Arg_i is an arginine, arginine derivative, arginine mimetic or a transition state analogue;

-Xaa₂ and Xaa₃ are any amino acids or stereoisomers thereof; and

-Y is absent or comprises the formula Z-Xaa_β-Xaa₇-Xaa₆-Xaa₅, wherein

Xaa₅, Xaa₆, Xaa₇ and Xaa₈ have an hydrophobicity score between about 4.5 to -0.4 based on a Kyte-Doolittle hydrophobicity plot, or

- 15 Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are Lys, His or Arg;

Z is absent or comprises an N-terminal acyl group linked to the N-terminal of the peptide sequence;

with the proviso that Xaa₅, Xaa₆, Xaa₇ and Xaa_β are not aromatic or negatively charged amino acids.

- 20 **[0065]** A Kyte-Doolittle hydrophobicity plot allows for the visualization of hydrophobicity over the length of a peptide sequence. A hydrophobicity scale which is based on the hydrophobic and hydrophilic properties of the 20 amino acids is used. Hydrophobicity (or hydrophilicity) plots are designed to display the distribution of polar and apolar residues along a protein sequence (Kyte and
25 Doolittle, 1982, J. Mol. Biol., 157: 105).

[0066] Xaa₅, Xaa₆, Xaa₇ and Xaa₈ can be positively charged amino acids or stereoisomers thereof. Xaa₅, Xaa₆, Xaa₇ and Xaa₈ can be Leu, He, Val or their analogues.

- 15 -

[0067] Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are thus not an aromatic amino acid which comprises a side chain which contains an aromatic ring system. Such amino acids are for example Phe, Trp, Tyr and His.

[0068] Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are thus not a negatively charged amino acids such as Glu and Asp.

[0069] In another embodiment, it is disclosed a composition comprising a PACE4 inhibitor as defined herein and a carrier.

[0070] In accordance with the present invention, a carrier or "pharmaceutical carrier" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more active compounds to an animal, and is typically liquid or solid. A pharmaceutical carrier is generally selected to provide for the desired bulk, consistency, etc., when combined with components of a given pharmaceutical composition, in view of the intended administration mode. Typical pharmaceutical carriers include, but are not limited to binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycotate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

[0071] A series of PC peptide inhibitors with varying degrees of selectivity and potency were tested for various PCs (see Figs. 5 and 6). One compound stand out: LLLL RVKR-N H₂ (four leucine or multi-leu peptide; SEQ ID NO: 5) was the most potent inhibitor of PACE4 (K_i of 6 nM) evaluated in this study and was significantly more effective on PACE4 than the other PCs (9-folds and more; see Fig. 5A as encircled and Fig. 6). Thus, LLLL RVKR-N H₂ (SEQ ID NO: 5) is a selective inhibitor of PACE4 ($K_i = 5$ nM), with next best inhibition against

- 16 -

PC7 ($K_1 = 50$ nM). An inhibitor having an affinity or selectivity in the nM range represents an indication of the potential efficacy of the inhibitor *in vivo*.

[0072] According to another aspect, it is disclosed a method of screening for a PACE4 inhibitor comprising the steps of contacting an agent with a PACE4
5 protein. Alternatively, a fragment of PACE4, wherein for example the Cys rich region has been removed, and has an activity similar to the wild-type PACE4 can also be used in the screening method (see ref Mains et al., 1997, *Biochem J.*, 321 : 587-593).

[0073] The agent can be firstly identified by techniques commonly used in
10 the art. As an example, but not restricted to, positional scanning synthetic peptide combinatorial libraries (PS-SPCL) and the incremental peptide assay (IPA) techniques can be used. Assessing the activity of the PACE4 protein can be accomplished by techniques known in the art.

[0074] Those skilled in the art can easily determine PACE4 activity using
15 routine experimentation. For example, the activity assay of PACE4 can be carried out in 96 well plates, and includes the use of a fluorogenic substrate, namely PyrRTKR-AMC (AMC is amino-methyl-coumarin). The substrate and the purified enzyme are placed in the wells, and depending on the units of enzyme present, the AMC moiety will be cleaved at a certain rate, such a pmoles/sec.
20 The resultant free AMC is now fluorescent and can be detected with a spectrofluorometer. The addition of inhibitors to the assay will yield progress curves that have lesser slopes. Based on these changes the inhibitory constants (K_i) is calculated (Fugere et al., 2002, *J. Biol. Chem.*, 277:7648-56).

[0075] Reduction of the activity of the PACE4 protein contacted by the agent
25 compared to the basal activity of the PACE4 protein without the agent is indicative that the agent is an inhibitor of PACE4. Basal enzyme activity in a cell is generally defined by the amount of protein or RNA present in a cell, assuming that more enzyme, protein or mRNA means more enzyme activity. Thus, for example but not restricted to, the basal activity of PACE4 can be evaluated by

RNA measurements, such as quantitative PCR or Northern blot analysis, or by protein measurements such as Western blots.

[0076] In alternate embodiment, it is described a method of identifying a cell proliferation inhibitor, comprising the steps of contacting an agent with a PACE4
5 protein in the cell and assessing the activity of the PACE4 protein, wherein reduction of the activity of the PACE4 protein contacted by the agent compared to the basal activity of the PACE4 protein without the agent is indicative that the agent is an inhibitor of PACE4 inhibiting cell proliferation. The proliferation rate of the cell can be compared to a control cell not contacted with the agent.

10 [0077] Further optimization of these inhibitors is described herein in cell-based assays or *in vivo*. N-terminal acylation and C-terminal amidation are valuable modifications to protect against amino- and carboxy-peptidases, respectively.

[0078] Other encompassed structural modifications are those enhancing cell
15 permeability, since PACE4 is an intracellular target. In an embodiment, N-terminus acylation can be with fatty omega amino acids or with steroid derivatives. In another embodiment, the fatty omega acids can be selected from the group consisting of 11-amino undecanoyl and 8-amino octanoyl, but not restricted to. The steroid derivatives can be, for example, cholyl.

20 [0079] Other known modifications are, but not restricted to: acyls other than acetyl group, alkyl groups including octyl and undecanyl, alkenes and poly alkenes saccharides (such as sugars, oligo and polysugars, as well as aminosugars, glucosamine and N-acetyl glucosamine), isoprenoids (e.g. farnesyl and geranyl), fatty amino acids, polyethylene glycols (PEGs), TAT peptide or
25 peptide-like sequences for cell mediated delivery.

[0080] Modifications to examine the cell penetration of inhibitors were carried out by adding of a fluorescent marker (such as FITC) to the N-terminus of the peptides (Fig. 7). These modifications can be tested in cell culture assays combined with flow cytometry analysis, to examine cell penetration. Cells were
30 treated with the cholyl-ML peptide linked to FITC (see Table 2). Following

- 18 -

treatment, there is a clear shift of the cells indicating that the choly-ML FITC peptide has penetrated the cells (Fig. 7A). As a further control, cells were treated with trypsin (Fig. 7B) to insure that the observed shift was not due to the choly-ML FITC peptide absorbed on the cell surface. It is demonstrated herein that substantial cell penetration of the peptide is most likely due to its very hydrophobic multi-leucine structure. In an alternate embodiment, cell penetration can be increased by the addition of fatty moieties to the peptidic sequences, such as cholesterol derivatives (cholic acid) or fatty amino acids (6-amino-caproic acid, 8-amino caprylic acid, 11-amino-dodecanoic acid).

10 **[0081]** The effects of the PACE4 inhibitors on cell proliferation were evaluated. MTT assay was used to evaluate the effects of PACE4 inhibitors on cell proliferation. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a standard colorimetric assay for measuring cellular proliferation. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active. Conversion is directly related to the number of viable cells. The MTT assay is quantitative and more sensitive than viability using trypan blue and can also be adapted to 96 well formats, whereas trypan blue tests must be read individually. Because the MTT assay requires less cell manipulation than [³H]thymidine incorporation assays (no cell harvesting or medium changes are necessary), the possibility of error is reduced and the standard deviation values are lower. Comparisons between [³H]thymidine incorporation and MTT assays have demonstrated less than 5% difference for determination of growth factor response. Other assays also known can be used to determine the effects of an inhibitor on the proliferation of a cell.

25 **[0082]** Various cell lines were tested, namely HT1060 (human fibrosarcoma), H345 (human SCLC-small cell lung carcinoma), U251 (human glioma) and DU145 cell lines (human prostatic cancer). PACE4 mRNA was expressed in each cell line. In all cases, both ML and acetyl-ML peptides had significant effects on the cell proliferation index (Fig. 8). Inhibitors can be used in any cell line expressing PACE4.

[0083] ML and acetyl-ML peptides with lipid or sterol N-terminal peptides were also compared with the prostatic cell line DU145. 8-amino octanoyl-ML ($\text{H}_2\text{N-CH}_2\text{-(CH}_2\text{)}_6\text{-CO-NH-LLLLLRVKR-CONH}_2$; or C8: $\text{CH}_3\text{-(CH}_2\text{)}_6\text{-CO-NH-LLLLLRVKR-CONH}_2$), 11-amino undecanoyl-ML ($\text{H}_2\text{N-CH}_2\text{-(CH}_2\text{)}_9\text{-CO-NH-LLLLLRVKR-CONH}_2$; or C11: $\text{CH}_3\text{-(CH}_2\text{)}_9\text{-CO-NH-LLLLLRVKR-CONH}_2$) or choyl-ML (choyl-NH-LLLLLRVKR-NH₂) peptides all had more potent effects than ML or acetyl-ML peptides, most likely due to their additional ability to penetrate the cell membranes (Fig. 9).

[0084] Accordingly, it is disclosed herein a method of lowering PACE4 activity in a cell, comprising contacting a PACE4 inhibitor as defined herein or with the cell, thereby lowering PACE4 activity in the cell. Preferably, the activity of PACE4 needs to be lowered by less than 50%, more preferably less than 40%, less than 30%, or less than 25%. Alternatively, the activity of PACE4 is lowered sufficiently to inhibit the activity of growth factors.

[0085] In another embodiment a method of reducing proliferation of a cell in a subject, comprising administering a PACE4 inhibitor to the subject is also encompassed.

[0086] A clonogenic assay was used to study the effectiveness of the inhibitors described herein on the colony forming potential of DU145 cells. The clonogenic assay or colony formation assay is a survival assay based on the ability of a single cell to grow into a colony. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. All ML peptides tested had important effects on the ability of DU145 cell lines to form colonies. The most potent effects were observed with lipid or sterol ML peptides (or octanoyl-ML, Fig. 10A). Other techniques to study the effectiveness of the inhibitors described herein include, but are not limited to, annexin assay, soft agar assay, Boyden chambers or crystal violet assay. Assays that measure the levels of caspase can also be useful to evaluate apoptosis.

[0087] The present invention further concerns the use of RNA interference (RNAi) to modulate PACE4 expression in target cells. "RNA interference" refers

- 20 -

to the process of sequence specific suppression of gene expression mediated by small interfering RNA (siRNA) without generalized suppression of protein synthesis. While the invention is not limited to a particular mode of action, RNAi may involve degradation of messenger RNA (e.g., PACE4 mRNA) by an RNA
5 induced silencing complex (RISC), preventing translation of the transcribed targeted mRNA. Alternatively, it may involve methylation of genomic DNA, which shuts down transcription of a targeted gene. The suppression of gene expression caused by RNAi may be transient or it may be more stable, even permanent.

