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(54) **Title**: COMPOUND INDUCING LBPA ACCUMULATION FOR INHIBITING CELL-TO-CELL TRANSMISSION OF HIV

(57) **Abstract**: The present invention concerns a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell, in an individual in need thereof. The invention further relates to products comprising a compound inducing LPBA accumulation in multi-vesicular bodies of a cell and an anti-HIV agent as combination products for simultaneous, sequential or separated use for the treatment and/or prevention of an infection with a HIV virus.

COMPOUND INDUCING LBPA ACCUMULATION FOR INHIBITING CELL-TO-CELL TRANSMISSION OF HIV

The present invention concerns a compound inducing Lyso-Bis Phosphatidic Acid (LBPA), also named Bis(Monoacylglycero)Phosphate (BMP), accumulation in multi-vesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell, in an individual in need thereof. The invention further relates to products comprising a compound inducing LPBA accumulation in multi-vesicular bodies of a cell and an anti-HIV agent as combination products for simultaneous, sequential or separated use for the treatment and/or prevention of an infection with a HIV virus.

BACKGROUND OF THE INVENTION

Persistent HIV reservoirs lead to the failure of potent antiretroviral therapy to eradicate the virus. Although long life-span quiescent T CD4+ memory cells containing the latent provirus are the major part of this reservoir, there is also a residual virus population in peripheral blood cells. This peripheral blood cell population was shown to be compartmentalized, indicating that there is persistent low-level replication (Delobel, Aids. 2005;19:1739-1750). An important compartment, the monocyte-macrophages, can store infectious virus particles for a long time. The mechanisms governing the biogenesis of these intracellular stores in monocytes-macrophages are still not clear. Some data indicate that HIV assembly takes place at the plasma membrane, while other findings point to the endosomal compartment. These intracellular stores can efficiently infect new cells by cell-to-cell contact, a process that was recently shown to involve the endocytosis of infecting virus particles by the target cell.

HIV budding involves membrane curvature out of the cytoplasm, either within the endosomal lumen or into the extracellular medium. This process occurs physiologically in the cell by intraluminal vesicle budding into multivesicular bodies (MVB) and results in the internalisation and breakdown of membrane proteins, or the back-fusion to deliver the contents of the intraluminal vesicle into the cytoplasm, or the release of the exosome into the extracellular medium. Lipid sorting is a key step in intracellular vesicle trafficking. The lipid lysobisphosphatidic acid (LBPA(BMP)), also called BMP (bis(monoacylglycero)phosphate), is specifically located in the MVB and is necessary for endosome internal budding (Matsuo, Science. 2004;303:531-534). LBPA(BMP) interacts

with Alix, an ESCRT-associated protein that also binds HIV. Two of the steps in LBPA (BMP) metabolism, synthesis of its precursor (Shinozaki, Biochemistry. 1999;38:1669-1675) and its hydrolysis (Ito, J Biol Chem. 2002;277:43674-43681), involve a MAFP-sensitive phospholipase A2 (PLA2) activity. MAFP (methyl arachidonyl fluorophosphonate) also inhibits the cytosolic calcium-dependent phospholipase A2 (cPLA2) that plays a major role in signal transduction and is involved in the trafficking of vesicle between membranes. In macrophages, this enzyme is translocated to the membranes of vesicles involved in the endocytosis pathway.

PLA2 is also involved in the sorting of cholesterol between intracellular compartments and LBPA (BMP) is functionally associated with cholesterol and influences the cellular distribution of cholesterol. Cholesterol is essential for HIV biogenesis and infectivity. The lipid content of the virus envelope evokes that of lipid rafts and the depletion of cholesterol with methyl-β-cyclodextrin reduces the infectivity of HIV. Cells can obtain free cholesterol by the internalization of LDL or by *de novo* synthesis. Another parameter of cellular lipid homeostasis is the intracellular distribution of cholesterol. High concentrations of progesterone retains cholesterol at the plasma membrane, so preventing its redistribution to intracellular membranes and, hence its esterification. The sterol analog, U18666A (3β-(2-Diethylaminoethoxy)androst-5-en-17-one, HCl), is also widely used to modify cholesterol synthesis and distribution, leading to cholesterol accumulation in the MVB compartment (Kobayashi, Nat Cell Biol. 1999;1:113-118). U18666A inhibits two enzymes in the cholesterol biosynthesis pathway, squalene open cyclase and C8-C7 isomerase.

The inventors have investigated the roles of cholesterol and PLA2 in HIV biogenesis, using U18666A and progesterone to modify the intracellular distribution and metabolism of cholesterol, and using the PLA2 inhibitor MAFP. The inventors evaluated the impact of these agents on the capacity of cells to support direct cell-to-cell HIV transfer. The inventors showed that progesterone and MAFP inhibit the egress of HIV, both involving the MVB compartment.

Prior Art:

Some viruses are known to bear a PLA2 activity. The human cytomegalovirus bears a host-cell derived PLA2 activity which is sensitive to inhibitors of cytosolic PLA2. Such inhibitors induce a decrease in de novo expression of certain viral proteins and of virus

production (Allal, J. Virol. 2004;7717-7726). Besides, sequence analysis has revealed PLA2 motifs in capsid proteins of parvovirus. Mutations reducing PLA2 activity decrease viral infectivity. More precisely, this PLA2 activity is critical for efficient transfer of the viral genome into the nucleus to initiate replication (Zadori, Developmental Cell. 2001;291-302). Patent application EP 1,791,858 further describes the use of a modified VP1-capsid protein of parvovirus B19 having a reduced PLA2 activity for use as a medicament for the treatment of a parvovirus infection. However, no role for a PLA2 activity has ever been shown in the HIV replication cycle.

Cholesterol biosynthesis has been shown to be important for the replication of norovirus. Indeed, the inhibition of cholesterol biosynthesis increases the level of norovirus proteins and RNA, whereas inhibitors of ACAT reduce the replication of norovirus (Chang, J. Virol. 2009;8587-8595). However, no effect of the inhibition of cholesterol biosynthesis has been shown on the HIV replication cycle.

TNF synthesis in placental cells is induced by HIV-1 infection that, in an autocrine manner, activates viral replication, because neutralizing anti-TNF antibodies block it. Progesterone is believed to inhibit the induction of TNF synthesis by viral infection and virus or gp-120–induced TNF transcription. Such inhibition leads to a decrease in the production of HIV-1 RNA in placental cells at the concentration found in the placental interface (Munoz, JID 2007;1294-1302). However, no effect of progesterone has been shown on the late phase of the viral cycle.

The international patent application WO 2004/112724 teaches that progesterone is an inhibitor of HIV replication, and probably acts as a steroid hormone receptor antagonist that blocks the steroid hormone receptor complex that comprises Rip-1. Rip-1 is a protein that binds to HIV Vpr and that has been shown to be partially responsible for mediating Vpr activity in the human host cell. WO 2004/112724 teaches the use of progesterone to therapeutically treat an individual infected with HIV in order to eliminate, reduce or stabilize viral titer and/or increase or stabilize CD4+ cell counts. WO 2004/112724 also teaches the use of progesterone to prevent a high risk individual from becoming infected with HIV. However, WO 2004/112724 does not teach that progesterone may interfere with cell to cell transmission of HIV.

Cocaine is associated with an increased risk for, and progression of, clinical disease associated with human immunodeficiency virus (HIV) infection. It has been shown that a

selective sigma-1 antagonist, BD1047, can block the effects of cocaine on HIV replication in the huPBL-SCID mouse (Roth, J. Leukoc. Biol. 2005;78:1198-203). More precisely, this blockage occurs before the viral traduction. However, no effect of this antagonist on the late phase of the viral cycle has been shown.

DESCRIPTION OF THE INVENTION

The inventors have investigated the influence of cellular lipid homeostasis on the spread of HIV between monocyte-macrophage cells, a major component of the HIV reservoir. Altering cholesterol metabolism and distribution, or inhibiting the phospholipase A2 activity, led to an increase in the MVB-specific lysolipid lyso-bis phosphatidic acid (LBPA (BMP)) content and to the formation of large MVB.

The inventors have shown that PLA2 inhibitors and progesterone cause virus to become trapped inside intracellular vesicles, thus reducing the release of HIV to the cell periphery and increasing its intracellular storage (Example 3-6). Moreover, PLA2 inhibitors and progesterone increase the LBPA (BMP) content in the MVB compartment (Example 8) and PLA2 inhibitors enhanced both the total number of MVB and their size (Example 7).

The inventors have finally shown that progesterone and MAFP strongly inhibit HIV proliferation in THP-1 cells and in MDM, and greatly reduce the transfer of the HIV reservoir from MDM to PBL (Example 9-10).

Progesterone is hypothesized to act via the Sigma-1 Receptor. Sigma-1R is a receptor chaperone located in the smooth endoplasmic reticulum. Progesterone is an antagonist of this receptor (Maurice, Pharmacol Ther. 2009;124:195-206), while cocain, which enhances HIV production, is an agonist. PLA2 is involved downstream of the Sigma-1Rs, and blocking these receptors with progesterone could, at least partly, inhibit PLA2 and so increase the cell LBPA (BMP) content (Figure 7). Thus Sigma-1Rs could regulate the “cross-talk” between cholesterol, progesterone, and PLA2, and account for the effects described by the inventors.

The inventors have remarkably shown that this increase in LBPA (BMP) content was accompanied by a decrease in virus particles release, the particles being sequestrated within intracellular vesicles. Moreover, the release of HIV from human monocyte-derived

macrophages (MDM) was inhibited as well as the transfer of HIV from MDM to autologous lymphocytes by direct cell-to-cell contact. Thus, compounds inducing LBPA (BMP) accumulation in MVB could represent a new way of altering the HIV reservoir.

Further, the inventors have shown that compounds inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell, in particular progesterone and MAFP, inhibit entry of HIV into a cell (in particular entry which involves cellular uptake by a macropinocytosis mechanism) (Figures 10-12, and Examples 11-12).

Therefore, the present invention pertains to a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell (more particularly entry which involves endocytosis, preferably which involves macropinocytosis), in an individual in need thereof.

The present invention also relates to a method for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell (more particularly entry which involves endocytosis, preferably which involves macropinocytosis), said method comprises a step wherein an individual in need thereof is administered with an effective amount of a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell.

“Endocytosis” is a process by which cells absorb molecules (such as virus) by engulfing them.

The term “macropinocytosis” is intended to mean a biological mechanism which involves the invagination of the cell membrane to form a pocket comprising the virus, said pochet then pinches off into the cell to form a vesicle. The vesicle then travels into the cytosol and fuses with other vesicles such as endosomes and lysosomes. The formation of the pocket occurs in a non-specific manner, then does not need any specific receptor.

In the context of the invention, Human Immunodeficiency Virus (HIV) denotes HIV-1 or HIV-2 of any group (M, including subtypes A-K, and their recombinant forms N, O or P). Preferably HIV is HIV-1.

It is generally considered that HIV assembly takes place at the plasma membrane, leading to the release of newly synthesized virions in the extracellular compartment and

indirect cell-to-cell transmission. However, other findings point to the endosomal compartment. These intracellular stores can efficiently infect new cells by direct cell-to-cell contact (Gousset, PLoS Pathog. 2008;4:e1000015 ; Groot, Blood; 2008;111:4660-4663 ; Sattentau, Nat Rev Microbiol. 2008;6:815-826). These cell-to-cell contacts take place at the interface between an infected cell and an uninfected cell, where a supramolecular structure termed a virological synapse is believed to form. In the case of direct cell-to-cell contacts, the newly synthesized virions are directly transmitted from an infected cell to another cell without between released in the extracellular compartment.

The compound of the invention is intended for use for inhibiting direct or indirect cell-to-cell transmission. Preferably, the compound of the invention is intended for use for inhibiting direct cell-to-cell transmission.

HIV budding involves membrane curvature out of the cytoplasm, either within the endosomal lumen or into the extracellular medium. This process occurs physiologically in the cell by intraluminal vesicle budding into multi-vesicular bodies (MVB). MVBs, also known as late endosomes, are mainly spherical, lack tubules, and contain many close-packed luminal vesicles. Markers include RAB7 and RAB9 and mannose 6-phosphate receptors. The inventors have shown that inhibiting the phospholipase A2 activity leads to the formation of large MVB.

The Lyso-Bis Phosphatidic Acid (LBPA (BMP)), also called Bis Mono-acyl-glycero Phosphate (BMP), is a lipid specifically located in the MVB that is necessary for endosome internal budding.