10 [0088] "Small interfering RNA" of the present invention refers to any nucleic acid molecule capable of mediating RNA interference "RNAi" or gene silencing. For example, siRNA of the present invention are double stranded RNA molecules from about ten to about 30 nucleotides long that are named for their ability to specifically interfere with protein expression. In one embodiment,
15 siRNA of the present invention are 12-28 nucleotides long, more preferably 15-25 nucleotides long, even more preferably 19-23 nucleotides long and most preferably 21-23 nucleotides long. Therefore preferred siRNA of the present invention are 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 nucleotides in length. As used herein, siRNA molecules need not to be limited to
20 those molecules containing only RNA, but further encompass chemically modified nucleotides and non-nucleotides.

[0089] siRNA of the present invention are designed to decrease PACE4 expression in a target cell by RNA interference. siRNA of the present invention comprise a sense region and an antisense region wherein the antisense region
25 comprises a sequence complementary to a PACE4 mRNA sequence and the sense region comprises a sequence complementary to the antisense sequence of PACE4 mRNA. A siRNA molecule can be assembled from two nucleic acid fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of siRNA molecule. The sense region
30 and antisense region can also be covalently connected via a linker molecule. The linker molecule can be a polynucleotide linker or a non-polynucleotide linker.

[0090] The binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed (e.g., RNAi activity). For example, the degree of complementarity between the sense and antisense region (or strand) of the siRNA construct can be the same or can be different from the degree of complementarity between the antisense region of the siRNA and the target RNA sequence (e.g., PACE4 RNA sequence). Complementarity to the target sequence of less than 100% in the antisense strand of the siRNA duplex (including deletions, insertions and point mutations) is tolerated when these differences are located between the 5'-end and the middle of the antisense siRNA. Determination of binding free energies for nucleic acid molecules is well known in the art. Examples of functional siRNA against PACE4 are disclosed in Table 1.

Table 1
siRNA probes against PACE4

siRNA name	Sequence
TRCN0000075243	CCGGGAGAGAAGTCTCCTCTGCATTCTCGAGAATGCAGAGGAGACTTCTCTTTTTG (SEQ ID NO 25) Clone ID NM_017573 2-1238s1c1 Accession Number(s) NM_017573 3 Region 3UTR
TRCN0000075244	CCGGCCTAGAGAACAAGGGCTACTACTCGAGTAGTAGCCCTTGTTCTAGGTTTTTG (SEQ ID NO 26) Clone ID NM_017573 2-469s1c1 Accession Number(s) NM_017573 3 Region CDS
TRCN0000075245	CCGGAGGCTACAACAACACTGGGTCTTCTCGAGAAGACCCAGTTGTTGTAGCCTTTTTG (SEQ ID NO 27) Clone ID NM_017573 2-397s1c1 Accession Number(s) NM_017573 3 Region CDS
TRCN0000075246	CCGGCCTCCCACTATACGCCTGGCTCTCGAGAGCCAGGCGTATAGTGGGAGGTTTTG (SEQ ID NO 28) Clone ID NM_017573 2-994s1c1 Accession Number(s) NM_017573 3 Region CDS
TRCN0000075247	CCGGCCCTTGGACGTCAGCACTGAACTCGAGTTCAGTGCTGACGTCCAAGGTTTTTG (SEQ ID NO 29) Clone ID NM_017573 2-377s1c1 Accession Number(s) NM_017573 3 Region CDS

[0091] To test the effects of PACE4 inhibitors *in vivo*, a nude mouse model was used in order to validate PACE4's role in tumor progression within an integrated system. A nude mouse is a genetic mutant that lacks a thymus gland,

- 22 -

resulting in an inhibited immune system due to a greatly reduced number of T cells. The genetic basis of the nude mouse mutation is a disruption of the *Foxni* gene. The nude mouse can receive many different types of tissue and tumor grafts, as it mounts no rejection response. These xenografts are commonly
5 used to test new methods of treating tumors. Nude mice were used to test the tumor progression of control DU145 cells compared to PACE4 silenced DU145 cells (clone 4-2) (Fig. 11). Control tumor received vehicle (DMSO) injections. Control tumor continued their growth pattern, reaching an average size of 160 mm³, while treated tumors only reached a size of 75 mm³ (Fig. 11A).
10 Consequently, PACE4 inhibition by the specific inhibitors described herein reduces tumors growth. The nude mouse model is well known and extensively tested (Naomoto et al., 1987, J. Cancer Res. Clin. Oncol., 113: 544-549; Taetle et al., 1987 Cancer Treat. Rep. 71: 297-304).

[0092] Accordingly to another embodiment, it is disclosed a method of
15 reducing tumor growth in a subject, comprising administering a PACE4 inhibitor as described herein to a subject. In a further embodiment, it is disclosed a method for the prophylaxis or treatment of a cancer in a subject, comprising administering to a subject in need of such treatment a therapeutically effective amount of a PACE4 inhibitor as defined herein. Preferably, the tumors are
20 completely blocked from growing *in vivo*. More preferably, tumors are completely blocked from growing by 75%, more preferably 66%, alternatively by 50%.

[0093] The method described herein can be used to treat prostate cancer. In addition, other model cell lines have also been reduced in their proliferative
25 index when treated with ML peptides. For example, SCLC cell line H345 (a small cell lung carcinoma), HT1080 cells (a fibrosarcoma), or in U251 (a glioblastoma) have also been tested. The ML peptides reduced their proliferation.

[0094] Tests were also conducted in order to determine if reductions in cell
30 proliferation was due to cell death occurring by apoptosis. The annexin V assay. is based on the observation that soon after initiating apoptosis, cells translocate

- 23 -

the membrane phosphatidylserine from the inner face of the plasma membrane to the cell surface. Once on the cell surface, phosphatidylserine can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for phosphatidylserine. Detection is analyzed by flow
5 cytometry. On DU145 cells at various concentrations (1-100 μ M), no changes on live, early apoptotic or late apoptotic/necrotic cells populations was seen (Fig. 12). This data re-enforces the notion that PACE4 inhibitors have effects through reductions of proliferation pathways and not through effects on apoptotic pathways. Other methods that can be used to measure apoptosis includes, but
10 not limited to, the annexin assay, measurement of caspases, DNA fragmentation assays, TUNEL assay or detection of apoptosis related molecules such as FAS or p53.

[0095] The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to
15 limit its scope.

EXAMPLE 1

PACE4 expression in clinically localized prostate tumors

[0096] Forty-seven primary prostate tumors samples obtained from patients
20 undergoing surgery were tested for PACE4 expression. Prostate tumor samples were obtained from patients either at St-Louis and Bichat Hospital (Paris, France), or Tournan's clinic (Tournan en Brie, France). Samples tissues from the thirty-four patients with clinically localized prostate tumors were obtained by removing clinically localized tumors by radical prostatectomy. The surgical
25 specimens were first sliced thickly, and samples were then cut from suspect areas. Part of the selected tissue was immediately placed in liquid nitrogen for RNA extraction, while adjacent sections were stained with H/E (hematoxylin and eosin) for histopathological examination. The sample tissues from hormone-refractory recurrent prostate carcinoma were obtained from patients with
30 metastatic disease at diagnosis. Since these patients were not amenable to radical surgery, they received endocrine therapy, either by classical androgen deprivation (orchidectomy or luteinizing-hormone-releasing hormone (LHRH)

agonist administration); or, by maximal androgen blockade (castration combined with antiandrogen therapy). These patients relapsed, and their tumors became clinically androgen-independent.

[0097] Only tissues where all epithelial cells were neoplastic were dissected and used. Suspect areas were examined histopathologically in the surgery suite, and a thick shave section was taken for research purposes. This pre-selected tumor specimen section was then sliced on each side in the laboratory and again subjected to pathological examination. Samples were considered suitable for molecular studies when all epithelial cells were neoplastic. Confirmed malignant areas were carefully dissected using a scalpel. This process yields a homogeneous cell population and thereby avoids dilution of tumor-specific genetic changes by nucleic acids from normal and reactive cells present in the same specimen. The tissues were grouped into similar clinical stages based on TNM system as: eighteen pT2 samples (tumors strictly confined to the organ), sixteen pT3 samples (tumors with extracapsular extension), and thirteen hormone-refractory samples (tumors no longer responsive to endocrine therapy).

[0098] Nine well-characterized matched normal prostate specimens from the thirty-four patients with clinically localized prostate who underwent radical prostatectomy were used to assess basal target-gene mRNA expression. Normal-looking areas of each surgical specimen were examined histologically for the absence of cancer cells and selected upon its microscopic pathological criteria to avoid including areas with benign hyperplasia.

[0099] A real-time PCR strategy was used to evaluate PACE4 mRNA expression levels in prostate tumor tissues using the nine matched normal prostate tissues as a reference (Fig. 1A). Total RNA was extracted from tissue specimens by using the acid-phenol guanidium method. The quality of RNA samples was determined by electrophoresis through agarose gels, staining with ethidium bromide, and visualization of the 18S and 28S RNA bands under ultraviolet light. RNA was reverse-transcribed.

- 25 -

[00100] All PCR reactions were performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and the SYBR[®] Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). Briefly, the thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 5 45 cycles at 95°C for 15 s and 65°C for 1 min. A genomic DNA and non-template control was included in each experiment. Samples and controls were tested in duplicate. Primers were chosen with the assistance of the computer programs Oligo 4.0 (National Biosciences, Plymouth, MN) and Primer Express (Perkin-Elmer Applied Biosystems). Primer sequences for endogenous control 10 genes *PPIA* (the peptidyl prolyl isomerase A gene encoding cyclophilin A) were described earlier (Chene et al., 2004, Int. J. Cancer, 111: 798-804). The PACE4 primer sequences are: sense, 5'-CAAGAGACCCAGGAGCATCCC-S' (SEQ ID NO: 8) and, antisense, 5'-ACCCGCTGGTCCGAGTGCT-3' (SEQ ID NO: 9). The threshold cycle (Ct) numbers obtained from PCR amplification were 15 expressed as N-fold differences in target gene expression relative to *PPIA* expression and termed "*Ntarget*" values.

[00101] The mean relative PACE4 mRNA expression levels (Fig. 1A) were significantly higher in both pT2 and pT3 groups (3.894 ± 0.4933 and 4.211 ± 0.5403 , respectively), when compared to the mean level found in normal 20 prostate tissues (2.243 ± 0.2613). However, the mean PACE4 expression level measured in hormone refractory tissues (2.79 ± 0.4359) was not significantly higher than the one measured in controls. Real-time PCR for the other PCs showed that furin, PACE4 and PC7 were the most expressed PCs in normal prostate tissues. However, only PACE4 mRNA levels increased in tumor 25 tissues, while the others showed little variation.

[00102] This higher PACE4 expression, particularly in epithelial cells, was directly assessed by an *in situ* hybridization using digoxigenin-labeled cRNA probes. Normal prostate tissues showed the expected epithelial cell distribution of PACE4 mRNA. However, tumor tissue showed a disorganization level of 30 tissue structure, with a higher PACE4 expression and even cells invading the stroma (Fig. 1C) compared to normal prostate tissue (Fig. 1B). The *in situ* was

- 26 -

done using a cRNA probe labelled with digoxigenin, as previously described (Dong et al., 1997, J. Neurosci. 17: 563-575).

EXAMPLE 2

Down regulation of PACE4 mRNA in DU145 cells by specific SOFA- δ Rz

5

[00103] An expression vector containing the tRNA^{Val} promoter to express the PACE4-SOFA- δ Rz into transfected cells was used. This promoter allows the transcription of a chimeric catalytic RNA containing a tRNA^{Val} motif, which drives the newly synthesized molecule into the cytoplasm of the cells, and the PACE4-SOFA- δ Rz, that catalyzes the cleavage of the targeted mRNA.