LBPA (BMP) accumulation in multi-vesicular bodies (MVB) may be measured by various methods well-known by one skilled in the art. For instance, it may be quantified by intracellular MVB LBPA (BMP) immuno-labeling using for instance the 6C4 anti-LBPA (BMP) antibody and microscopy or FACS analysis, as described in Example 8. Alternatively, LBPA (BMP) may be separated by two-dimensional thin-layer chromatography followed by an HPLC isolation on Nucleosil-NH2 support and biochemical quantification of its fatty acid content may be performed by gas chromatography.

In a preferred embodiment, an increase in the LBPA (BMP) content in the MVB compartment of at least 10%, 20%, 30%, 40% or 50% in cells treated with the compound compared to untreated cells is indicative of a LBPA (BMP) accumulation in the MVB.

In some embodiments, the compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell for use for inhibiting cell-to-cell transmission of a HIV in an individual in need thereof is a Phospholipase A2 (PLA2) inhibitor or a Sigma-1 Receptor antagonist. By PLA2 inhibitor is meant a compound that inhibits the activity and/or the expression of the Phospholipase A2. By Sigma-1 Receptor antagonist is meant a compound that inhibits the activity and/or the expression of the Sigma-1 Receptor. Preferably, the inhibitors or antagonists according to the invention are direct inhibitors or antagonists that bind to their targets.

The compound inducing LBPA (BMP) accumulation may be a small molecule, an organic or inorganic compound, a peptide inhibitor, a peptidomimetic, an antibody, a lipid, an antisense, an RNA interference, an aptamer, or a ribozyme blocking PLA2 or Sigma-1R activity and/or expression.

In preferred embodiments, said compound is selected from the group consisting of MAFP (methyl arachidonyl fluorophosphonate), ATMK / AACOCF3 (arachidonyl trifluoromethyl ketone), Pyrrolidine-2 (N-{(2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl), Pyrrophenone (N-[[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide), BEL / HELSS (BromoEnolLactone or HaloEnolLactone Suicide Substrate), FKGK11 (1.1.1.2.2-pentafluoro-7-phenyl-3-heptanone), Me-INDOXAM (2-(8-((2-Carboxyethyl)oxy)-2-ethyl-3-(o-phenylbenzyl)-indolizin-1-yl)glyoxylamide), Progesterone, NE100 (N,N-diethyl-2-(4-methoxy-3-phenethyloxyphenyl)ethanamine hydrochloride, N-[2-(4-methoxy-3-phenethyloxyphenyl)ethyl]-N-propylpropan-1-amine-hydrochloride), BD1063 (1-[2-(3,4-dichlorophenyl)-ethyl]-4-methylpiperazine dihydrochloride), BD1047 (N-[2-(3,4-dichlorophenylethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide), Haloperidol (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one) and BMY-14,802 (1-(4-fluorophenyl)-4-[4-(5-fluoropyrimidin-2-yl)piperazin-1-yl]butan-1-ol).

In a much preferred embodiment, the compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) is MAFP or progesterone.

In another preferred embodiment, the compound inducing LPBA accumulation in multi-vesicular bodies (MVB) is not progesterone.

Interfering with cell to cell transmission of HIV prevents viral load from increasing. It may also result in a decrease of viral load if the cells infected with HIV are eliminated by the immune system or if an anti-HIV is used in combination with the compound inducing LPBA accumulation in multi-vesicular bodies (MVB).

According to some embodiments, the invention also pertains to a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) for use for decreasing the viral load of a patient infected with HIV. The term “viral load” refer to the amount of virus present in an involved body fluid. For example, it can be given in RNA copies per milliliter of blood plasma. Different techniques for measuring the viral load are well-known by the one skilled in the art. These include PCR, RT-PCR, ELISA and/or determination of virus-associated reverse transcriptase (RT) activity.

Preferably, the compound inducing LPBA accumulation in multi-vesicular bodies (MVB) does not interfere or inhibit HIV replication cycle in cells.

The expression “individual in need thereof” is intended for a human or non-human mammal infected or likely to be infected with HIV. Preferably the patient is a human. More preferably, the individual in need of a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell for use for inhibiting cell-to-cell transmission of a HIV is a symptomatic individual suffering from a HIV infection.

A “HIV infection” refers to the condition of an individual who is infected with HIV. HIV infection indifferently denotes an asymptomatic HIV infection or a symptomatic HIV infection including the acquired immunodeficiency syndrome (AIDS), whatever its stage of development. Therefore, according to some embodiment, the individual is a symptomatic individual suffering from AIDS.

In another embodiment, the individual is an asymptomatic individual who has been exposed or is at risk of having been exposed to HIV. Such individuals include health care

or other individuals who may have accidentally exchanged blood or other biological fluids with an HIV-infected individual, such as through an accidental needle stick, injuries that occur during emergency medical care, rescue or arrest and unprotected sexual contact. Additionally, the present invention is particularly useful to prevent recurrence of infection in patients who have been previously diagnosed as HIV positive but show no indication of infection.

HIV virus may infect different types of cell including monocytes, macrophages, and lymphocytes. Therefore, in a preferred embodiment the cell wherein the compound of the invention is intended to induce LPBA accumulation in MVB is a monocyte, a macrophage or a lymphocyte. More preferably, the cell is a T lymphocyte. Even more preferably, the cell is a T helper lymphocyte (LT4).

In the context of the invention, inhibiting cell to cell transmission of HIV means inhibiting HIV transmission from a monocyte to a monocyte and/or to a macrophage and/or to a lymphocyte. Inhibiting cell to cell transmission of HIV can also mean inhibiting HIV transmission from a macrophage to a monocyte and/or to a macrophage and/or to a lymphocyte. Alternatively, inhibiting cell to cell transmission of HIV can also mean inhibiting HIV transmission from a lymphocyte to a monocyte and/or to a macrophage and/or to a lymphocyte.

The inventors have shown that progesterone and inhibitors of PLA2 strongly inhibit HIV proliferation in THP-1 cells and in MDM, and greatly reduce the transfer of the HIV reservoir from MDM to PBL (Example 9-10). Therefore, the compound of the invention is preferably for use for inhibiting cell-to-cell transmission of HIV from a monocyte, or a macrophage, or a lymphocyte to a lymphocyte.

The compound of the present invention may be administered as a pharmaceutical composition. Thus, another aspect of the invention pertains to a pharmaceutical composition comprising a compound inducing LPBA accumulation in multi-vesicular bodies of a cell for the treatment and/or prevention of HIV infection.

As used herein, the term "treatment", means the action of reversing, alleviating, inhibiting the progress of the HIV infection, or one or more symptoms of such infection.

In addition to treating HIV-infected individual, the present combination products may be used for preventing HIV infection in high risk individuals who, for example, are suspected of having been exposed to the virus.

Pharmaceutical compositions comprising a compound of the invention include all compositions wherein the peptide is contained in an amount effective to achieve the intended purpose. In addition, the pharmaceutical compositions may contain suitable physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

The term "physiologically acceptable carrier" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. Suitable physiologically acceptable carriers are well known in the art and are described for example in Remington's Pharmaceutical Sciences (Mack Publishing Company, Easton, USA, 1985)., which is a standard reference text in this field. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

Besides the physiologically acceptable carrier, the compositions of the invention can also comprise minor amounts of additives, such as stabilizers, excipients, buffers and preservatives.

The composition of the invention may further comprise a second active principle, in particular an anti-HIV therapeutic agent. When formulated within the same composition, the compound of the invention and the anti-HIV therapeutic agent may have a complementary action.

The compound of the present invention may be administered either as an individual therapeutic agent or in combination with other therapeutic agents, in particular anti-HIV therapeutic agents. Thus, another aspect of the invention pertains to products comprising a compound inducing LPBA accumulation in multi-vesicular bodies of a cell and an anti-HIV agent as combination products for the treatment and/or prevention of HIV infection.

The combination products comprising the compound of the invention and an anti-HIV agent may be formulated as separate pharmaceutical compositions and may be administered sequentially or simultaneously or used separated.

An "anti-HIV agent" denotes a compound which leads to decreased HIV replication. There are different classes of anti-HIV drugs that act at different stages of the HIV life-cycle.

Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. Examples of integrase inhibitors include raltegravir and elvitegravir which is currently under clinical trial.

Reverse transcriptase inhibitors include nucleoside and nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI).

NRTI inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation. Abacavir (ABC), emtricitabine (FTC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), zidovudine (AZT) and tenofovir (TDF) are examples of NRTI.

NNRTI inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function. Delavirdine, efavirenz (EFV), nevirapine (NVP), etravirine (ETV) are four currently available agents in this class.

Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virions. Examples of protease inhibitors include atazanavir, darunavir, indinavir, fosamprenavir, lopinavir, nelfinavir, saquinavir, tripanavir and ritonavir.

Entry inhibitors include fusion inhibitors and CCR5 inhibitors. They interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets. Maraviroc and vicriviroc (which is currently under clinical trial) are CCR5 inhibitors and enfuvirtide is one currently available fusion inhibitor.

Maturation inhibitors inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein. Because these viral particles have a defective core, the virions released consist mainly of non-infectious particles. Two drugs in this class are under investigation, bevirimat and Vivecon.

Therefore, the invention also pertains to products comprising a compound inducing LPBA accumulation in multi-vesicular bodies of a cell and an anti-HIV agent selected from the group consisting of integrase inhibitors, reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, fusion inhibitors, CCR5 inhibitors, and maturation inhibitors as combination products for simultaneous, sequential or separated use for the treatment and/or prevention of an infection with a HIV virus.

According to another embodiment, the invention pertains to a pharmaceutical composition comprising a compound inducing LPBA accumulation in multi-vesicular bodies of a cell and an anti-HIV agent selected from the group consisting of integrase inhibitors, reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, fusion inhibitors, CCR5 inhibitors, and maturation inhibitors.

The compound of the present invention may be administered by any means that achieve the intended purpose. For example, administration may be achieved by a number of different routes including, but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intracerebral, intrathecal, intranasal, oral, rectal, transdermal, buccal, topical, local, inhalant or subcutaneous use. Parenteral and topical routes are particularly preferred.

Dosages to be administered depend on individual needs, on the desired effect and the chosen route of administration. It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

Generally, multiple administrations are performed. According to preferred embodiments of the invention, the compounds are provided over a course of time in which a therapeutically effective amount of compound is present in the individual's body so as to reduce the viral load to essentially undetectable levels or essentially undetectable levels

such that an asymptomatic individual will not develop symptoms or the onset of such symptoms shall be delayed.

Depending on the intended route of delivery, the compounds may be formulated as liquid (e.g., solutions, suspensions), solid (e.g., pills, tablets, suppositories) or semisolid (e.g., creams, gels) forms.

By "effective amount" is meant an amount sufficient to achieve a concentration of compound which is capable of preventing, treating or slowing down the disease to be treated. Such concentrations can be routinely determined by those of skilled in the art. The amount of the compound actually administered will typically be determined by a physician or a veterinarian, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the subject, the severity of the subject's symptoms, and the like. It will also be appreciated by those of skilled in the art that the dosage may be dependent on the stability of the administered compound.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Effect of U18666A and progesterone on HIV infection of THP1 cells.

Data are expressed as HIV RNA $\log_{10}$ copies / million viable cells.

(A) As compared to control (■), U18666A (7 µM) (▲) was added on day 0 to cells with low (1:1000, panel A1) or high (1:2, panel A2) infection ratios.

(B) Identical experiments with progesterone (▲), 10 µM final, added to infected cells (1:1000 and 1:2 infection ratios in panel B1 and panel B2 respectively) on day 0, as compared to control (■) .[(*) *p<0.01*]

Figure 2. Influence of lipid disturbing agents on HIV-1 release and HIV-1 intracellular stores in THP-1 cells.

Infected and uninfected THP-1 cells were mixed in a 1:1000 ratio and drugs added to the culture each day. The final concentration on day 4 was 20 µM for MAFP, 40 µM for progesterone (PROG), 28 µM for U18666A (U18), 20 µM for zidovudine (AZT) and 40 µM for lopinavir (LPV).

(A) Cells were counted after staining with Trypan blue and the result expressed as millions of cells per ml. The graph represents the mean of 11 independent experiments and the bars the standard error of the mean.

(B) The ATP content was quantified for 75,000 viable cells using the Cell Titer Glo® kit (Promega). The graph represents the mean of 4 independent experiments and the bars the standard error of the mean.

(C) HIV-1 RNA in the culture supernatant was quantified as HIV-1 RNA log10 copies / million viable cells. The graph represents the mean of 8 independent experiments and the bars the standard error of the mean. Differences between each drug and the control are significant ($p<0.05$).

Figure 3. Effect of lipid disturbing agents on HIV-1 production by co-cultures of primary macrophages (MDM).

Monocytes-derived macrophages (MDM) were prepared as described in "Material and Methods", infected by coculture (ratio 1:100) and treated by repeated additions of the three drugs to reach final concentrations at day 4 of 15 µM for MAFP, 30 µM for progesterone, 21 µM for U18666A.

The HIV-1 RNA in the culture medium was normalized to the signal (relative luminescence units) obtained for viable cells with the Cell Titer Glo ® Promega kit and expressed relative to the control. The graph represents the mean of 5 independent experiments and the bars the standard error of the mean. Differences are significant between MAFP, PROG, U18 and the control ($p<0.05$).

Figure 4. Effect of methyl arachidonyl fluorophosphonate (MAFP), a cytosolic phospholipase A2 inhibitor, on HIV production by THP1 cells.

Data are expressed as HIV RNA $\log_{10}$ copies / million viable cells.

Cells infected at low (A) or high (B) infection ratios were incubated with 5 µM MAFP (▲) (added on day 0) as compared to control (■).(Panel A, ratio 1:1000, *p=0.07* on day 8; Panel B, ratio 1:2, *p=0.10* on day 8).

(C) Effect of repeated additions of MAFP added to the cultures every day (except at day 5) giving concentration ranges of 0.6 µM to 35 µM. Between day 0 and day 8, MAFP concentration increased of 0.6 µM to 4.2 µM (●); 2.5 µM to 17.5 µM (▼) and 5 µM to 35 µM (■). Untreated cells (▲). [(*) *p< 0.001*]

Figure 5. Effect of HIV infection, U18666A, progesterone and PLA2 inhibitor on the MVB compartment of THP1 cells

The integrity of the overall MVB compartment was evaluated, qualitatively by immunofluorescence, and quantitatively by FACS by incubating cells with Rh-PE, a fluorescent lipid that specifically targets the MVBs. Immunofluorescence was examined

with a Zeiss wide-field microscope (objective lens 63X) Bar 10 µm. Cells were examined on day 4.

(A) Uninfected THP-1 cells

(B-E) Infected (1:2 ratio) THP-1 cells treated with vehicle (B) or repeated additions of 7 µM U18666A (C), 10 µM progesterone (D) or 5 µM MAFP (E).

(F) Percentage of labeled cells of a representative experiment. Open bars: uninfected cells. Black bars: infected cells (ratio 1:2).

Figure 6. Effect of progesterone and PLA2 inhibitor on the LBPA (BMP) content of infected THP1 cells

Cells at low infection ratio (1:1000) were cultured for 4 days in the presence of daily additions of ethanol (vehicle), MAFP (5 µM), or progesterone (10 µM). After 4 days of coculture, i.e on cells in presence of 20 µM MAFP or 40 µM progesterone. Cells were then collected by centrifugation and the LBPA (BMP) content in a lipid extract was measured. The LBPA (BMP) content is expressed as nmol/50x10^6 viable cells. Each type of symbol represents an independent experiment (Expt 1 △, Expt 2 +, Expt 3 x).

Figure 7. Effect of progesterone and PLA2 inhibitor on the LBPA (BMP) detected in infected THP1 cells

Cells at low infection ratio (1:1000) were cultured for 4 days in the presence of daily additions of ethanol (vehicle), MAFP (5 µM), or progesterone (10 µM). After 4 days of coculture, i.e on cells in presence of 20 µM MAFP or 40 µM progesterone. Cells were then immunolabeled with anti-LBPA (BMP) antibody and the fluorescent signal quantified on a BD FACS Calibur. Each symbol represents an independent experiment. Data are expressed as mean fluorescence intensity (MFI) (Expt 4 ○, Expt 5 ◇, Expt 6 □).

Figure 8. Effect of progesterone and PLA2 inhibitor MAFP on HIV production by cell-to-cell infection of human MDM.

MDM were prepared as described in Example 1, infected by coculture (ratio 1:100) and treated by repeated additions (every two days) of 10 µM progesterone, 5 µM MAFP or 5 µM AZT. HIV RNA was measured on day 4 of treatment (i.e at final concentrations of 15 µM of MAFP or AZT and 30 µM progesterone). HIV RNA was normalized to the signal obtained from viable cells with the Cell Titer Glo ® Promega kit. $p < 0.005$ for MAFP, progesterone or AZT, versus control.

Figure 9. Effect of MAFP and progesterone on direct cell-to-cell transfer.

A PCR experiment was performed on HIV DNA extracts from unadherent autologous leukocytes cocultivated with MDM, following repeated additions (every two days) of ethanol (CTL), MAFP 5 µM or 10 µM progesterone and 10 µM lopinavir. Quantification of the effect of MAFP and progesterone is expressed as the $2^{[-delta(deltaCt)]}$ count for a single addition on day 0 (Expts 1 and 2), or for repeated additions (Expts 3-5, *p< 0.0001*) of MAFP or progesterone, versus control.

Figure 10. Influence of Lipid disturbing agents on endocytosis.

THP-1 cells were treated each day to obtain final concentrations on day 4 of 20 µM for MAFP, 40 µM for progesterone (PROG) and 28 µM for U18666A (U18). Endocytosis was monitored by the internalization of FITC dextran. Histograms represent the fluorescence measured at 4°C (dashed lines) and at 37°C (solid lines). Similar results were obtained in three independent experiments.

Figure 11. Influence of lipid disturbing agents on surface molecules involved in HIV-1 spread.

Infected and uninfected THP-1 cells were mixed in a 1:1000 ratio and drugs added to the culture each day. The final concentration on day 4 was 20 µM for MAFP, 40 µM for progesterone (PROG) and 28 µM for U18666A (U18). The cells were labeled with anti-CD4, anti-CCR5, anti-CXCR4, anti-LFA-1 and anti- ICAM-1 antibodies and analysed using a BD Biosciences LSRII Flow Cytometer™. The Mean Fluorescence Intensity (MFI) was calculated using the DIVA™ software. The graph represents the mean of 3 independent experiments and the bars the standard error of the mean.

Figure 12. Influence of lipid disturbing agents on the cell content of BMP and on sterol distribution in THP1 monocytes

Infected and uninfected THP-1 cells were mixed in a 1:1000 ratio and drugs added each day to obtain the final concentrations of MAFP, progesterone (PROG) and U18666A (U18) on day 4. The cells were stained with the 6C4 anti-LBPA (BMP) antibody and analysed by FACS.

(A) BMP quantified by the mean fluorescence intensity (MFI) of the cell population and expressed as percentage of that of the controls. The graph represents the mean of independent experiments and the bars the standard error of the mean (CTL and MAFP: n= 5, CTL and PROG: n=7, CTL and U18: n=8). Differences are significant between PROG or U18 and the control ($p<0.05$).

(B) Representative FACS profiles for each drug. The percentage of labeled cells and the MFI are shown.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 shows the sequence of a forward primer specific for the gag-LTR sequence

SEQ ID NO: 2 shows the sequence of a reverse primer specific for the gag-LTR sequence

SEQ ID NO: 3 shows the sequence of a Taqman® probe

SEQ ID NO: 4 shows the sequence of a forward primer specific for the beta-actin gene

SEQ ID NO: 5 shows the sequence of a reverse primer specific for the beta-actin gene

EXAMPLES

Example 1: Materials and Methods

Material

Rhodamine–PE (Rh-PE) [(1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)] was purchased from Avanti Polar Lipids, Birmingham, Alabama, USA. Progesterone and filipin were supplied by Sigma-Aldrich Chimie S.a.r.l. Lyon, France. MAFP was obtained from Biomol International (Enzo Life Sciences, Villeurbanne, France). Macrophage colony stimulating factor (M-CSF) was purchased from Miltenyi Biotec SAS, Paris, France. QIAamp Viral mini kits and QIAamp DNA blood mini kits were purchased from Qiagen SA, Courtaboeuf, France. RT-PCR kits were from Roche Diagnostics, Meylan, France. Culture media and FITC-dextran (MW 10,000) were supplied by Invitrogen SARL, Cergy Pontoise, France. Lopinavir (LPV) and zidovudine (AZT) were obtained from the NIH Research Reagents Program, Germantown, MD, USA. U18666A (3β-(2-Diethylaminoethoxy)androst-5-en-17-one, HCl) was purchased from Sigma. PE-labeled goat anti-mouse IgG antibody was from AbD Serotec, Colmar, France. Anti-LBPA (BMP) antibody was a generous gift from T Kobayashi (Lipid Biology Laboratory, RIKEN Institute, Saitama, Japan). PE-anti-CD206, Pacific blue anti-CD4, APC-anti-CD54 (ICAM-1), FITC anti-CD11a (LFA-1), PE- anti-184 (CXCR4) and PerCP-Cy5.5 anti-CD195 (CCR5) and compensation beads were obtained from BD biosciences, Le Pont de Claix, France. anti-HIV-1 (KC-57 FITC) from Beckman Coulter, Fullerton, USA.

Cell culture

The human promonocytic THP-1 cells were purchased from the ATCC (ATCC number TIB-202™) and maintained at 5.10^5 cells/ml in RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (10% FCS RPMI).

Peripheral blood mononuclear cells were prepared from buffy coats from healthy donors (Etablissement français du sang, Toulouse), plated in 6-well plates (10^7 cells/well) and incubated for 2 hours at 37°C. Adherent monocytes were washed three times with phosphate-buffered saline (PBS) and cultured for 7 days in RPMI -10% FCS containing 25 ng/ml M-CSF to trigger their differentiation into macrophages (MDM, monocyte-derived macrophages). The differentiation from monocytes to macrophages was monitored by quantifying CD206 by FACS.

The viability of THP-1 cells was analysed by trypan blue staining and that of MDM using the luminescent assay, Cell Titer Glo kit® (Promega).

Infection

Infection from virus suspensions. THP-1 cells were infected with the HIV_{LAI} strain and MDM were infected with the HIV_{Ba-L} strain. This was done by incubating the cells with a titrated suspension of virus for 3 hours at 37°C. The ratio of infection was 10 copies of virus RNA per THP-1 cell and 100 copies per MDM. The cells were then washed 5 times with RPMI and placed in RPMI-10% FCS. The infected THP-1 cell line was maintained by adding 3 ml of uninfected THP-1 cells to 20 ml of the infected cell suspension every week.

Infection by coculture of infected and uninfected cells. THP-1 cells infected as described above on day 0, collected by centrifugation, and mixed with uninfected THP-1 at various ratios. Similarly, infected MDM were scraped off the plate, collected, and added to uninfected MDM in a ratio of 1:100.

For immunofluorescence experiments, a glass coverslip was placed in the wells.

Cell treatments

All the molecules used were dissolved in ethanol (0.1% final v/v) before adding them to cell cultures.

Treatment of THP1-cells on day 0. Molecules were added to the culture medium containing 1% FCS of mixed infected and uninfected cells on day 0 to give the indicated final concentration.

Repeated treatment of THP1 cells or MDM. Molecules were added to the culture medium containing 1% FCS on day 0 to give the indicated final concentration. A fraction of the culture medium was removed and replaced by fresh medium containing the same

amount of test molecules as on day 0. This was done daily for THP1 and every two days for MDM.

Analysis of direct cell-to-cell transfer of HIV

MDM were infected with the HIV_{Ba-L} strain as described above. Treatment was repeated every two days. Lopinavir (10 µM) was added on day 6, and treatment continued for 4 more days. The cells were washed on day 10 incubated with autologous PBL for three hours for contact with MDM containing potentially infectious virus stores. Non-adherent cells were then cultivated for two days in RPMI 10% FCS and their infection evaluated by measuring their HIV DNA content.

HIV quantification

RNA was extracted from 140 µl aliquots of culture supernatant using QIAamp viral RNA mini kits (Qiagen) and quantified by real time RT-PCR targeting the LTR gene, as described by Mavigner et al 28. Each run was 45 cycles and the standard curve established from the supernatant of a culture of infected PBMC that had been quantified by a routine automated method (Roche Diagnostics).

DNA was extracted from 10^6 cells using QIAamp DNA blood mini kits (Qiagen) and quantified by real time PCR targeting a gag-LTR sequence using the following primers, forward 5'-GCCTCAATAAAGCTTGCCTTGA (SEQ ID NO: 1) and reverse 5'-GCTCTCTCGACGCAGGACT (SEQ ID NO: 2), and detected with the Taqman® probe 5'-6Fam-AAGTAGTGTGTGCCCGTCTGTTRTKTGACT-Tamra-3' (SEQ ID NO: 3). Each run was for 45 cycles. The beta-actin gene in each sample was amplified using the primers: forward 5'-GATGAGATTGGCATGGCTTT (SEQ ID NO: 4) and reverse 5'-AGAGAAGTGGGGTGGCTTTT (SEQ ID NO: 5), and the products were detected using SyBRgreen®. The HIV/beta-actin DNA ratio was evaluated using the $2^{[-delta(deltaCt)]}$ count.