[00104] The expression vector ptRNA^{Val}/hygromycin, containing the RNA polymerase III promoter tRNA^{Val} promoter for cellular applications was used (see D'Anjou et al., 2004, J. Biol. Chem., 279: 14232-14239). A PCR strategy was used to create a DNA template containing a 5'-KpnI restriction site and a 3'-blunt end. The sequences of the two complementary and overlapping DNA oligodeoxynucleotides (ODNs) used were: sense, 5'-ATCCATCG GGTACC GGGCCAGCTAGTTT(GGCCTCTGCTAC) _BS (CA-AC) _{BL} CAGGGTCCACC-3' (SEQ ID NO: 10) and, antisense, 5'-CCAGCTAGAAAGGGTCCCTT-AGCCATCCGCGAACGGATGCCCA(ATCAAC) _{P1} ACCGCGAGGAGGTGGACCCTG(GTTG) _BL-3 (SEQ ID NO: 11). The underlined nucleotides (nt) correspond to the KpnI restriction site, and those in parenthesis to the PACE4 specific biosensor (BS), blocker (BL) and P1 stem (P1) of the PACE4-SOFA- δ Rz. The purified and KpnI-digested PCR product was cloned in the expression vector previously digested with KpnI and EcoRV restriction enzyme. The vector used to restore PACE4 mRNA levels contained the full length PACE4 cDNA and a neomycin resistance gene.

[00105] Radiolabeled PACE4 RNA was obtained from transcription of a XhoI-digested pcDNA3 vector containing a chimeric cDNA composed of the PC5/6A signal peptide linked to proPACE4 coding sequence using T7 RNA polymerase with 50 μ Ci of [α -³²P]GTP. The catalytic RNAs were synthesized using a PCR-based strategy with the expression vectors to generate DNA templates

30

- 27 -

containing a 5'-T7 RNA polymerase promoter. The sense primer 5'-TTAATACGACTCACTATA CAAAACCAACTTTGGTACC-S' (SEQ ID NO: 12) or 5'-TTAATACGACTCACTATA GGGCCAGCTAGTTT-S' (SEQ ID NO: 13), complementary to either the tRNA^{Val} promoter or the PACE4-SOFA- δ Rz, were
5 use. The underlined nucleotides correspond to the T7 RNA polymerase promoter sequence. The antisense ODN sequence used for both PCR was 5'-CCAGCTAGAAAGGGTCCCTTA-3' (SEQ ID NO: 14). After PCR, the purified products were used as templates for T7 RNA polymerase transcription of tRNA^{Val}-PACE4-SOFA- δ Rz or PACE4-SOFA- δ Rz. All products were purified
10 on either denaturing 5% or 7.5% PAGE, for PACE4 or PACE4-SOFA- δ Rz transcripts, respectively.

[00106] One of the major advantages of δ Rz technology is the reduced number of "off-target effects" which sometimes hinders the interpretation of data obtained with siRNA technology. However, even a simple δ Rz (see D'Anjou et
15 al., 2004, J. Biol. Chem., 279: 14232-14239) can result in a certain number of predicted "off-target" effects due to the limited recognition sequence (i.e., 7 nucleotides). Thus, a second-generation δ Rz was designed with a "specific on/off adapter" (SOFA adapter). This new design allows a stronger effect on in vitro cleavage assays and a higher specificity for the targeted sequence, with no
20 "off targets" effects. Without wishing to be bound to theory, the SOFA δ Rz used herein was designed against human PACE4 mRNA, which was used in DU145 cells, and provides an important "proof of concept" for the role of PACE4 in tumor progression.

[00107] Before transfecting the vector, a cleavage assay was performed. The
25 SOFA- δ Rz cleavage assays under single turnover conditions ([SOFA- δ Rz] > [PACE4 RNA]) were done at 37 °C for 3 hours in a 10 μ l reaction containing trace amount of radiolabeled PACE4 RNA and 1 μ M of SOFA- δ Rz in reaction buffer containing 50 mM Tris-HCl, pH 7.5, and 10 mM MgCl₂. The reactions were stopped by the addition of loading buffer (97% formamide, 1 mM EDTA
30 (pH 8.0), 0.025% xylene cyanol and 0.025% bromophenol blue), electrophoresed on denaturing 5% PAGE gel, and analysed with a

- 28 -

PhosphorImager™ (Amersham Biosciences). This molecule had the same cleavage capacity than the PACE4-SOFA- δ Rz itself by performing an *in vitro* cleavage assay before transfecting DU145 cells.

[00108] PACE4-SOFA- δ Rz expression vector was transfected into DU145, a highly invasive, androgen-independent prostate epithelial tumor cell line. Human cancer prostate cell lines DU145 were obtained from ATCC. Cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 5% fetal bovine serum (Wisent Bioproducts). Cells were grown at 37 C in a water-saturated atmosphere in air/CU₂ (5%). Cells were transfected using lipofectamine2000™ (Invitrogen), and were selected for resistance to hygromycin B (Invitrogen) at 125 μ g/ml, with 200 μ g/ml of neomycin for double-transfected cells. The stable cell line transfected with the SOFA- δ Rz expression vector was named 4-2, while the 4-2 cell line stably transfected with the PACE4 expression vector was named 4-2+PACE4. Stable cell lines transfected with the ptRNA^{Val}-PACE4-SOFA- δ Rz were established by the selection of clones resistant to hygromycin B.

[00109] Northern blot analyses on total RNA extracts were performed for wild-type DU145 (DU145), DU145 transfected with ptRNA^{Val}-PACE4-SOFA- δ Rz (4-2) and, on 4-2 cells co-transfected with PACE4 cDNA expression vector (4-2+PACE4). Total RNA was isolated from DU145 cells using guanidinium isothiocyanate followed by lithium chloride precipitation. RNA migration (5 μ g) on denaturing agarose gel, membrane transfer and ³²P-labeled RNA probe transcriptions were performed. Linearized vectors were used as DNA template for complementary RNA probe transcription using either T7 or SP6 RNA polymerase. The 1066-base pair (bp) cDNA for human furin probe was obtained by digestion of the full-length clone with XhoI enzyme. A 456-bp cDNA fragment of PACE4 was cloned in pGEM-T™ easy vector system (Promega) by RT-PCR reaction on DU145 total RNA with specific primers. This vector was subsequently used for probe transcription. For PC7 probe, a 285-bp rat cDNA was used, and for bovine 18S ribosomal RNA probe, a 600-bp cDNA was used. The ImageJ software™ 1.37v was used for all densitometric analysis.

- 29 -

[00110] As seen in figure 2A, the PACE4 mRNA levels in the SOFA- δ Rz transfected cell line are significantly reduced when compared to the untransfected cells. These levels were partially re-established by the overexpression of PACE4 cDNA. A densitometric analysis using 18S ribosomal RNA as loading control was performed to quantify the mRNA levels in those clonal cell lines using wild type DU145 cells as reference (0.31 ± 0.11 and 0.75 ± 0.06 for 4-2 and 4-2+PACE4, respectively; Fig. 2B). The mRNA levels of two others endogenous expressed PCs were also verified to confirm the specificity of the PACE4-SOFA-(Rz cleavage. Levels of furin and PC7 mRNAs (Figs. 2C and 2D, respectively) remained mostly unchanged in the 4-2 cells, confirming the reduction of PACE4 expression without significantly affecting the expression of other endogenous PCs.

EXAMPLE 3

The reduction of PACE4 expression slows DU145 proliferation in vitro

[00111] The total cell numbers of the stable cell lines of Example 2 were counted at different times. The cell proliferation was measured by the colorimetric MTT assay (thiazolyl blue tetrazolium bromide; Sigma-Aldrich). Briefly, cells were seeded in 96-well plate (BD Biosciences) in triplicate with 100 μ l of a 3.5×10^4 cells/ml cell suspension in complete growth medium (RPMI 1640 media supplemented with 5% fetal bovine serum). The following day, cells were carefully washed twice with PBS and media were replaced with 100 μ l of either RPMI or conditioned growth media. 48 hours later, 20 μ l of a MTT solution (5 mg/ml in PBS 1x) was added to each well for 4.5h at 37 $^{\circ}$ C/5% CO₂. The media was then discarded and the cells were solubilized with 100 μ l isopropanol/0.04 N HCl solution. The absorbance was measured at a wavelength of 550 nm with a reference at 650 nm in microplate reader (SpectraMax190TM; Molecular Devices). Cells were plated at a density of 5.0×10^4 /well in 6-well plates (BD Biosciences) in duplicates. Complete growth medium was changed after 48 hours. After incubation, cells were washed in PBS, trypsinized and counted in after staining in 0.4% (w/v) trypan blue solution (Sigma). Only viable cells were counted in duplicate.

- 30 -

[00112] As seen in figure 3A, the results showed a significant reduction of proliferation for the 4-2 cells (~ 200 000 ± 14 000 cells) when compared to untransfected DU145 (≈ 375 000 ± 40 000 cells) 96 hours after the initial plating. This reduction was partially reversed in the cell line 4-2+PACE4 (≈ 280 000 ± 25
5 000 cells). An in vitro clonogenic assay was also performed on the same cell lines to detect the proportion of cells that retained the capacity to grow into a colony (Fig. 3B). The results of this assay confirmed the lower proliferation of DU145 with lowered PACE4 expression (4-2), as a 68% reduction was observed of cell growth when compared to wild-type DU145. The colony
10 formation capacity of DU145 cells was partially restored (16% less than untransfected cells) in 4-2+PACE4 cells.

EXAMPLE 4

PACE4 inhibition prevents tumor growth in xenograft tumor model

15 [00113] The ability of the experimental cell lines to grow as tumors in mouse model was tested. Four-week-old female athymic nude mice (NU/NU; Charles River Laboratories) were inoculated subcutaneously at the opposite sides of the flank with 3.0×10^6 cells per inoculums. Cells were grown in complete media and harvested at their exponential growing state. Mice were housed under
20 pathogen free conditions and the implantations were done under anesthesia conditions in laminar flow hood. Xenografts were measured three times per week and volume (V) was determined by this equation: $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of a xenograft. As shown in figure 4A, the reduction of PACE4 mRNA levels (see (in Fig. 4A) reduced dramatically the
25 ability of 4-2 cells to induce tumor growth, while untransfected DU145 cells (see (in Fig. 4A) were able to develop into well-defined tumor masses. Histological analysis (Fig. 4B) shows the well define tumor masses when DU145 cells are implanted (see panels A and B in Fig. 4B), however, no such compact and well
30 define structure is obtained with the 4-2 cells (see panels C and D in Fig. 4B), confirming that the lack of PACE4 has significant effects on tumor progression.

EXAMPLE 5**Generation of potent inhibitors of PACE4 and PC7**

[00114] One of the keys to the development of potent and selective PC inhibitors is an understanding of the substrate-binding pocket. The deepest region of the substrate-binding pocket accommodates the consensus motif RXKR (P4-P3-P2-P0 nearly identical in all PCs. Using an incremental peptide assay (IPA), the core warhead sequence, **RVKR** (SEQ ID NO: 1), was extended one amino acid at a time. In the N-terminal version of this assay, peptides bearing the 20 natural L-amino acids at the P₅ position were synthesized and tested. The most efficient inhibitory peptides (pentapeptides) were modified further, by individually adding the 20 L-amino acid at the P₆ position, and so forth creating inhibitor peptides with multi-leucines (see Table 2). Thus, the effect of extending the N-terminal side of the core sequence **RVKR-NH₂** with multiple leucines on the inhibition potency and specificity of PCs was tested. **RVKR-NH₂** was a poor micromolar inhibitor of all PCs, but was most potent on PC1/3 (Fig. 5 and as schematized in Fig. 6).