Peripheral cellular receptor labeling

Cells (10^6) that had been treated for 4 days were collected by centrifugation at 105Xg for 5 min, washed in PBS and incubated with PBS 5% human serum, 1% BSA for 15 min at 4°C. They were then labeled with anti-CD11a, anti-CD195, anti-CD184, anti-CD54 and anti-CD4 antibody for 30 min at 4°C, washed twice in PBS and fixed with a PBS-3.7% PFA solution for 15 min at 4°C. They were finally analysed using a BD Biosciences LSRII Flow Cytometer™ and DIVA™ software. Compensation was done using single-stained compensation beads.

MVB characterization using Rh-PE

Rh-PE labeling: Appropriate amounts of Rh-PE stored in in chloroform:methanol (2:1) were dried and suspended in absolute ethanol. The ethanolic solutions were diluted with PBS to give the final concentration (3µM) and vortexed vigorously. An aliquot (1 ml) of this mixture was then added to 2.10^6 cells per condition. These cells had been treated for 4 days and collected by centrifugation. The cells were incubated with Rh-PE for 60 min at 4°C. The medium was then removed, the cells extensively washed with PBS containing 3% w/v BSA, and they were incubated in 1% FCS RPMI for 3 hours at 37°C. A sample of 3.10^5 labeled cells was washed in PBS and fixed with PFA 3.7% for 20 min.

FACS analysis: labeled cells were analysed using a BD Biosciences FACSCalibur Flow Cytometer™ and WinMDI™ software.

Wide field microscopy: Washed cells were made to adhere onto L-polylysine-coated microscope slides using a cytospin at 600 rpm 6 min and mounted between sealed glass coverslips and microscope slides with Fluorsave®. They were then examined under a Zeiss wide field microscope on the RIO cellular platform of IFR 150, Toulouse, France.

Lyso bis phosphatidic acid (LBPA (BMP), or bis(monoacylglycero)phosphate [BMP]) quantification

Intracellular MVB LBPA (BMP) labeling with anti-LBPA (BMP) (6C4) and FACS analysis: A sample (10^6) of cells that had been treated for 4 days were collected by centrifugation (1200 rpm/ 5 min), washed with PBS and fixed by incubation in 3.7% PFA for 20 min at 4°C. They were rinsed with PBS and permeabilized by incubation with PBS containing 0.05% saponin and 1% BSA for 10 min at 4°C. The permeablized cells were incubated with Fc blocking reagent for 10 min at 4°C and then with the anti-LBPA (BMP) antibody for 45 min at 4°C. The cells were washed again and incubated with PE-conjugated goat anti-mouse antibodies for 45 min at 4°C. Finally, they were washed and analysed in a BD Biosciences FACSCalibur Flow Cytometer™ and WinMDI™ software.

Biochemical quantification of LBPA (BMP): Lipids were extracted from 50.10^6 cells with chloroform and methanol plus the antioxidant bromohydroxytoluene (BHT). Internal standard (C_{15} BMP) was added and LBPA (BMP) was measured by chromatography.

Macropinocytosis measurement

Macropinocytosis of THP-1 cells was measured by their uptake of FITC-dextran. Cells (5.105) were incubated with 1 mg/ml FITC-dextran in PBS with 2% FCS for 30 min either at 4°C to block endocytosis and to measure nonspecific binding, or at 37°C to

measure specific uptake. The cells were then washed three times in cold PBS-FCS and analysed using a BD Biosciences FACSCalibur Flow Cytometer™ and FlowJo™ software.

Transmission electron microscopy (TEM)

Cocultures (1:2 ratio) of infected and uninfected cells were fixed by incubation with 2% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH = 7.4) for 1 hour and washed with the Sorensen phosphate buffer for 12 hours. These cells were postfixed with 1% OsO_4 in Sorensen phosphate buffer (0.05 M Sorensen phosphate, 0.25 M glucose, 1% OsO_4) for 1 hour, washed twice with distilled water and prestained with 2% aqueous uranyl acetate for 12 hours. The samples were dehydrated in an ascending ethanol series, embedded in Epon 812 (Electron Microscopy Sciences) and the resin polymerised for 24 hours at 60°C. Ultrathin sections (70 nm thick) were cut and mounted on 150 mesh collodion-coated copper grids and poststained with 3% uranyl acetate in 50% ethanol and with 8.5% lead citrate. The embedded cells were examined in a HU12A Hitachi electron microscope at an accelerating voltage of 75 KV.

Statistical analyses

Graphs were prepared with GraphPad Prism software and statistical analyses were done using StatView software (SAS Institute Inc), including the F-test for comparing variances and Student's *t*-test for comparing results.

Example 2: HIV infection between monocytic THP1 cells

Because HIV can spread by direct cell-to-cell transmission, the inventors used cocultures of infected and uninfected cells. Infected monocytic THP1 cells were mixed with uninfected ones in various ratios, and the amounts of virus released from the cells over time was measured. The ratios between cells varied from 1 infected cell to 2 uninfected ones ("high infection ratio"), to 1 infected cell to 1000 uninfected ones ("low infection ratio") and reached a plateau on day 4 in all the conditions tested. Cell proliferation was at a steady-state. A very high titer (10^9 HIV RNA copies / ml culture medium) was obtained with a ratio of 1:2 and infected cells had to be diluted 1000-fold to obtain a virus titer of 10^6 RNA copies / ml. The virus titer on day 4 was correlated with the ratio of infected to uninfected cells. Therefore, imaging analyses and LBPA (BMP) measurements were performed on day 4. AZT inhibited the infection by 1 log even at a high infection ratio (1:2), which is consistent with its effect *in vitro*.

Electron microscopy indicated that infected cells present on day 4 differed from control uninfected cells in that they contained large amounts of virus that appeared

agglutinated around the cells. There was budding of the virus from the plasma membrane but also virus was present in intracellular vesicles.

Example 3: Modification of cholesterol distribution and viral infection

Intracellular trafficking requires constant changes in the lipid composition of membranes and cholesterol is critical because any impairment of its distribution leads to cellular defects. HIV itself modulates the host cell cholesterol content. The inventors therefore investigated the influence of the cellular cholesterol distribution on HIV production under the experimental conditions outlined in Example 2. U18666A, a cationic amphiphilic compound causes the cholesterol precursors desmosterol and squalene epoxyde to accumulate in endosomes. U18666A had a small, transient effect on day 2 which no longer persisted for longer periods (Figure 1A) even when the ratio of infected /uninfected cells was low (Figure 1A1), or when repeated amounts of U18666A were added. Progesterone causes cholesterol to accumulate at the cell periphery, a different distribution to that caused by U18666A. Progesterone (10 µM) added on day 0 inhibited HIV production by one log by THP1 cells in a low ratio of infected/uninfected cells (1:1000) (Figure 1B), and by about 0.5 log at a high ratio (1:2) of infected/uninfected cells (Figure 1B2). Adding progesterone each day to a high ratio (1:2) of infected/uninfected cells was no more effective than a single addition at day 0.

The inventors used filipin staining to monitor the distribution of free sterols in infected cells. There was no apparent difference between infected and uninfected cells. The cholesterol in cells cultured at an infected/uninfected cells ratio of 1:1000 was diffusely distributed through the cytoplasm. U18666A increased the accumulation of free cholesterol and its precursors in the intracellular vesicles of these cells, while progesterone caused free cholesterol to accumulate at the cell periphery. Similar results were obtained when cells were cultured at an infected/uninfected cell ratio of 1:2.

Electron microscope examination of cells cultured with U18666A for four days showed that the virus was mainly around the cells, while cells cultured with progesterone showed no accumulation of virus around the cell, but HIV was inside intracellular vesicles. Therefore, the redistribution of cellular cholesterol appears to play a key role in the control of virus release.

Example 4: Influence of MAFP and progesterone on HIV-1 release from THP-1 cells and on HIV-1 intracellular stores

HIV release in the supernatant of cocultures of HIV-infected and non infected THP-1 cells depends on the ratio of these infected to non infected cells, (data not shown).

In order to evaluate the effect of MAFP and progesterone on HIV-1 release and storage, the inventors chose concentrations given preliminary results in the laboratory. Cultures were treated from day 0 to 3 with selected drugs and analyzed at day 4. The cytotoxicity observed after cell staining was not higher for MAFP and progesterone than for antiretroviral treatments already used in HAART, especially lopinavir, and was similar in non infected cultures and in cocultures of infected and uninfected cells mixed in a ratio of 1:1000 (Figure 2A). Moreover, the ATP content of viable cells was quantified as a control of their global metabolic activity, which was in the same range whatever the treatment was (Figure 2B), and was similar between infected and uninfected cells. In these conditions, HIV-1 release was measured in the culture supernatant by quantification of HIV-1 RNA relatively to the number of viable cells. MAFP and progesterone induced a significant decrease in the HIV-1 released into the culture supernatant on day 4, the virus titer being 0.5 to 0.75 $\log_{10}$ lower than that of the control (Figure 2C).

Example 5: Influence of MAFP and progesterone on HIV-1 release from primary cultures of human monocyte-derived macrophages (MDM)

The effects of MAFP and progesterone on primary human monocyte-derived macrophages (MDM) were confirmed. Compared to control and whatever the treatment was, around 75 % of cells were viable at day 4 of coculture of infected and non infected cells (Figure 3A, left bars). MAFP and progesterone strongly inhibited HIV-1 infection of MDM. Compared to control, 15% and 10% of HIV-1 RNA were retrieved in the cell supernatant for MAFP and progesterone respectively (Figure 3A, right bars). Also, confocal microscopy experiments showed that this decrease of HIV-1 release was associated to the accumulation of BMP in treated cells (data not shown). Like in THP-1 cells, control MDM cells were faintly stained by the anti-BMP antibody, whereas the treated ones had accumulated BMP in intracellular vesicles.

Example 6: Effect of a cPLA2 inhibitor on HIV infection of THP1 cells

PLA2 activity regulates cholesterol-induced Golgi vesiculation and its lipolytic activity enables the cell to generate outward-bending membranes and to regulate membrane trafficking events. The inventors therefore examined the influence of PLA2 inhibition on HIV production by determining the effect of the PLA2 inhibitor, methyl arachidonyl fluoro phosphonate (MAFP) on the spread of HIV in cell cultures. MAFP added to cultures on day 0 inhibited by 0.5 log the HIV production at a low ratio of infected to uninfected cells (1:1000, Figure 4A) and to a lesser extent at a high infection ratio (Figure 4B). Repeated treatment inhibited the HIV production by 1 log at high ratio of infected cells (Figure 4C);

the most efficient concentrations were 5-35 µM. Electron microscopy of cells after 4 days of infection showed that peripheral virus aggregates had almost completely disappeared, whereas there were viruses in the large intracellular compartments, and these compartments contained more virus particles than untreated control infected cells. This suggests that the PLA2 inhibitor caused virus to become trapped inside intracellular vesicles.

Example 7: Effect of the lipid molecules on the MVB compartment of THP1 cells

As treatment with progesterone or MAFP caused the virus to accumulate in the large intracellular compartments, the inventors examined the influence of these molecules on the MVB compartment using Rh-PE labeling. The fluorescent probe Rh-PE accumulates specifically in the MVB compartment when it is added to cells. The Rh-PE in MVBs was quantified by FACS, so providing an estimation of the total volume of the MVBs in cells. In comparison, immunofluorescence microscopy of Rh-PE labeled cells was used to monitor the morphology of MVBs (Figure 5).

FACS quantification showed that HIV-infected cells had more MVBs per cell (Figure 5B) than did control ones (Figure 5A), and the percentage of labeled infected cells was double that of uninfected ones (Figure 5F, "CTL"). FACS quantification showed that neither U18666A nor progesterone modified the total volume of the MVB compartment (Figure 5C-D) from that of untreated infected cells (Figure 5B). MAFP caused the formation of large MVBs, as quantified by FACS (Figure 5E and 5B), and greatly increased the percentage of labeled cells independently of infection (Figure 5F).

Therefore MAFP enhanced both the total number of MVB (Figure 5F) and their size (Figure 5E), which can act as a storage compartment for HIV, as the virus is no longer released from the cell. Although the MVB compartment in infected progesterone-treated cells was not modified (Figure 5D), the effect of progesterone on HIV was similar to that of MAFP; they both reduced the release of HIV to the cell periphery and increased its intracellular storage (Figures 1 and 4).