Table 2

Designed peptides / PACE4 inhibitors

20	<u>Peptides inhibitors</u>	
	OLeu	RVKR-NH ₂ (SEQ ID NO: 1)
	1 Leu	LRVKR-NH ₂ (SEQ ID NO: 2)
	2 Leu	LLRVKR-NH ₂ (SEQ ID NO: 3)
	3 Leu	LLLRVKR-NH ₂ (SEQ ID NO: 4)
25	4 Leu	LLLLRVKR-NH ₂ (SEQ ID NO: 5)
	5 Leu	LLLLLRVKR-NH ₂ (SEQ ID NO: 6)
	6 Leu	LLLLLLRVKR-NH ₂ (SEQ ID NO: 7)
	Multi-Leu (ML)	LLLLRVKR-NH ₂
	<u>Peptide with optimized stability</u>	
30	Acetyl-ML	CH ₃ CO-NH-LLLLRVKR-NH ₂
	<u>Peptides with optimized penetration</u>	
	8-amino-octanoyl-ML	H ₂ N-CH ₂ -(CH ₂) ₆ -CO-NH-LLLLRVKR-NH ₂
	11-amino-undecanoyl-ML	H ₂ N-CH ₂ -(CH ₂) ₉ -CO-NH-LLLLRVKR-NH ₂
	Cholyl-ML	ChOlyl-NH-LLLLRVKR-NH ₂
35		

- 32 -

[00115] Enzyme inhibition assays for furin (Fig. 5D) were performed in 100 mM Hepes pH 7.5, 1 mM CaCl₂, 1 mM β-mercaptoethanol, 0.5 μg/μL BSA. Assays for PC2 (Fig. 5E) were performed in 20 mM Bis-Tris pH 5.7, 1 mM CaCl₂, 0.1% Brij-30. Assays for PC1/3, PC4, PACE4, PC5/6 and PC7 (Figs. 5F, 5 G, A, B and C respectively) were performed in 20 mM Bis-Tis pH 6.5, 1 mM CaCl₂. All assays were performed with the substrate pyroGlu-Arg-Val-Lys-Arg-methyl-coumaryl-7-amide (PyrRTKR-MCA) (Bachem, CA) at 100 μM for furin, PC1/3, PC4, PACE4 and PC5/6, 200 μM for PC2 and 250 μM for PC7. Assays were carried out at 37°C for 30-60 min and real-time fluorescence was measured with an excitation wavelength of 370 nm and an emission wavelength of 460 nM using a Gemini XST[™] 96-well spectrofluorometer and SoftMaxPro4[™] software (Molecular Devices, CA). Inhibitory peptides were added to the enzymes at decreasing concentrations from 100 μM to 50 nM and incubated 5 minutes prior to the addition of substrate. Kinetics were analyzed using SoftMaxPro4[™] and K_i values were determined as previously described, using K_m values of 8, 131, 20, 18, 21, 9 and 62 μM for furin, PC2, PC1/3, PC4, PACE4, PC5/6 and PC7, respectively. Each K_i value is the mean of 2 to 10 independent experiments.

[00116] As shown in figure 5 (peptides are disclosed in Table 2), LRVKR-NH₂ (SEQ ID NO: 2) and LLRVKR-NH₂ (SEQ ID NO: 3) were mid-nanomolar inhibitors of furin, but the progressive extension by additional leucines decreased the inhibition potency to the micromolar range (Fig. 5D). All multi-leucine peptides were poor micromolar inhibitors of PC2 (Fig. 5E). PC1/3 was best inhibited by LLRVKR-NH₂ (SEQ ID NO: 3), but the progressive extension with leucine caused a decrease in potency to the low micromolar range (Fig. 5F). PC4 inhibition potency by multi-leucine peptides generally increased with length (Fig. 5A). The multi-leucine peptide containing five leucines (SEQ ID NO: 6) is the best inhibitor of PC4 evaluated in this study (K_i of 164 nM; Fig. 5G). For PACE4, the progressive extension by multiple leucines caused an increase in inhibition potency to the low nanomolar range (Fig. 5A). LLLLRVKR-NH₂ (four leucine or multi-leu peptide; SEQ ID NO: 5) was the most potent inhibitor of PACE4 (K_i of 6 nM) evaluated in this study and was significantly more effective

- 33 -

on PACE4 than the other PCs (9-folds and more; Fig. 5A as encircled and in Fig. 6). PC5/6 inhibition increased when adding one or two leucines (SEQ ID NOs: 6 and 7), but the addition of more leucine had a decreasing effect on inhibition potency (Fig. 5B). PC5/6 was best inhibited by LLRVKR-NH₂ (SEQ ID NO: 3) in the mid-low nanomolar range. Finally, progressive leucine extensions caused an increase in inhibition potency for PC7 (Fig. 5C). Peptides of four, five and six leucines (SEQ ID NOs: 5, 6 and 7) were similar in potency (K₁ values of -35-50 nM).

[00117] Consequently, the multi-leu peptide (SEQ ID NO: 5) represents not only the most potent inhibitor of PACE4, but since the K₁ is in the nanomolar range, it also represents a promising inhibitor for *in vivo* efficacy because of its high selectivity for PACE4.

EXAMPLE 6

Cell penetration analysis of PACE4 inhibitors

[00118] Improving the penetration efficacy of identified PACE4 inhibitors was also tested. ML peptide (LLLLRVKR-NH₂, see Table 2) was tested for its ability to enter DU145 cells. Cells were treated with the cholyl-ML peptide linked to FITC. Following FACS scan analysis, control cells are observed in the red spectra. Following treatment, there is a clear shift of the cells indicating that the cholyl-ML FITC peptide has penetrated the cells (Fig. 7A). As a further control, cells were treated with trypsin (Fig. 7B) to insure that the observed shift was not due to the cholyl-ML FITC peptide absorbed on the cell surface. Since, the shifted spectra remains intact, this shows that cholyl-ML FITC peptide has penetrated the cell membranes.

EXAMPLE 7

PACE4 inhibitors effects on cell proliferation index

[00119] The index of cellular proliferation of cells treated with the ML and acetyl-ML (CH₃CO-NH-LLLLRVKR-CONH₂, see Table 2) peptides were measured using the colorimetric MTT assay (thiazolyl blue tetrazolium bromide; Sigma-Aldrich). Briefly, cells were seeded in 96-well plate (BD Biosciences) in

- 34 -

triplicate with 100 μ l of a 3.5×10^4 cells/ml cell suspension in complete growth medium. The following day, cells were carefully washed twice with PBS and media were replaced with 100 μ l of either RPMI or conditioned growth media. Conditioned growth medium preparation consists in 1.2×10^5 cells seeded in 6-well plates with complete growth media. The next day, cells are washed twice with PBS and the media are replaced with 1 ml RPMI growth medium. 48 hours later, the conditioned media are collected, filtered through 0.45 μ M syringe filter units and incubated on different cell lines.

[00120] 48 hours later, 20 μ l of a MTT solution (5 mg/ml in PBS 1x) was added to each well for 4.5h at 37 °C/5% CO₂. The media was then discarded and the cells were solubilized with 100 μ l isopropanol/0.04 N HCl solution. The absorbance was measured at a wavelength of 550 nm with a reference at 650 nm in microplate reader (SpectraMax190™; Molecular Devices).

[00121] Four human cell lines were tested, including the small cell carcinoma cell line H345, a glioblastoma cell line U251, the prostatic cell line DU145 and a sarcofibroma cell line HT1080. In all cases, both ML and acetyl-ML peptides had significant effects on the cell proliferation index (Fig. 8 and Table 3). However, acetyl-ML peptides were more potent due to the added protection of the N-terminal acylation.

20

Table 3
Cell proliferation index

DU145	ML	acetyl ML
Control	100%	100%
1 μ M	97.73%	63.84%
10 μ M	92.03%	41.91%
50 μ M	72.57%	25.79%
100 μ M	54.70%	24.05%

[00122] ML and acetyl-ML peptides with lipid or steroid N-terminal peptides were also compared with the prostatic cell line DU145. As described in figure 9,

- 35 -

8-amino-octanoyl-ML ($H_2N-CH_2-(CH_2)_6-CO-NH-LLLLRVKR-NH_2$) or 11-amino undecanoyl-ML ($H_2N-CH_2-(CH_2)_9-CO-NH-LLLLRVKR-NH_2$) or cholyl-ML (ChOlyl-NH-LLLLRVKR-NH₂) peptides all had more potent effects than ML or acetyl-ML peptides, most likely due to their additional ability to penetrate the cell membranes (Fig. 9 and Table 4).

Table 4

Cell proliferation index ML and acetyl-ML peptides

DU145	ML	acetyl ML	8-amino-octanoyl ML	11-amino-undecanoyl ML	cholyl ML
Control	100%	100%	100%	100%	100%
1 μ M	97.73%	63.84%	106.05%	92.44%	86.65%
10 μ M	92.03%	41.91%	97.69%	35.94%	38.70%
50 μ M	72.57%	25.79%	36.30%	---	---
100 μ M	54.70%	24.05%	33.54%	1.42%	4.36%

EXAMPLE 8**PACE4 inhibitors effects on the clonogenic assay**

[00123] All ML peptides tested had important effects on the ability of DU145 cell lines to form colonies. Cell lines were seeded in 6-well plates (BD Biosciences) at a density of 300 cells/well in triplicate. DU145 cells were treated for 24 hours with acetyl-ML, 8-amino octanoyl-ML, 11-amino undecanoyl-ML and cholyl-ML at concentrations of 10 and 100 μ M. After colony formation, media was discarded and cells were washed once with PBS. Colonies were fixed and stained in 5 mg/ml methylene blue/50% methanol solution for 10 min. Excess of staining solution was removed carefully with distilled water and the plates were dried overnight before scanning with Li-Cor Odyssey Infrared Imaging System™ (Li-Cor Biosciences). Scanned images were analyzed with ImageJ™ software 1.37v to measure the total particule area. The assay was performed in duplicate. As shown on figures 10A and C, the most potent effects were observed with lipid or sterol ML peptides (or octanoyl-ML, white histogram in Fig. 10A).

EXAMPLE 9**PACE4 inhibitors effects on *in vivo* formation of tumor**

[00124] DU145 cells were implanted subcutaneously (sc) at two sites on the backs of Nu/Nu mice, which lack an immune system. A nude mouse is a genetic mutant that lacks a thymus gland, resulting in an inhibited immune system due to a greatly reduced number of T cells. The genetic basis of the nude mouse mutation is a disruption of the *Foxn1* gene. The nude mouse can receive many different types of tissue and tumor grafts, as it mounts no rejection response. These xenografts are commonly used to test new methods of treating tumors. Twenty days after implantation and once tumor had reached an average size of 50 mm³, intra-tumoral cholyl-ML peptide (see Table 2) was injected at a dose of 30 mg/kg, at a frequency of once every 2 days. Control tumor received vehicle (DMSO) injections at the same frequency. Control tumor continued their growth pattern, reaching an average size of 160 mm³, while treated tumors only reached a size of 75 mm³ (Fig. 11A). Representative mice are shown on the B panel of figure 11, while the histology of the control and treated tumors are shown in panels C and D of figure 11.

EXAMPLE 10**PACE4 inhibitors effects on apoptosis**

[00125] To determine if the reduced cell number observed in the 4-2 cell line, described previously in Example 3, was a consequence of the induction of the apoptosis, the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane was analyzed. This analysis was performed with a FITC-conjugated annexin-V, which has a strong affinity for these extracellular phosphatidylserines, and the fluorescent intercalating agent propidium iodide (PI). Cell lines were seeded in a 6-well plate at a density of 8×10^4 cells/well in complete growth medium. The next day, cells were washed twice with PBS and complete growth media with or without cisplatin (Sigma) at final concentration of 66 μ M were added. After a 48 hours incubation period, growth media were collected and combined to the harvested cells obtained after trypsin treatment. The collected pellets were washed with PBS before staining.

- 37 -

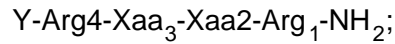
Then, cells were stained with the Annexin-V-FLUOS™ Staining Kit (Roche Applied science), which double labeled cells with annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Stained cells were then analyzed with FACScan flow cytometer (BD Biosciences).

5 [00126] Figure 12 shows the percentage of annexin-V/PI-labeled cells determined by flow cytometry. Both DU145 and 4-2 cell lines exhibited a low level of annexin-V positivity (lower and upper right quadrants; 2% and 3%, respectively) and a similar PI positivity for necrotic cells (upper left quadrant; 6% and 7%, respectively). Treatment with the cytotoxic compound cisplatin induced
10 the apoptosis in both cell lines, since a higher annexin-V positivity was measured for both cell lines (30% and 17% for DU145 and 4-2 cells, respectively). A higher PI staining was also observed, indicating a higher number of dead cells following cisplatin treatment (12% and 16% for DU145 and 4-2 cells, respectively). Thus, these results indicate that apoptosis pathway is
15 still functional, although it is not induced by reduction of PACE4 expression levels.

[00127] 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
20 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 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.

WHAT IS CLAIMED IS:

1- A PACE4 inhibitor comprising a peptide sequence comprising the following formula:



wherein

-Arg₁ is an arginine, arginine derivative, arginine mimetic or a transition state analogue;

-Xaa₂ and Xaa₃ are any amino acids or stereoisomers thereof; and

-Y is absent or comprises the formula Z-Xaa₈-Xaa₇-Xaa₆-Xaa₅, wherein

Xaa₅, Xaa₆, Xaa₇ and Xaa₈ have a hydrophobicity score between about 4.5 to -0.4 based on a Kyte-Doolittle hydrophobicity plot, or

Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are independently selected from the group consisting of Lys, His and Arg;

Z is absent or comprises an N-terminal acyl group linked to the N-terminal of the peptide sequence;

with the proviso that Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are not aromatic or negatively charged amino acids.

- 2- The PACE4 inhibitor of claim 1, wherein Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are positively charged amino acids or stereoisomers thereof.
- 3- The PACE4 inhibitor of claim 1, wherein Xaa₃ is Val.
- 4- The PACE4 inhibitor of any one of claims 1-3, wherein Xaa₂ and Xaa₃ are independently selected from Gly and Ala.
- 5- The PACE4 inhibitor of any one of claims 1-4, wherein Xaa₂ is Lys or Arg.
- 6- The PACE4 inhibitor of any one of claims 1-5, wherein Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are aliphatic hydrophobic amino acids.

- 39 -

- 7- The PACE4 inhibitor of claim 6, wherein said aliphatic hydrophobic amino acids are Leu, Iso or Val.
- 8- The PACE4 inhibitor of any one of claims 1-7, wherein Xaa₅, Xaa₆, Xaa₇ and Xaa₈ are Leu.
- 9- The PACE4 inhibitor of claim 1, wherein Xaa₇ and Xaa₈ are small amino acids.
- 10- The PACE4 inhibitor of claim 1, said inhibitor comprises at least one of the following amino acid sequences: SEQ ID NO: 2, 3, 4, 5, 6 and 7.
- 11- The PACE4 inhibitor of any one of claims 1-10, wherein the N terminus is acylated.
- 12- The PACE4 inhibitor of claim 11, wherein the N terminus acylation is with fatty omega amino acids or with steroid derivatives.
- 13- The PACE4 inhibitor of claim 12, wherein the fatty omega amino acids are C2 to C18.
- 14- The PACE4 inhibitor of claim 13, wherein the fatty omega amino acids are C2 to C11.
- 15- The PACE4 inhibitor of claim 13 or 14, wherein the fatty omega amino acids are selected from the group consisting of 11-amino undecanoyl and 8-amino octanoyl.
- 16- The PACE4 inhibitor of claim 12, wherein the steroid derivatives are choyl.
- 17- A composition comprising the PACE4 inhibitor as defined in claim 1 and a carrier.

- 18- The composition of claim 17 further comprising at least one anti-cancer drug.
- 19- The composition of claim 17 or 18 adapted for delivery by at least one of the following route selected from the group consisting of oral, mucosal, intranasal, intraocular, intratracheal, intrabronchial, intrapleural, intraperitoneal, intracranial, intramuscular, intravenous, intraarterial, intralymphatic, subcutaneous, intratumoral, gastric, enteral, colonic, rectal, urethral and intravesical route.
- 20- A method of lowering PACE4 activity in a cell, comprising contacting the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 18 with the cell, thereby lowering PACE4 activity in the cell.
- 21- The method of claim 20, wherein the cell is in a subject.
- 22- The method of claim 20, wherein the cell is a cancer cell.
- 23- The method of claim 20, wherein the cell has increased PACE4 activity.
- 24- A method of reducing the proliferation of a cell in a subject, comprising administering the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 to the subject, thereby reducing the proliferation of the cell in the subject.
- 25- The method of claim 24, wherein the cell is a cancer cell.
- 26- The method of claim 24, wherein the cell has increased PACE4 activity.
- 27- A method of reducing tumor growth in a subject, comprising administering the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 to the subject, thereby reducing tumor growth in the subject.

- 41 -

- 28- The method of claim 27, wherein said tumor is derived from a prostate cancer or a metastasis thereof.
- 29- A method for the prophylaxis or treatment of a cancer in a subject, comprising administering to said subject a therapeutically effective amount of the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17, thereby preventing or treating the cancer in the subject.
- 30- The method of claim 29, wherein said cancer is a prostate cancer.
- 31- Use of the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 in the manufacture of a medicament for preventing or treating cancer in a subject.
- 32- The use of claim 31, wherein said cancer is a prostate cancer.
- 33- The use of claim 31, wherein said PACE4 inhibitor or composition reduces cell proliferation, tumor growth or metastasis formation.
- 34- Use of the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 for preventing or treating cancer in a subject.
- 35- The use of claim 34, wherein said cancer is a prostate cancer.
- 36- The use of claim 34, wherein said PACE4 inhibitor or the composition reduces cell proliferation, tumor growth or metastasis formation.
- 37- Use of the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 for lowering PACE4 activity in a cell.
- 38- Use of the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 for reducing proliferation of a cell in a subject.

- 39- Use of the PACE4 inhibitor as defined in claim 1 or the composition as defined in claim 17 for reducing tumor growth in a subject.
- 40- A method of screening for a PACE4 inhibitor comprising the steps of:
- a) contacting an agent with a PACE4 protein;
 - b) assessing the activity of the PACE4 protein of step a);
- wherein a reduction of the activity of the PACE4 protein of step b) compared to the basal activity of a PACE4 protein that has not been in contact with the agent of step a) is indicative that the agent is an inhibitor of PACE4.
- 41- A method of identifying a cell proliferation inhibitor, comprising the steps of:
- a) contacting an agent with a PACE4 protein in a cell;
 - b) assessing the activity of the PACE4 protein of step a);
- wherein a reduction of the activity of the PACE4 protein of step b) compared to the basal activity of the PACE4 protein that has been in contact with the agent of step a) is indicative that the agent is an inhibitor of PACE4 inhibiting cell proliferation.
- 42- The method of claim 41, further comprising the steps of:
- c) comparing the proliferation rate of the cell to a control cell not contacted with the agent.