Example 8: LBPA (BMP) (BMP) content

A change in the MVB content of cells should also lead to changes in the amount of LBPA (BMP) that accumulates in MVB. The inventors used two experimental methods to quantify cellular LBPA (BMP). One was lipid extraction and chromatography and the other was FACS analysis of cells incubated with an anti-LBPA (BMP) antibody (Figures 6-7). Although there was great inter-experimental variability, both methods showed similar

results (Figures 6-7). MAFP increased the LBPA (BMP) content 1.5-fold to 7-fold, while progesterone produced an even greater increase in the LBPA (BMP) in the MVB compartment, 3-fold to 10-fold (Figure 7). While the increase in LBPA (BMP) produced by progesterone involved the whole cell population, MAFP acted on a subpopulation of cells.

Example 9: Effect of progesterone and PLA2 inhibition on MDM cell infection

The inventors checked the effects of progesterone and MAFP observed on THP1 cells by repeating the tests on human monocyte-derived macrophages and comparing the responses to that of AZT-treated cells (Figure 8). The test compounds were added to the cells every two days (repeated treatment) because MAFP has more effect under these conditions (Figure 4C). Progesterone and MAFP both strongly inhibited HIV proliferation in MDM, as they did in THP-1 cells (Figure 8).

Example 10: Effect of progesterone and PLA2 inhibition on direct HIV cell-to-cell transfer

Thus MAFP and progesterone increase the LBPA (BMP) content of cells, leading to the sequestration of HIV in large intracellular stores that have many of the features of MVB. This is associated with decreased HIV secretion. This retention of HIV in cells prompted us to examine the effects of MAFP and progesterone on the direct transfer of HIV from one cell to another. The inventors first cultured the MDM for six days to ensure that the cells were filled with mature virus particles. The intracellular stores of virus constituted in these first days after infection remain infectious for several weeks. The production of new infectious virus particles was then blocked by incubating the cells with the protease inhibitor lopinavir for four days. These MDM were then cultured with autologous non-adherent leukocytes for three hours at day 10. The inventors thus analyzed the impact of MAFP and progesterone on direct cell-to-cell transfer. Both MAFP and progesterone greatly reduced the transfer of the HIV reservoir from MDM to PBL (Figure 9).

Example 11: Effects of MAFP and progesterone on early endocytosis and on the surface molecules involved in HIV-1 spread

Endocytosis is involved in HIV entry and components of late endosomes are recruited for HIV budding (Benaroch et al., Retrovirology, 7: 29, 2010). The inventors investigatd whether HIV trapping into intracellular compartments upon MAFP or progesterone treatment could result of their effect at some steps of the endocytosis pathway. First, the inventors monitored endocytosis using FITC-dextran. After uptake,

dextrans move through the endocytic track up to the lysosomes and thus, they are good tracers of membrane-impermeable cargo along the endocytic track. The inventors found that endocytosis monitored by dextran was blocked by MAFP or progesterone (Figure 10).

However, inhibition of endocytosis was not accompanied by significant variations of the surface receptors involved in HIV uptake (Figure 11). Consistent with literature data (Pelchen-Matthews et al., Immunol. Rev., 168:33-49, 1999), HIV-1 infection tended to decrease the expression of CD4, CCR5, CXCR4 as well as LFA-1 and ICAM-1 while, at the opposite, treatments led rather to a slight increase of the surface expression of these markers, both in non infected and infected cells (Figure 11). This effect of MAFP and progesterone could indicate a blockade on the internalization for recycling of peripheral receptors, consistent with the endocytosis inhibition observed with dextran.

Example 12: Influence of MAFP and progesterone on the BMP content and sterol distribution inTHP-1 cells

To go further in the exploration of the endocytosis pathway, the inventors investigated the variations of BMP content upon MAFP or progesterone treatment, a key lipid of endocytic processes.

To do that, they labeled BMP using a specific monoclonal antibody (6C4) and quantified it by FACS (Figure 12 A, B). The cells were also examined using confocal imaging (data not shown).

For progesterone and U18 treated cells, FACS dot plots show a shift to the high values of fluorescence of the whole population of cells, with a significant increase of the mean fluorescence intensity (MFI) compared to control ($p<0.01$, Figure 12A). In MAFP treated cells, a population of highly labeled cells could be evidenced: although MFI was not different from control one, the number of highly labeled cells (defined as cells having a fluorescence exceeding the MFI of control of two standard deviations), was around 3-fold higher than in control ($p<0.05$), when it was around 7-fold higher for progesterone ($p<0.05$; Figure 12B). Confocal microscopy showed that control cells were faintly stained by the anti-BMP antibody, whereas the treated cells had accumulated BMP in intracellular vesicles (data not shown).

A functional link has been described between BMP and cholesterol (Chevallier et al., J. Biol. Chem., 283: 27871-27880, 2008), so filipin staining was used to label free sterols and monitor their distribution. It has been found that free sterols accumulated in intracellular vesicles in THP-1 cells treated with progesterone. In contrast, the whole population of MAFP-treated cells showed faint labeling, as did the control cells (data not

shown). In summary, MAFP triggered the accumulation of BMP into vesicles without vesicular accumulation of filipin-stained sterols including cholesterol.

Therefore, the inventors have shown that MAFP and progesterone inhibit the macropinocytosis. This inhibition might occur by modification of raft formation in the plasma membrane as reported for high concentration of progesterone.

CLAIMS

1. A compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

2. The compound for the use according to claim 1, wherein the compound is a Phospholipase A2 (PLA2) inhibitor or a Sigma-1 Receptor antagonist.

3. The compound for the use according to claim 1 or 2, wherein the compound is selected from the group consisting of MAFP (methyl arachidonyl fluorophosphonate), ATMK / AACOCF3 (arachidonyl trifluoromethyl ketone), Pyrrolidine-2 (N-{(2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl), Pyrrophenone (N-[[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide), BEL / HELSS (BromoEnolLactone / HaloEnolLactone Suicide Substrate), FKGK11 (1.1.1.2.2-pentafluoro-7-phenyl-3-heptanone), Me-INDOXAM (2-(8-((2-Carboxyethyl)oxy)-2-ethyl-3-(o-phenylbenzyl)-indolizin-1-yl)glyoxylamide), Progesterone, NE100 (N,N-diethyl-2-(4-methoxy-3-phenethyloxyphenyl)ethanamine hydrochloride, N-[2-(4-methoxy-3-phenethyloxyphenyl)ethyl]-N-propylpropan-1-amine-hydrochloride), BD1063 (1-[2-(3,4-dichlorophenyl)-ethyl]-4-methylpiperazine dihydrochloride), BD1047 (N-[2-(3,4-dichlorophenylethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide), Haloperidol (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one) and BMY-14,802 (1-(4-fluorophenyl)-4-[4-(5-fluoropyrimidin-2-yl)piperazin-1-yl]butan-1-ol).

4. The compound for the use according to any of claims 1 to 3, wherein the compound is MAFP.

5. The compound for the use according to any of claims 1 to 3, wherein the compound is progesterone.

6. The compound for the use according to any of claims 1 to 5, wherein the individual is a symptomatic individual suffering from a HIV infection, or an asymptomatic individual who has been exposed or is at risk of having been exposed to HIV.

7. The compound for the use according to any of claims 1 to 6, wherein the cell involved in cell-to-cell transmission of a HIV is a monocyte, a macrophage or a lymphocyte.

8. The compound for the use according to any of claims 1 to 7, for use for inhibiting cell-to-cell transmission of HIV from a monocyte, or a macrophage, or a lymphocyte to a lymphocyte.

9. Products comprising a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell and an anti-HIV agent as combination products for simultaneous, sequential or separated use for the treatment and/or prevention of an infection with a HIV virus.

10. A pharmaceutical composition comprising a compound inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multi-vesicular bodies (MVB) of a cell and an anti-HIV agent.

11. The products according to claim 9, or the pharmaceutical composition according to claim 10, wherein the compound inducing LPBA accumulation in MVB of a cell is a Phospholipase A2 (PLA2) inhibitor or a Sigma-1 Receptor antagonist.

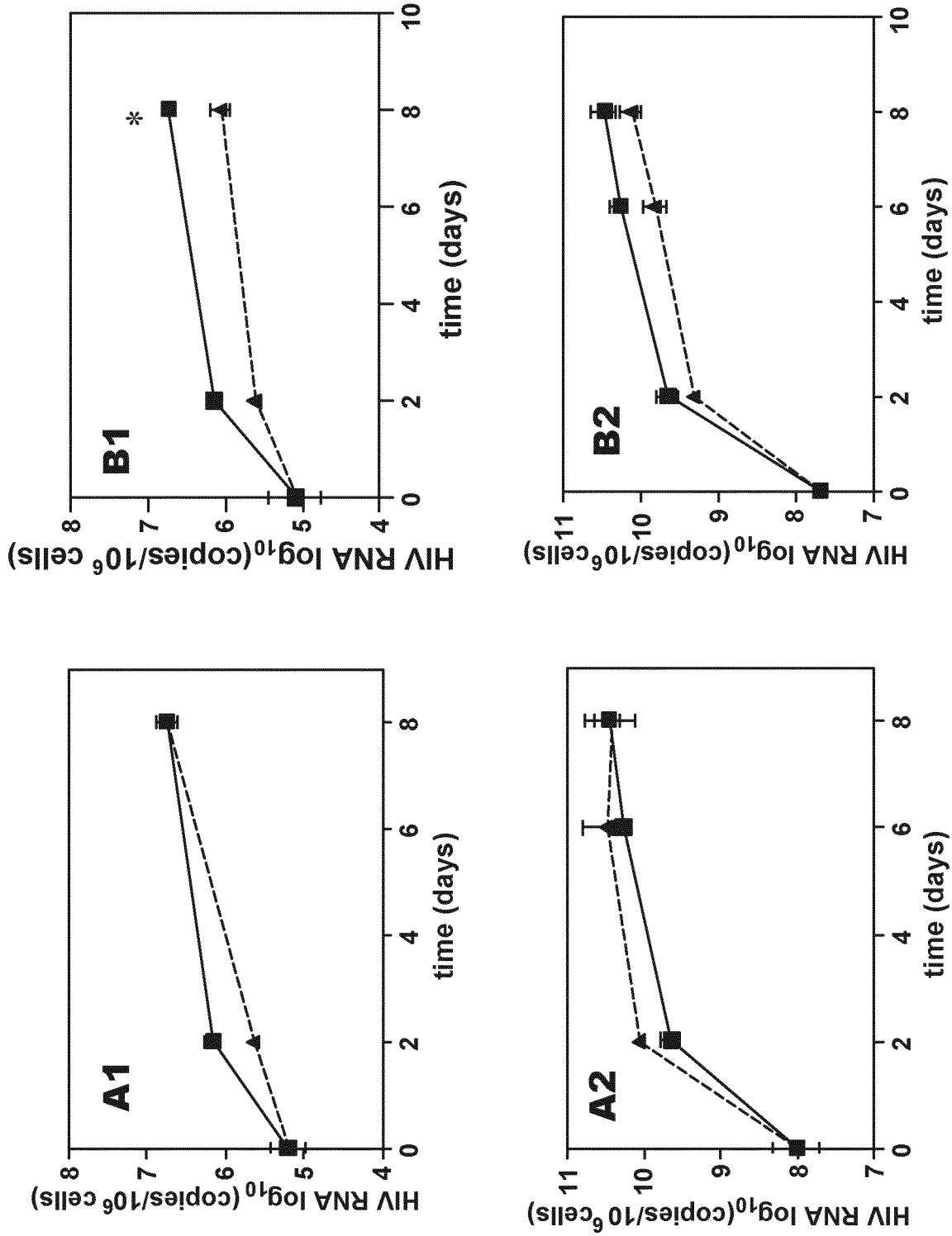
12. The products according to claim 9 or 11, or the pharmaceutical composition according to claim 10, wherein the compound inducing LPBA accumulation in MVB of a cell is selected from the group consisting of MAFP (methyl arachidonyl fluorophosphonate), ATMK / AACOCF3 (arachidonyl trifluoromethyl ketone), Pyrrolidine-2 (N-{(2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl), Pyrrophenone (N-[[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(triphenylmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thiazolidinylidene)methyl]-benzamide), BEL / HELSS (BromoEnolLactone or HaloEnolLactone Suicide Substrate), FKGK11 (1.1.1.2.2-pentafluoro-7-phenyl-3-heptanone), Me-INDOXAM (2-(8-((2-Carboxyethyl)oxy)-2-ethyl-3-(o-phenylbenzyl)-indolizin-1-yl)glyoxylamide), Progesterone, NE100 (N,N-diethyl-2-(4-methoxy-3-phenethyloxyphenyl)ethanamine hydrochloride, N-[2-(4-methoxy-3-phenethyloxyphenyl)ethyl]-N-propylpropan-1-amine-hydrochloride), BD1063 (1-[2-(3,4-dichlorophenyl)-ethyl]-4-methylpiperazine dihydrochloride), BD1047 (N-[2-(3,4-dichlorophenylethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide), Haloperidol

(4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one) and BMY-14,802 (1-(4-fluorophenyl)-4-[4-(5-fluoropyrimidin-2-yl)piperazin-1-yl]butan-1-ol).