1 / 12

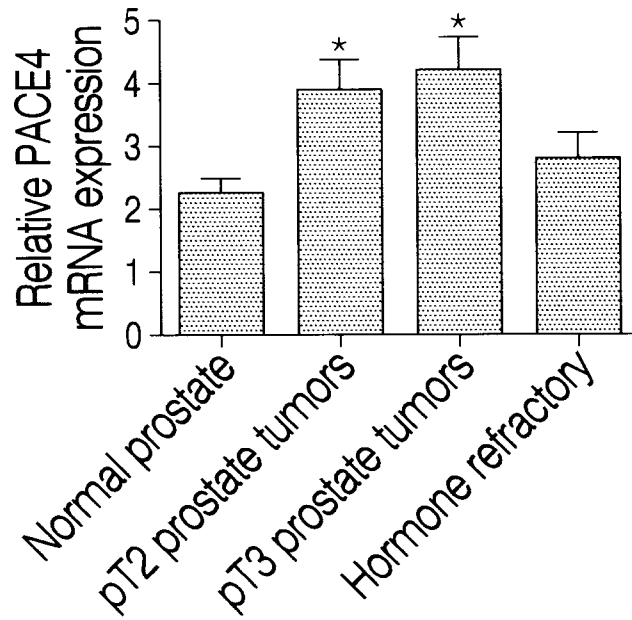


Fig. 1A



Fig. 1B

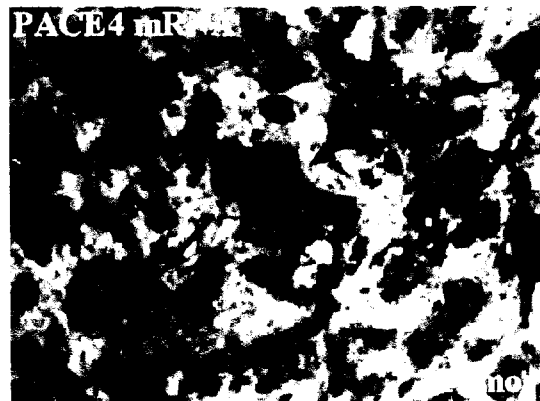


Fig. 1C

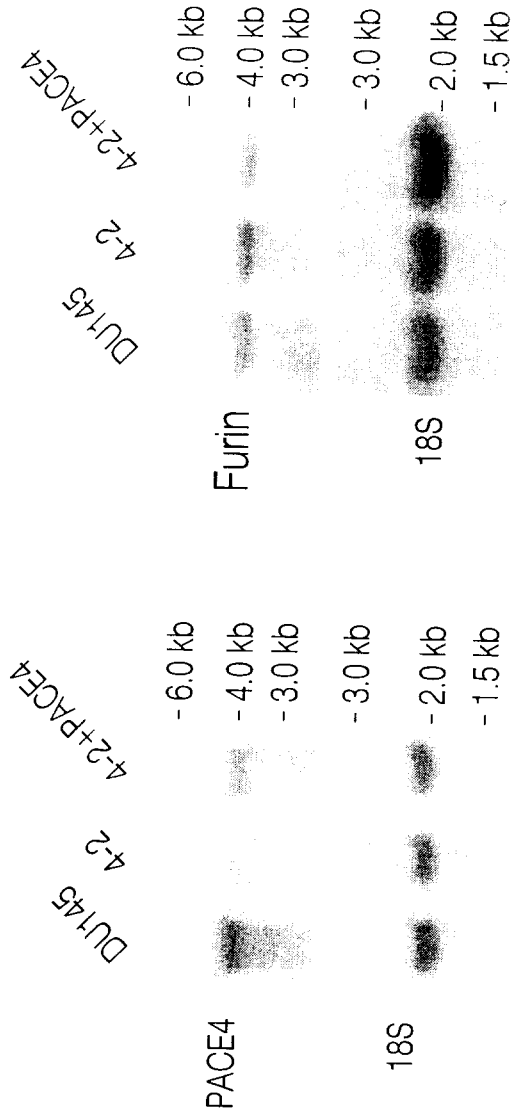


Fig. 2A

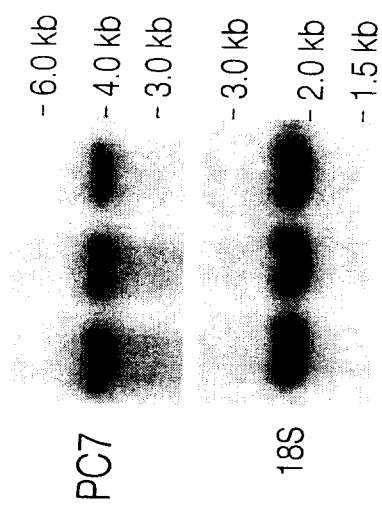


Fig. 2C

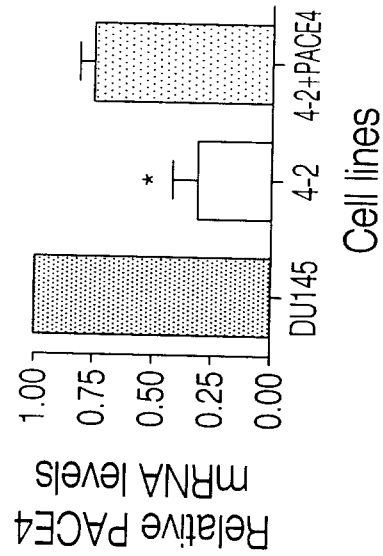


Fig. 2B

3 / 12

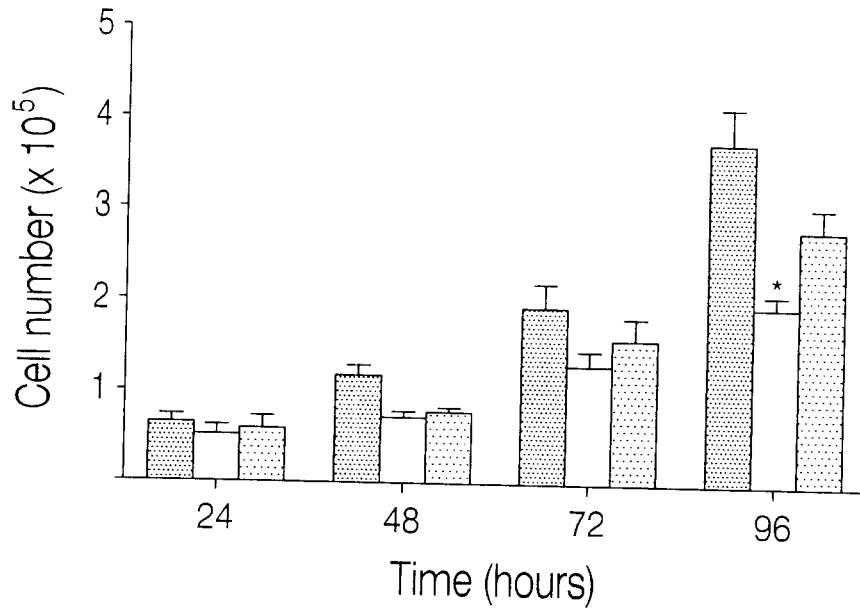


Fig. 3A

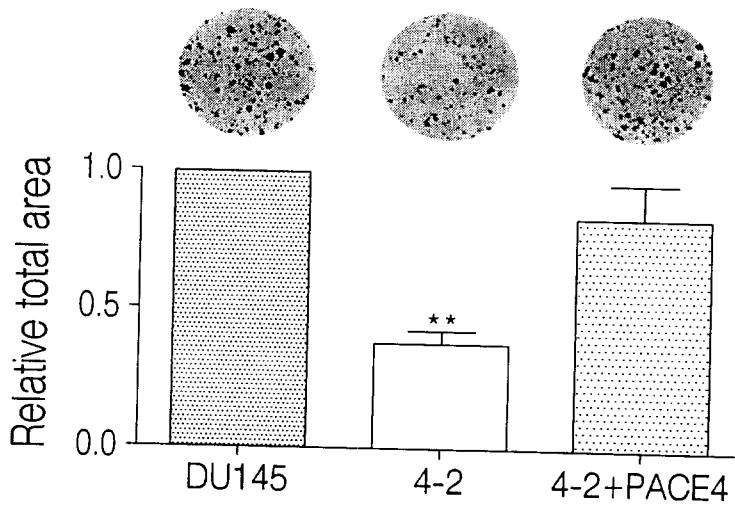


Fig. 3B

4 / 12

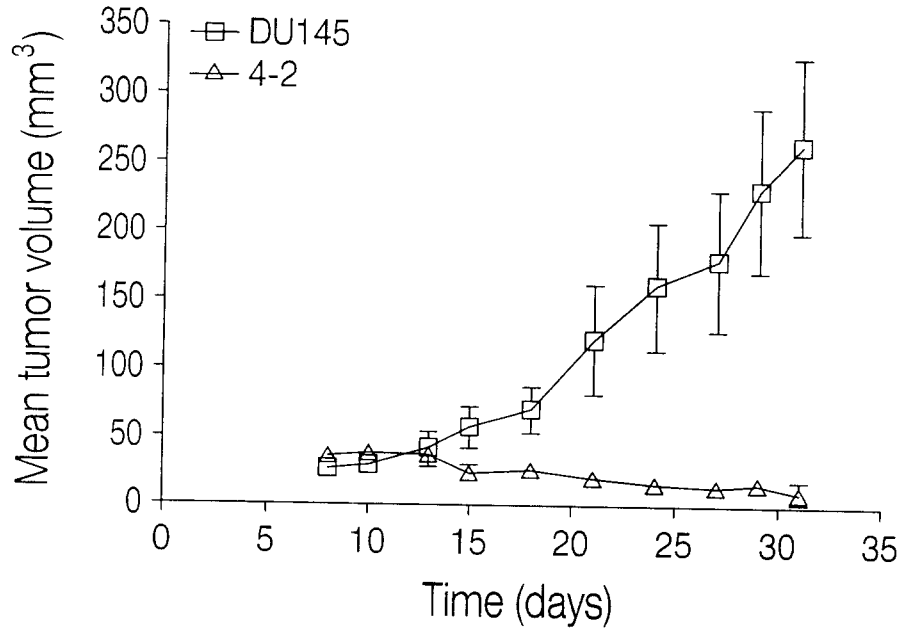


Fig. 4A

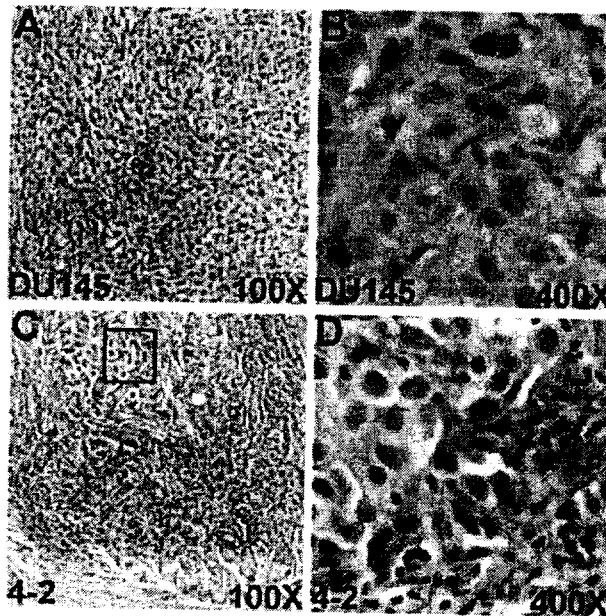


Fig. 4B

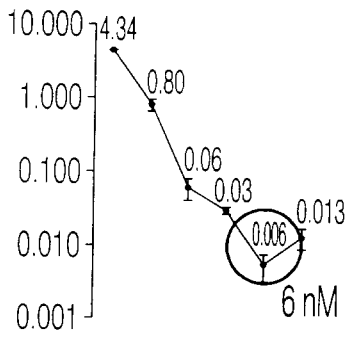


Fig. 5A

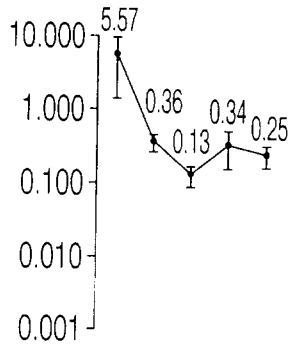


Fig. 5B

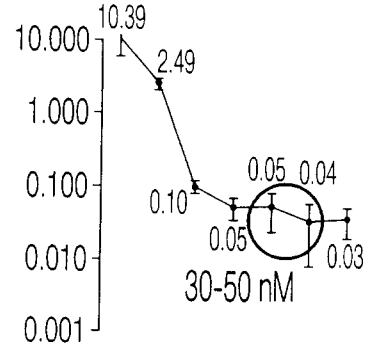


Fig. 5C

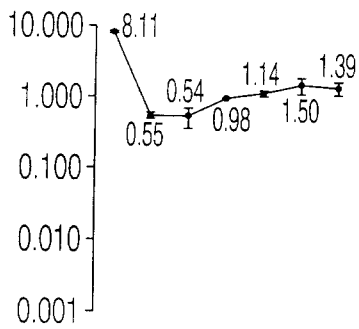


Fig. 5D

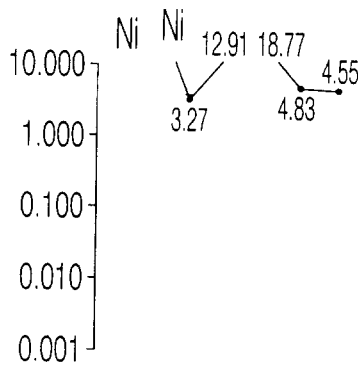


Fig. 5E

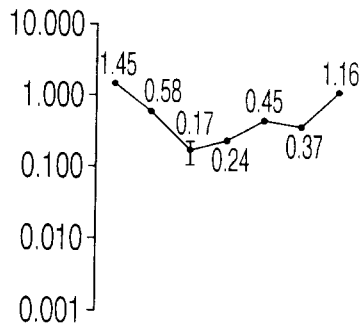


Fig. 5F

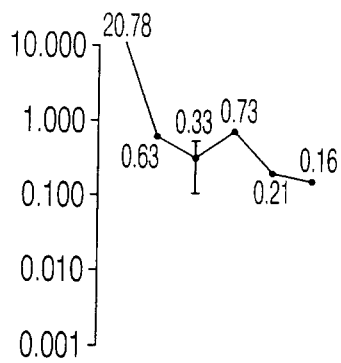


Fig. 5G

6 / 12

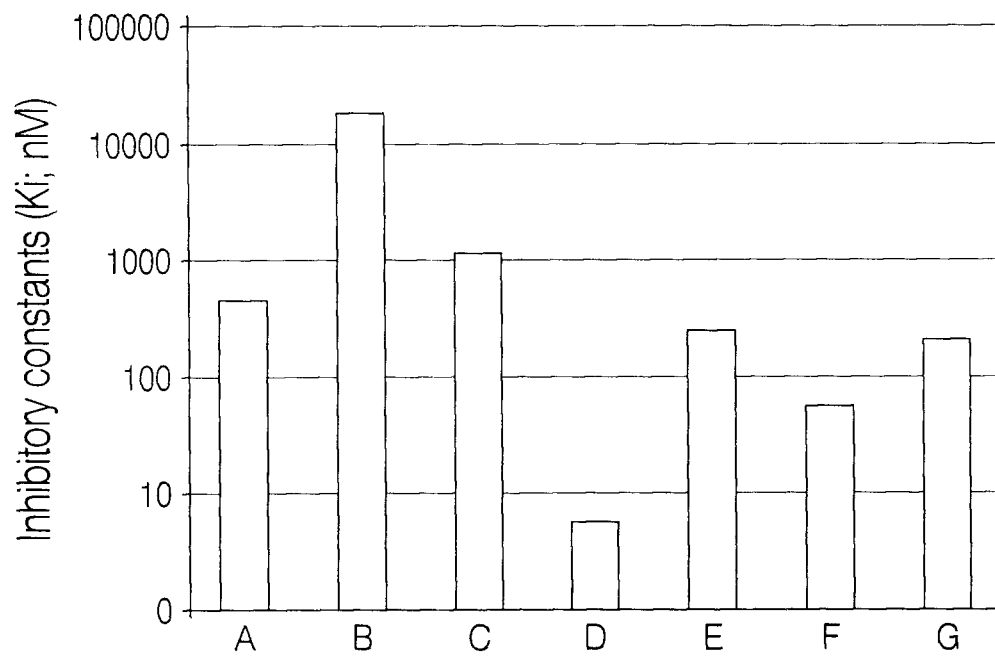


Fig. 6

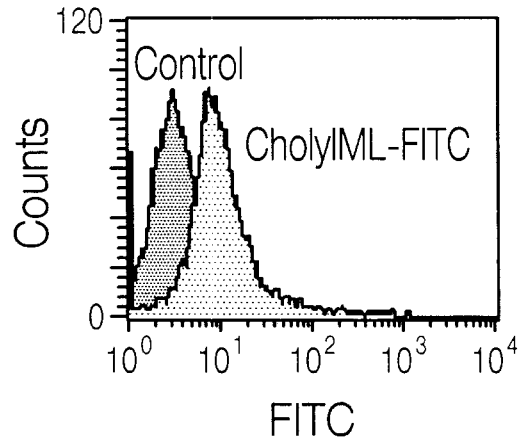


Fig. 7A

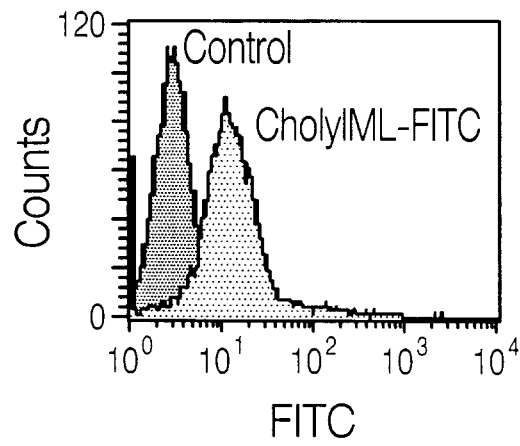


Fig. 7B

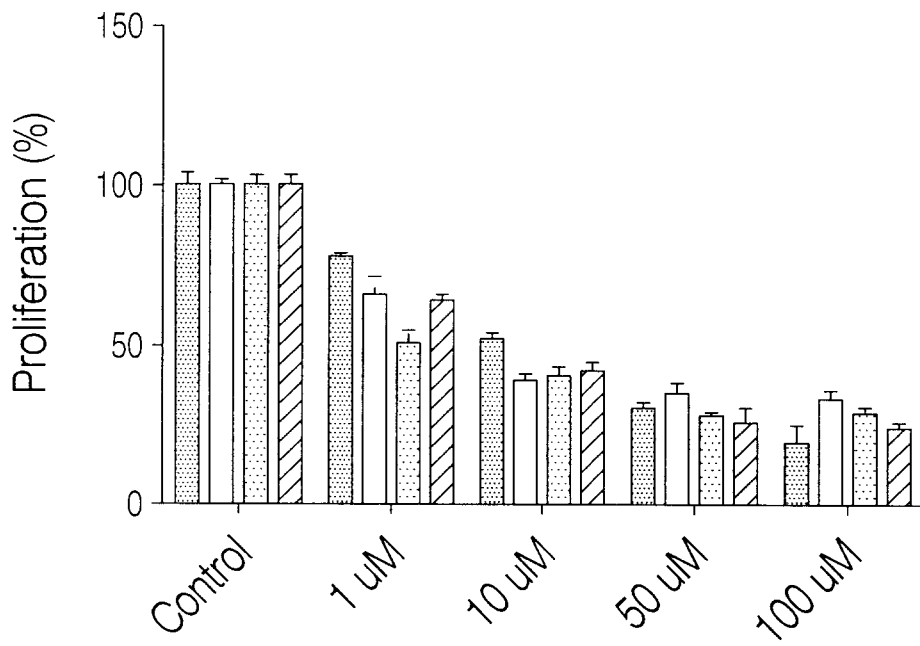


Fig. 8

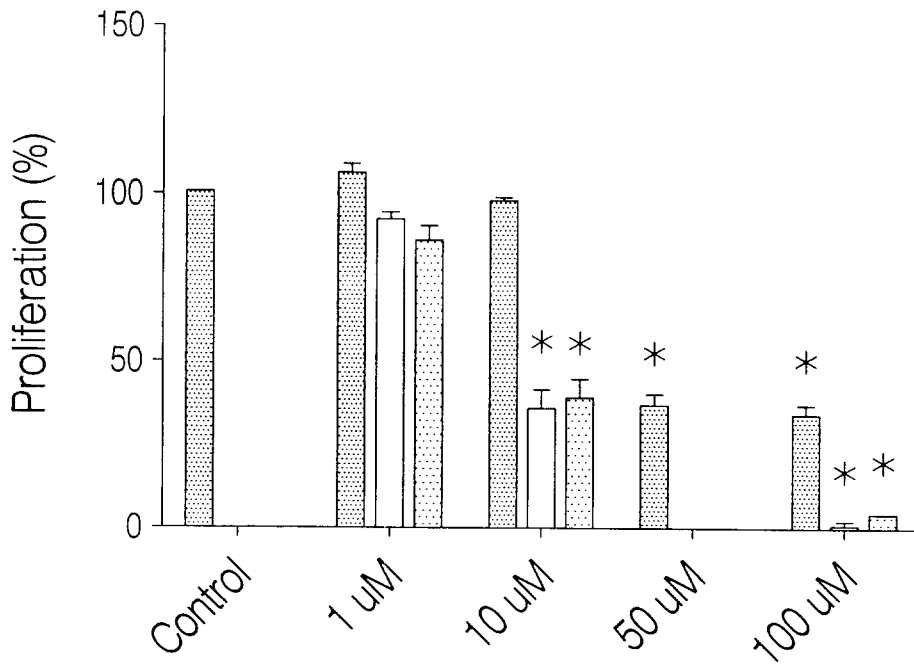


Fig. 9

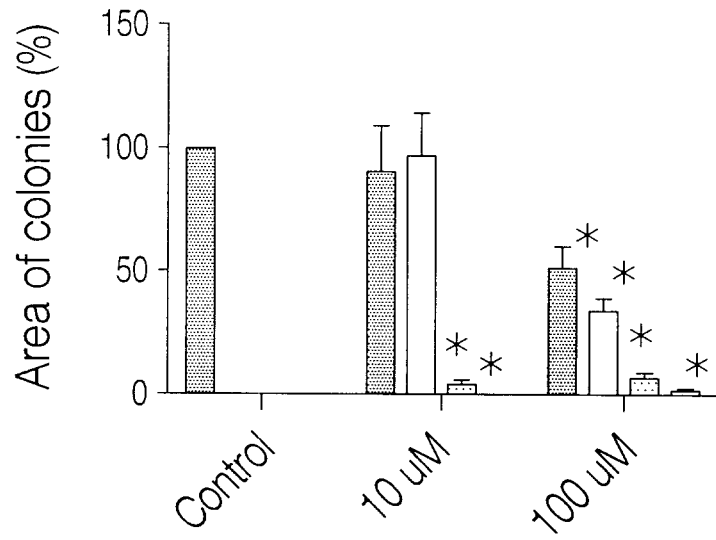


Fig. 10A

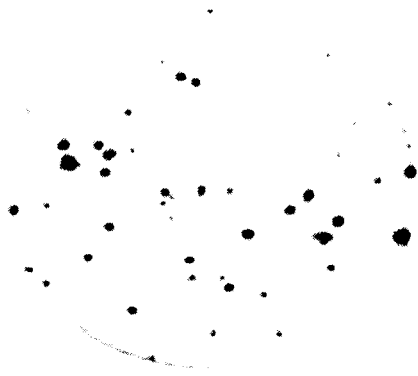


Fig. 10B

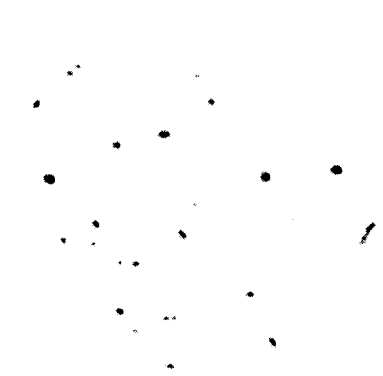


Fig. 10C

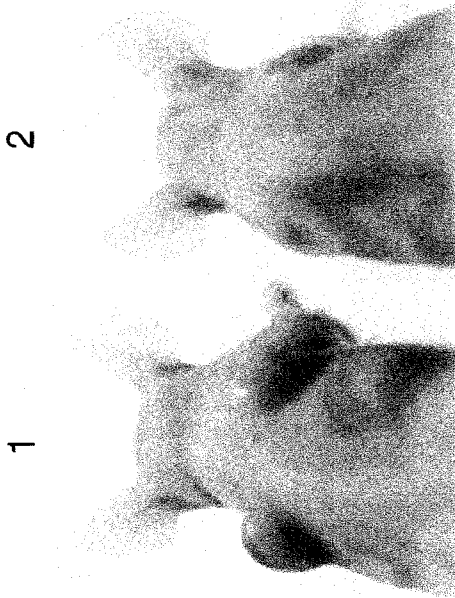


Fig. 11B

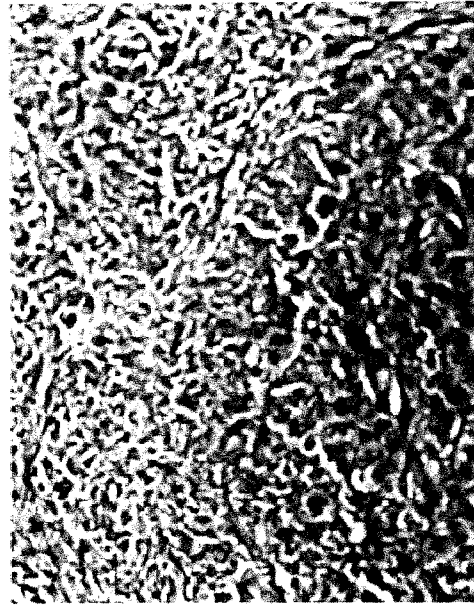


Fig. 11D

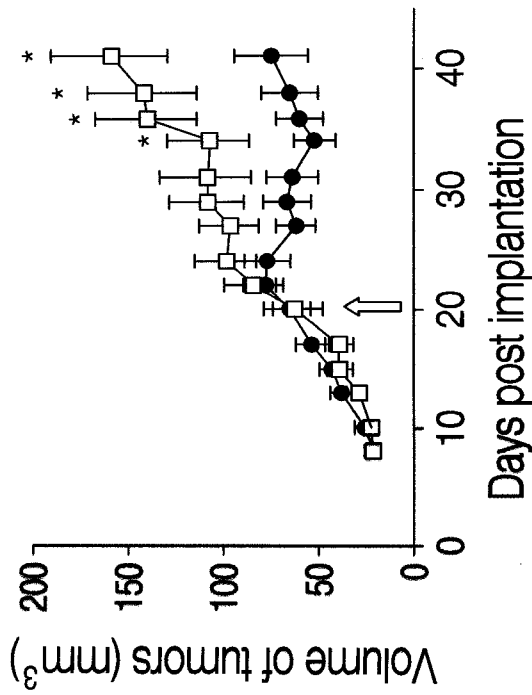


Fig. 11A

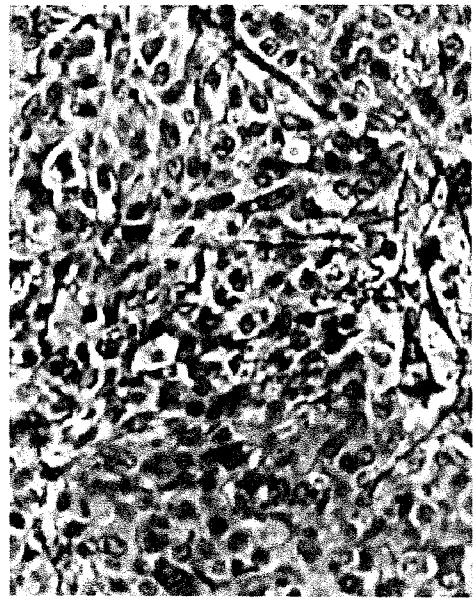


Fig. 11C

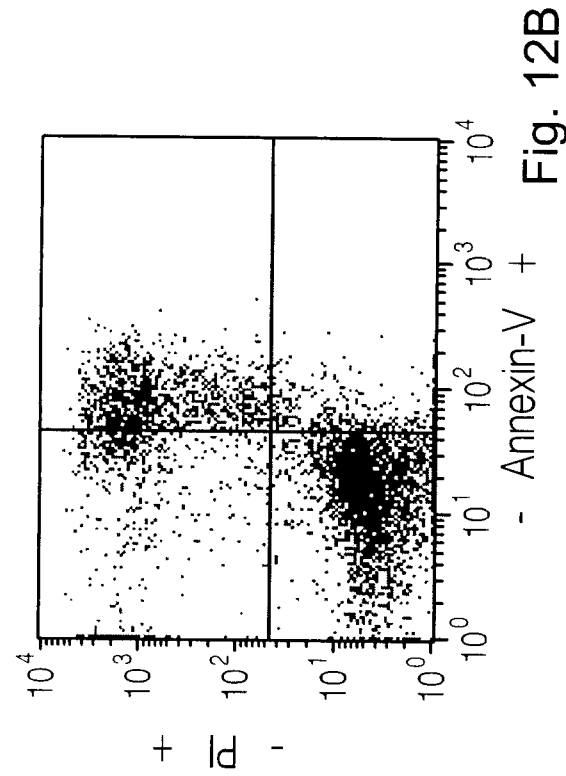


Fig. 12B

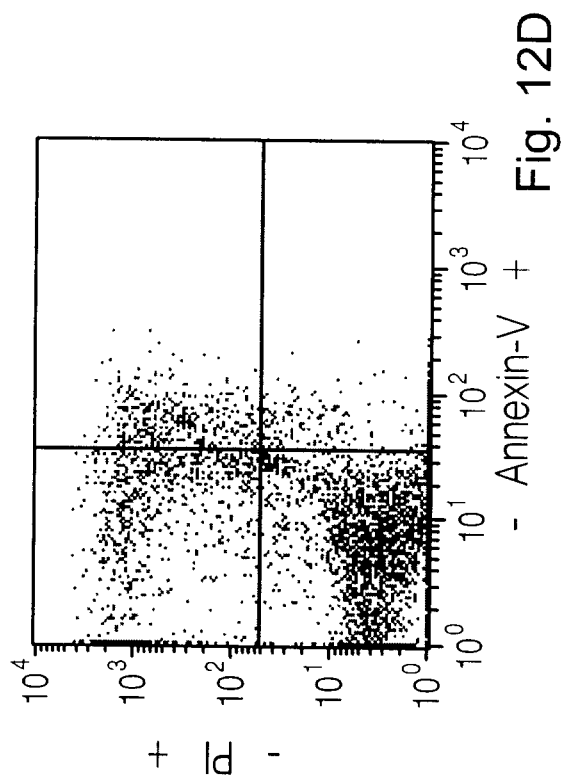


Fig. 12D

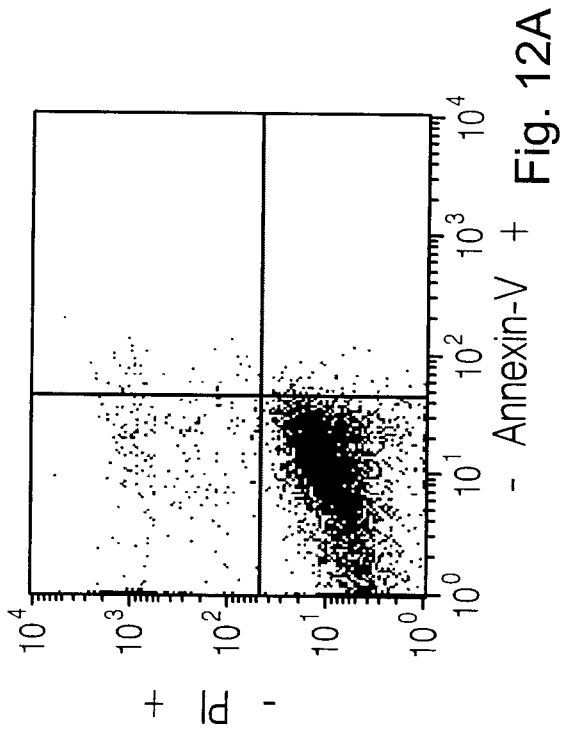


Fig. 12A

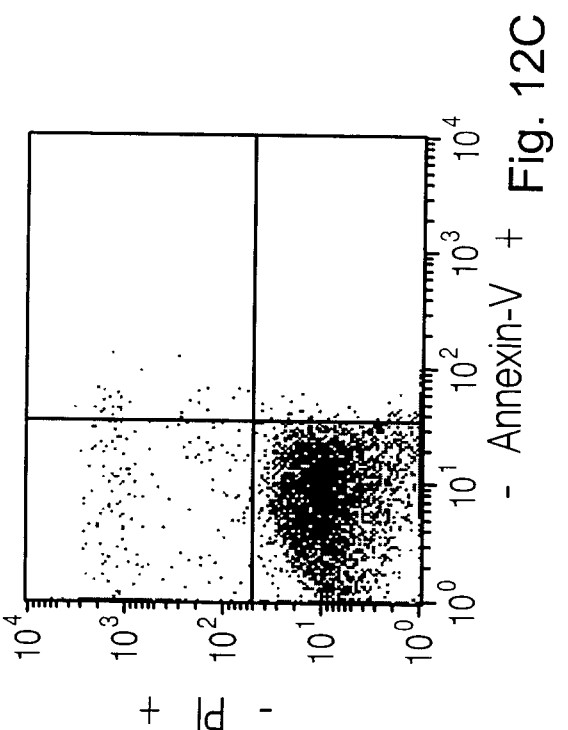


Fig. 12C

INTERNATIONAL SEARCH REPORT

International application No
PCT/CA2009/000935

A CLASSIFICATION OF SUBJECT MATTER IPC C07K5/11 (2006 01) , A61K 38/07 (2006 01) , A61K 38/08 (2006 01) , A61P 35/00 (2006 01) , C07K5/10 (2006 01) , C07K 7/06 (2006 01) (more IPCs on the last page) 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) C07K5/11 (2006 01), A61K 38/07 (2006 01), A61K 38/08 (2006 01), A61P 35/00 (2006 01), C07K 5/10 (2006 01), C07K 7/06 (2006 01), C12Q 1/02 (2006 01), GOIN33/53 (2006 01), GOIN33/68 (2006 01), C07K 14/81 (2006 01), C12N9/64 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Canadian Patent Database, STN (Registry and CPlus), Delphion, NCBI database, Google Patents, Genome Quest Key Words SEQ ID NOs 2-7, PACE4, inhibitor, stereoisomer, acylation, choly, steroid, anti-cancer, proliferation, therapeutic, drug		
C DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	TSUJI, A et al Development of selectivity of α 1-antitrypsin variant by mutagenesis in its reactive site loop against proprotem convertase A crucial role of the P4 arginine in PACE4 inhibition Protein Engineering February 2002, Volume 15 (2), 123-130 ISSN 0269-2139 Whole document	1, 3, 5, 11 and 17-42
X	CAMERON, A et al Polyarginines are potent furin inhibitors The Journal of Biological Chemistry 24 November 2000, Volume 275 (47), 36741-36749 ISSN 0021-9258 Whole document	1, 5, 11 and 17-42
P, A	BASAK, A et al Recombinant proprotem convertase 4 (PC4) from <i>Leishmania tarentolae</i> expression system Purification, biochemical study and inhibitor design Protein Expression and Purification August 2008, Volume 60 (2), 117-126 ISSN 1046-5928	
A	WO 2004/0091 13 A1 (FERGUSON, M et al) 29 January 2004	
A	WO 2005/02561 1 A1 (MALFAIT, A et al) 24 March 2005	
<input type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
*	Special categories of cited documents	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
A	document defining the general state of the art which is not considered to be of particular relevance	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
E	earlier application or patent but published on or after the international filing date	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
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)	& document member of the same patent family
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	
Date of the actual completion of the international search		Date of mailing of the international search report