13. The products according to any of claim 9 or 11, or the pharmaceutical composition according to claim 10, wherein the compound inducing LPBA accumulation in MVB of a cell is methyl arachidonyl fluoro phosphonate (MAFP) or progesterone.

14. The products according to any of claim 9 or claims 11 to 13, or the pharmaceutical composition according to claim 10, wherein the anti-HIV agent is selected from the group consisting of integrase inhibitors, reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, fusion inhibitors, CCR5 inhibitors, and maturation inhibitors.

FIG.1



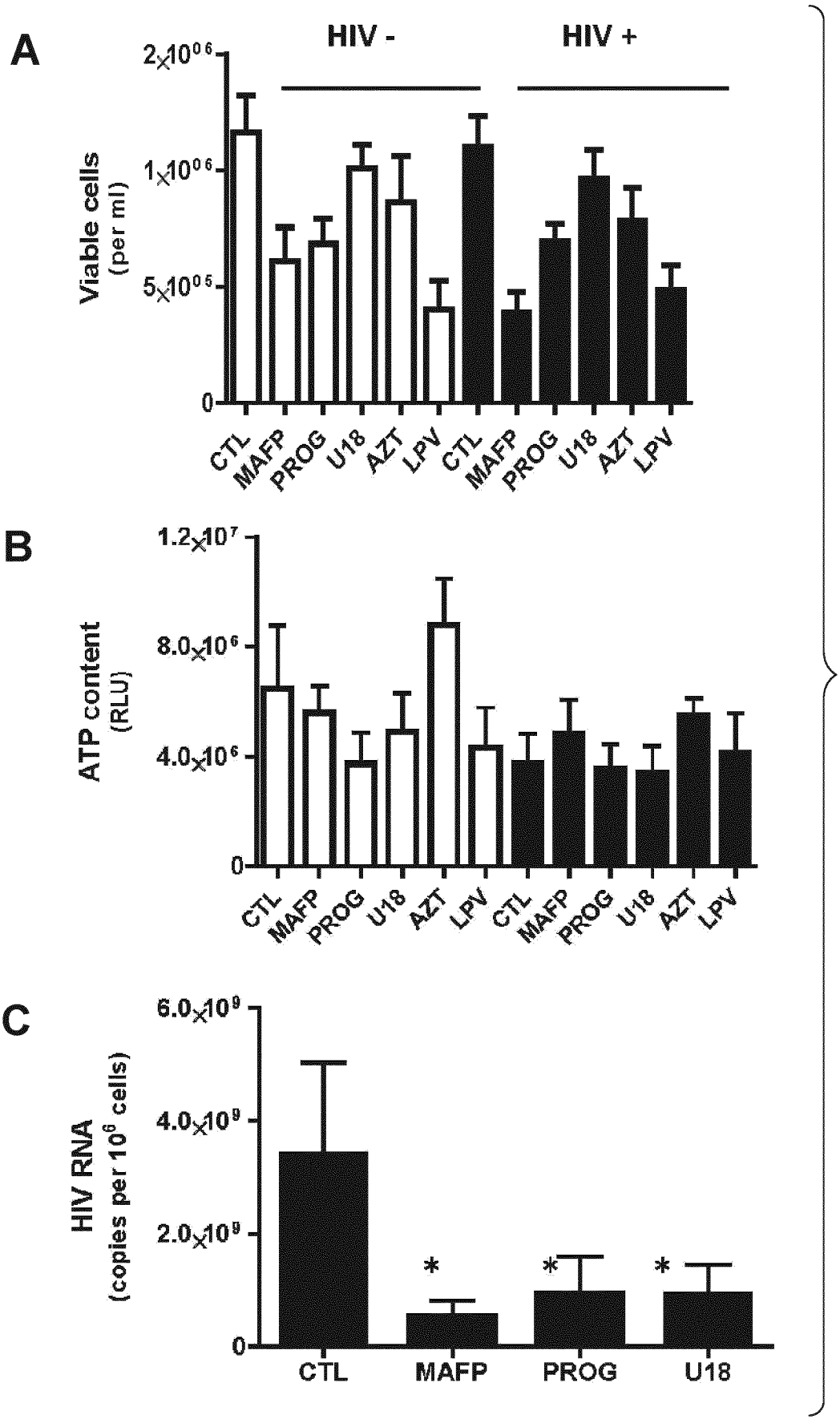

A
HIV -
HIV +
Viable cells (per ml)
2x10^6
1x10^6
5x10^5
0
CTL
MAFP
PROG
U18
AZT
LPV
B
ATP content (RLU)
1.2x10^7
8.0x10^6
4.0x10^6
0
C
HIV RNA (copies per 10^6 cells)
6.0x10^9
4.0x10^9
2.0x10^9
*

FIG.2

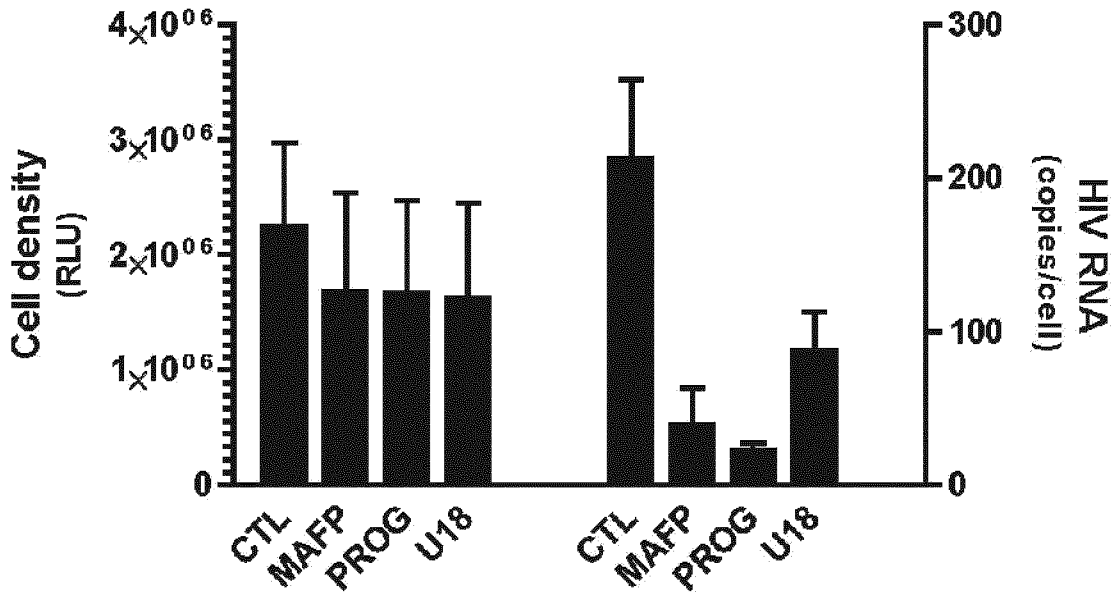

Cell density
(RLU)
4x10^06
3x10^06
2x10^06
1x10^06
0
300
200
100
0
HIV RNA
(copies/cell)
CTL
MAFP
PROG
U18
CTL
MAFP
PROG
U18

FIG.3

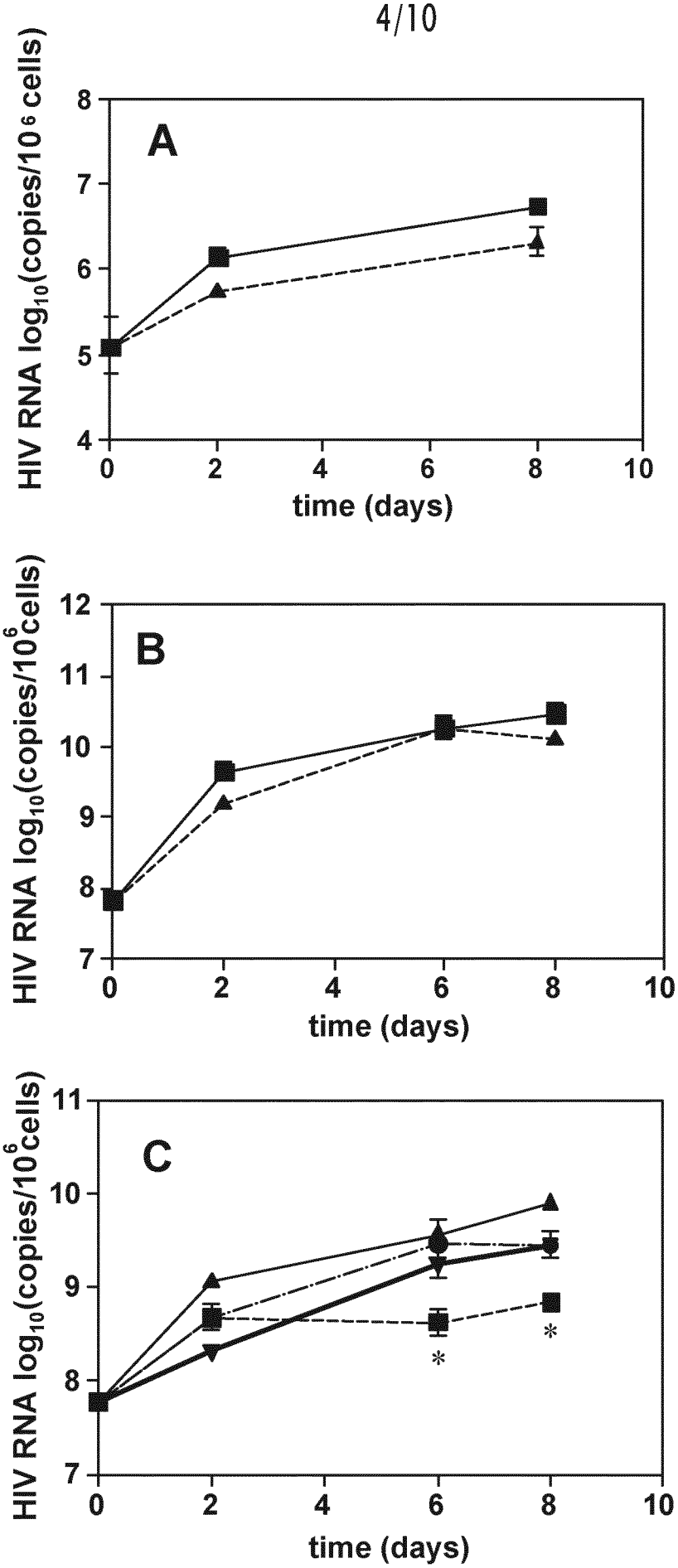

A
HIV RNA log10(copies/10 6 cells)
8
7
6
5
4
0
2
4
6
8
10
time (days)
B
HIV RNA log10(copies/10^6cells)
12
11
10
9
8
7
0
2
4
6
8
10
time (days)
C
HIV RNA log10(copies/10^6cells)
11
10
9
8
7
*
*
0
2
4
6
8
10
time (days)