22 September 2009 (22-09-2009)		19 October 2009 (19-10-2009)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, CI 14 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No 001-819-953-2476		Authorized officer Adrian All (819) 934-7930

INTERNATIONAL SEARCH REPORT

International application No
PCT/CA2009/000935

C12Q 1/02 (2006.01) , *G01N 33/53 (2006.01)* , *G01N 33/68 (2006.01)* , *C07K 14/81 (2006 01)* , *C12N 9/64 (2006.01)*

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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 Claim Nos 20-30 and 40-42

because they relate to subject matter not required to be searched by this Authority, namely

Although, claims 20-30 and 40-42 of the present application encompass a method of medical treatment which this Authority is not obliged to search under Rule 39 I(iv) of the PCT, the search has been carried out on the basis of the alleged effects of the compounds referred to therein. Claims 40-42 are directed to *in vivo* methods which appear to encompass medical treatment

2 Claim 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 Claim Nos

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a)

Box No. 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 additional fees, this Authority did not invite payment of additional fees

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 claim 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 claim Nos

Remark on Protest The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation

No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No
PCT/CA2009/000935

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO 20040091 13A1	29-01-2004	AU 2003254473A1	09-02-2004
		AU 2003254473B2	01-05-2008
		CA 2492331AA	29-01-2004
		EP 1556080A1	27-07-2005
		GB 0217136D0	04-09-2002
		JP 2005535674T	24-1 1-2005
		US 2006052309A1	09-03-2006

WO 200502561 1A1	24-03-2005	BR PI0414423A	14-1 1-2006
		CA 2539300AA	24-03-2005
		EP 1663300A1	07-06-2006
		JP 2007505887T	15-03-2007
		MX PA06002916A	31-05-2006
		US 2005090466A1	28-04-2005