FIG.4

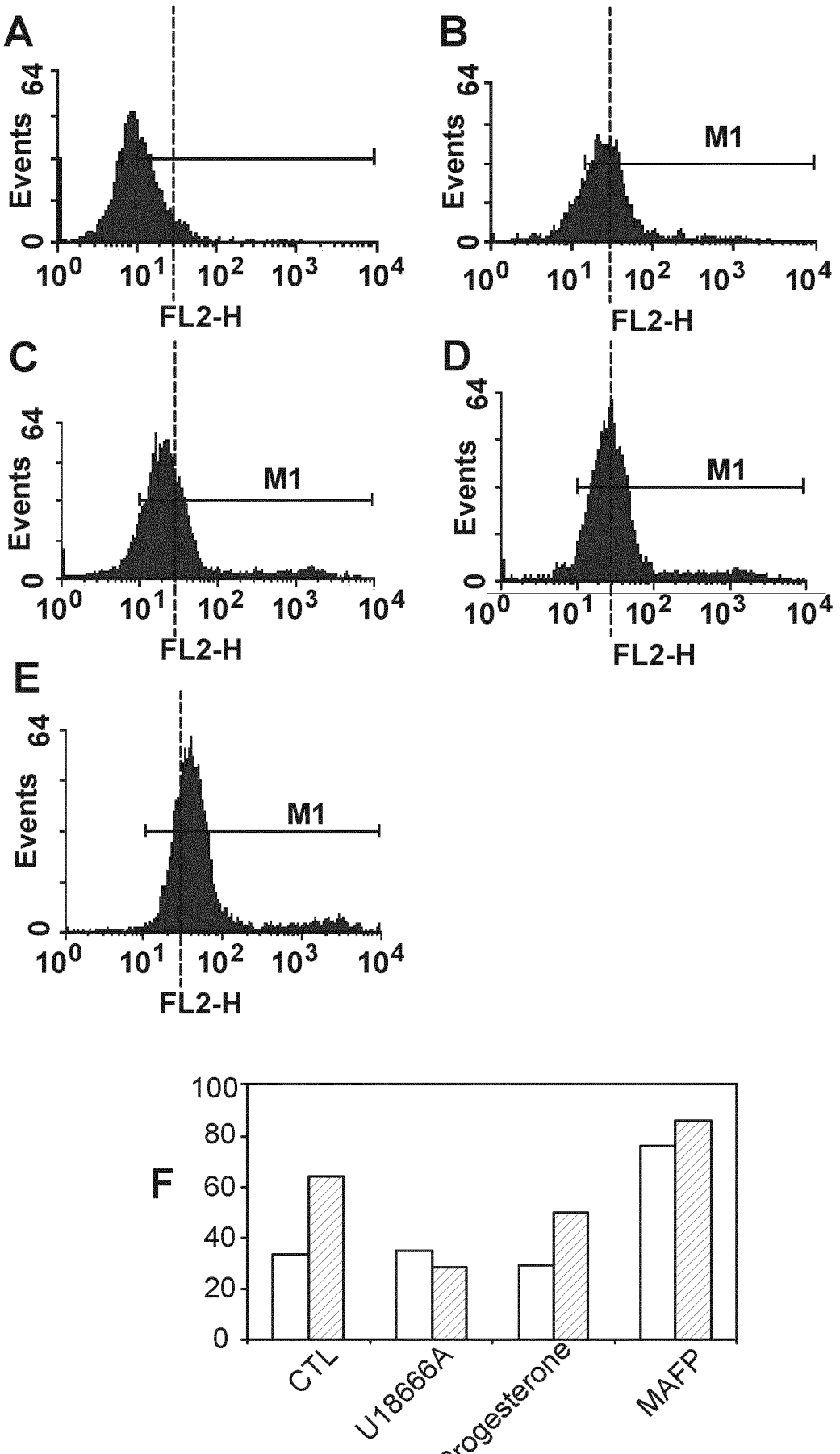

A
B
C
D
E
F
Events
64
0
M1
FL2-H
10^0 10^1 10^2 10^3 10^4
100
80
60
40
20
0
CTL
U18666A
Progesterone
MAFP

FIG.5

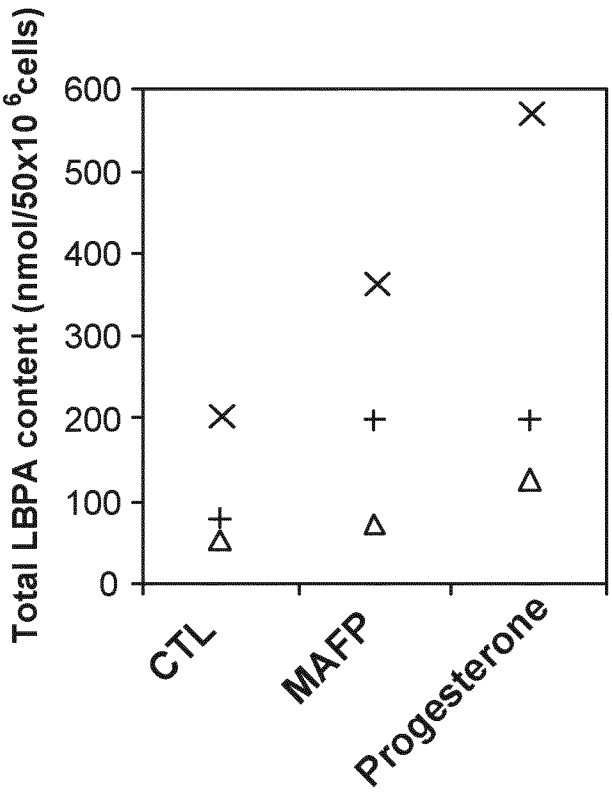

Total LBPA content (nmol/50x10 6cells)
600
500
400
300
200
100
0
CTL
MAFP
Progesterone

FIG.6

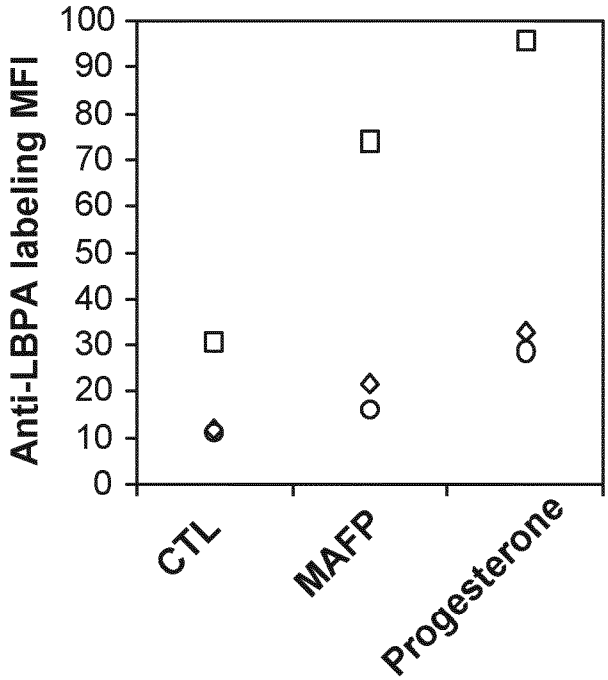

Anti-LBPA labeling MFI
100
90
80
70
60
50
40
30
20
10
0
CTL
MAFP
Progesterone

FIG.7

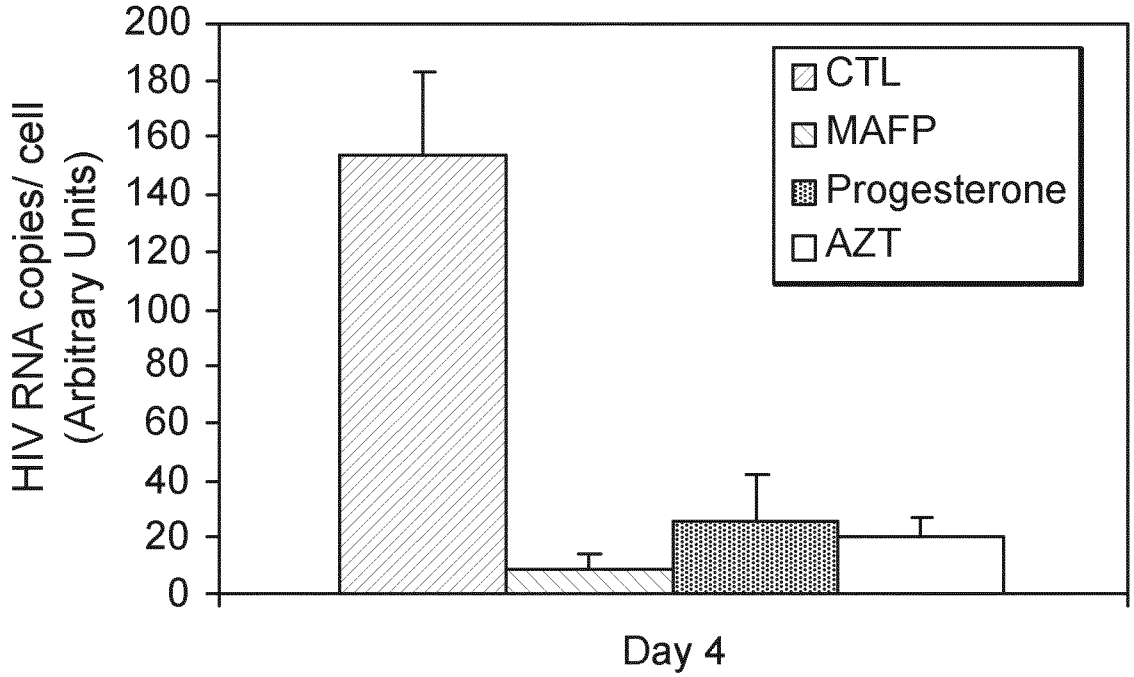


FIG.8

Treatment	Experiment	MAFP	Progesterone
		Fold expression relative to CTL	
At day 0	1	2.17×10^{-1}	ND
	2	8.33×10^{-1}	ND
Repetitive	1	2.33×10^{-5}	2.05×10^{-5}
	2	7.78×10^{-3}	1.33×10^{-4}
	3	3.48×10^{-2}	9.66×10^{-3}

FIG.9

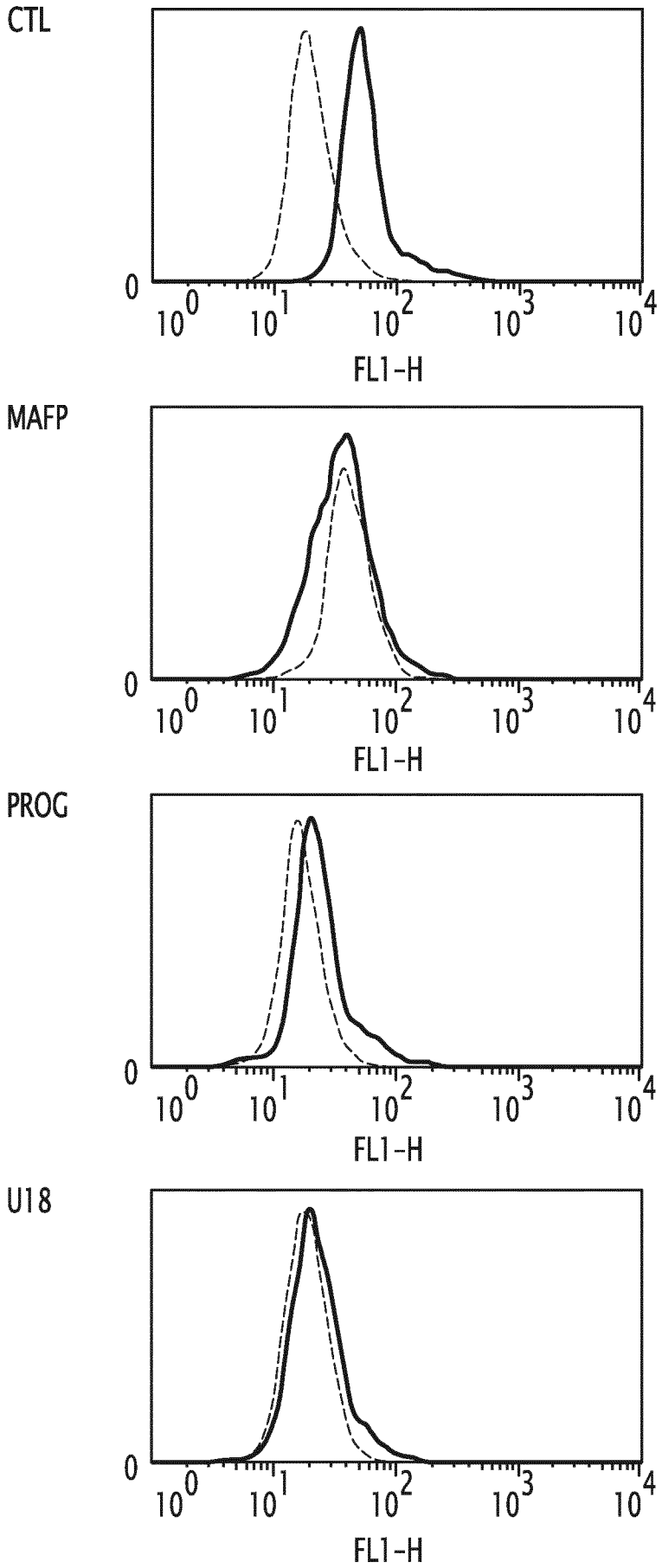

CTL
0
10^0 10^1 10^2 10^3 10^4
FL1-H
MAFP
0
10^0 10^1 10^2 10^3 10^4
FL1-H
PROG
0
10^0 10^1 10^2 10^3 10^4
FL1-H
U18
0
10^0 10^1 10^2 10^3 10^4
FL1-H

FIG.10

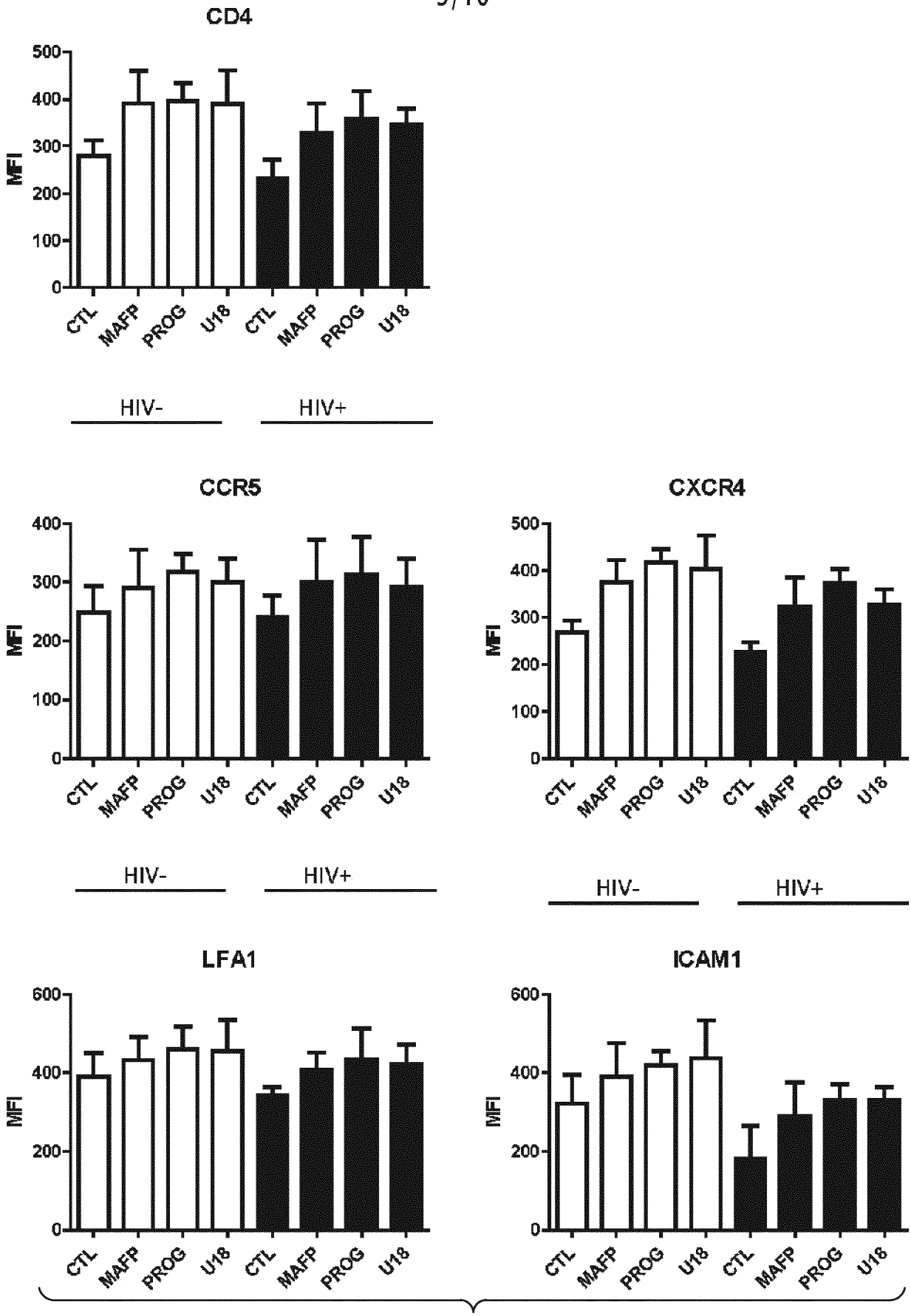
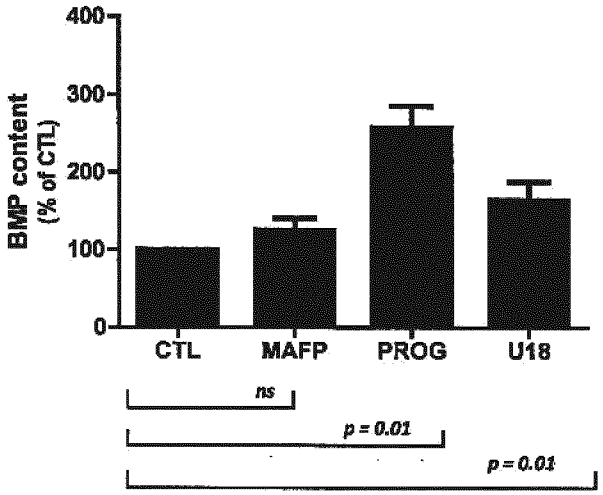

CD4
MFI
500
400
300
200
100
0
CTL
MAFP
PROG
U18
CTL
MAFP
PROG
U18
HIV-
HIV+
CCR5
MFI
400
300
200
100
0
CTL
MAFP
PROG
U18
CTL
MAFP
PROG
U18
HIV-
HIV+
CXCR4
MFI
500
400
300
200
100
0
CTL
MAFP
PROG
U18
CTL
MAFP
PROG
U18
HIV-
HIV+
LFA1
MFI
600
400
200
0
CTL
MAFP
PROG
U18
CTL
MAFP
PROG
U18
ICAM1
MFI
600
400
200
0
CTL
MAFP
PROG
U18
CTL
MAFP
PROG
U18

FIG.11


BMP content (% of CTL)
400
300
200
100
0
CTL
MAFP
PROG
U18
ns
p = 0.01
p = 0.01

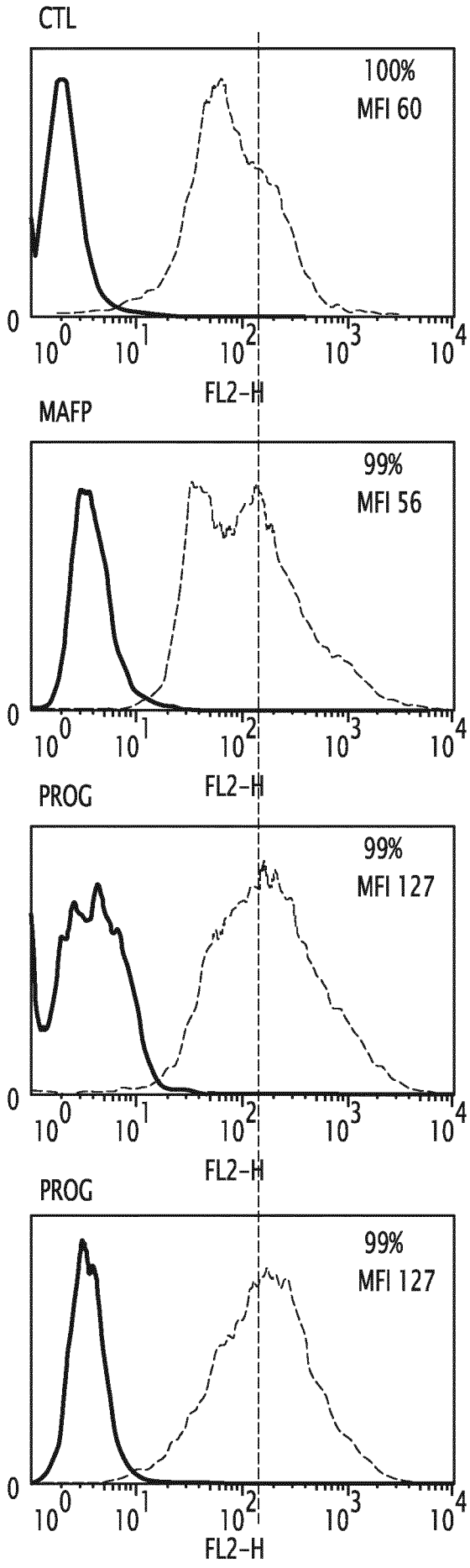

CTL
100%
MFI 60
MAFP
99%
MFI 56
PROG
99%
MFI 127
PROG
99%
MFI 127
0
10^0
10^1
10^2
10^3
10^4
FL2-H

FIG.12

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/068538

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00 A61K31/121 A61K31/137 A61K31/366 A61K31/4045
A61K31/427 A61K31/445 A61K31/47 A61K31/495 A61K31/496
A61K31/505 A61K31/506 A61K31/513 A61K31/52 A61K31/522

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)
A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BEILSTEIN Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/202439 A1 (CHATTERJEE SUBROTO [US]) 13 August 2009 (2009-08-13) page 3, paragraph 41 page 31; claim 8 page 18, paragraph 233	1-4,6-14
A	WO 2004/112724 A2 (VIRAL GENOMIX INC [US]; KIM JONG JOSEPH [US]) 29 December 2004 (2004-12-29) page 29, paragraph 3rd page 7 page 11, paragraph 2nd	1,9,10

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 2011	15/02/2012

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/068538

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GEKKER G ET AL: "Cocaine-induced HIV-1 expression in microglia involves sigma-1 receptors and transforming growth factor-beta1", INTERNATIONAL IMMUNOPHARMACOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 6, no. 6, 1 June 2006 (2006-06-01), pages 1029-1033, XP024976856, ISSN: 1567-5769, DOI: DOI:10.1016/J.INTIMP.2005.12.005 [retrieved on 2006-06-01] abstract page 1032	1

A	DUNCAN ROBIN E ET AL: "Identification and functional characterization of adipose-specific phospholipase A2 (AdPLA).", THE JOURNAL OF BIOLOGICAL CHEMISTRY 12 SEP 2008 LNKD- PUBMED:18614531, vol. 283, no. 37, 12 September 2008 (2008-09-12), pages 25428-25436, XP002643856, ISSN: 0021-9258 abstract	1-4,6-8

A	EP 0 526 258 A2 (LILLY CO ELI [US] LILLY CO ELI [DE]) 3 February 1993 (1993-02-03) page 3, line 24 - line 29	1-4,6-8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/068538

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box 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:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional 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 claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

4(completely); 1-3, 6-14(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 4(completely); 1-3, 6-14(partially)

A compound, i.e. MAFP (methyl arachidonyl fluorophosphonate), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

2. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. ATMK / AACOCF3 (arachidonyl trifluoromethyl ketone), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

3. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. Pyrrolidine-2 (N-{(2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCI), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

4. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. Pyrrophenone (N-[[(2S,4R)-1-[2-(2,4-difluorobenzoyl)benzoyl]-4-[(tripheny lmethyl)thio]-2-pyrrolidinyl]methyl]-4-[(Z)-(2,4-dioxo-5-thi azolidinylidene)methyl]-benzamide), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof..

---

5. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. BEL / HELSS (BromoEnolLactone / HaloEnolLactone Suicide Substrate), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i)

cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

6. claims: 1-3, 6-13(all partially)

A compound, i.e. FKGK11 (1.1.1.2.2-pentafluoro-7-phenyl-3-heptanone), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

7. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. Me-INDOXAM (2-(8-((2-Carboxyethyl)oxy)-2-ethyl-3-(o-phenylbenzyl)-indolizin-1-yl)glyoxylamide), inducing Lyso-Bis Phosphatidic Acid (LPBA), accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

8. claims: 5(completely); 1-3, 6-14(partially)

A compound, i.e. progesterone, inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

9. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. NE100 (N,N-diethyl-2-(4-methoxy-3-phenethyloxyphenyl)ethanamine hydrochloride, inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

10. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. N-[2-(4-methoxy-3-phenethyloxyphenyl)ethyl]-N-propylpropan-1-amine-hydrochloride), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a

Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

11. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. BD1063 (1-[2-(3,4-dichlorophenyl)-ethyl]-4-methylpiperazine dihydrochloride), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

12. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. BD1047 (N-[2-(3,4-dichlorophenylethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

---

13. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. Haloperidol (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

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14. claims: 1-3, 6-12, 14(all partially)

A compound, i.e. BMY-14,802 (1-(4-fluorophenyl)-4-[4-(5-fluoropyrimidin-2-yl)piperazin-1-yl]butan-1-ol), inducing Lyso-Bis Phosphatidic Acid (LPBA) accumulation in multivesicular bodies (MVB) of a cell for use for inhibiting i) cell-to-cell transmission of a Human Immunodeficiency Virus (HIV), and/or ii) entry of HIV into a cell in an individual in need thereof.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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