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(54) **Title:** FOXO1 INHIBITOR FOR USE IN THE TREATMENT OF LATENT VIRUS INFECTION

(57) **Abstract:** The present invention relates the treatment of latent virus infection. A pharmacological and specific inhibitor of FOXO1, the AS1842856 compound, has been used both in vitro and in vivo to uncover the role of FOXO1 in various cellular models (14–20). Using this drug the inventors were able to show that FOXO1 inhibition alone was sufficient to trigger a G0→G1 transition of human peripheral blood T cells (PBT) upstream of the R restriction point of the cell cycle leading to a reactivation of the latent T cells. This transition was characterized by a parallel increase in cell size, metabolic activity and RNA synthesis. The inventors also showed that FOXO1 inhibition was accompanied by the inactivation of the SAMHD1 HIV restriction factor together with a permissiveness of resting human CD4+ T cells to lentiviral infection. They finally demonstrated using HIV-1 latency models that the drug reactivates HIV-1 provirus. Thus, the present invention relates to a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs for use in the treatment of latent viruses' infection in a subject in need thereof.

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FOXO1 INHIBITOR FOR USE IN THE TREATMENT OF LATENT VIRUS INFECTION

FIELD OF THE INVENTION:

The present invention relates to the treatment of latent viruses' infection.

BACKGROUND OF THE INVENTION:

HIV-1 (Human Immunodeficiency Virus of type 1) and HIV-2 are both responsible for AIDS (Acquired Immunodeficiency Syndromes). However, HIV-2 displays low viremia and restrained transmission compared to HIV-1, suggesting a post-integration restriction. HIV-1 is entirely dependent on the host cell for providing the metabolic resources for completion of its viral replication cycle. As a matter of fact, HIV-1 replicates efficiently only in activated CD4+ T cells. Besides those activated CD4+ T cells, human T lymphocytes possess an ability to remain quiescent over long periods of time. Upon recognition of a foreign antigen only a very small fraction of these cells actively divide and clonally expand to give rise to antigen-specific effector and long-lived non-dividing memory T cells. As a consequence, a large proportion of circulating T lymphocytes in the peripheral blood are naïve or memory quiescent T cells at the G0 state of the cell cycle (1). These cells are characterized by a very low metabolic rate, low levels of transcriptional activity, small cell size and very long periods of survival (2-4). The HIV-1 reservoir is defined as the cell population where the virus persists during therapy. The main reservoir resides in latently infected quiescent CD4+ memory T cell, established during the first days of infection. These cells carry stably integrated and transcriptionally silent but replication-competent proviruses. They do not produce virus particles while in the resting state, but can give rise to infectious virions following activation by various stimuli (Pitman MC et al. (2018)).

To counter the HIV-1 replication cycle and to allow patients to live decently, considerable combinatory antiretroviral therapy (cART) have been developed. However, due to HIV latency (both HIV-1 and HIV-2), such cART treatment cannot be stopped for now, since it does not lead to a full eradication of the infection corresponding to a re-emergence of a detectable level of viremia in infected patients when interrupting the treatment. This is because the HIV provirus remains integrated in the host genome of resting CD4+ T cells from viral reservoirs and can be reactivated at any time.

Forkhead box class O (FOXO) transcription factors have been reported as being key players to regulate and maintain cell quiescence in various cell types. In unstimulated cells, these transcription factor are in the nucleus, unphosphorylated and active, thereby maintaining the transcription of numerous genes. This includes genes such as CDK1NB (also known as p27), a cyclin-dependent kinase inhibitor that blocks the cell cycle in the G0/G1 phase. However, after activation (i.e. by growth factors), FOXOs are phosphorylated by the serine/threonine kinase Akt downstream of PI3-kinase and rapidly excluded from the nucleus, resulting in the interruption of their transcriptional activity (5). Thus, FOXO1 act as key regulators to coordinate signals delivered by growth factors to molecular events leading to cell growth and cell division. A similar role of FOXOs in human T cells was initially suspected because activation of quiescent T cells strongly induces the PI3-kinase/Akt axis and the rapid nuclear exclusion of FOXO1, the most abundant FOXO molecule present in T cells (6). Consistently, overexpression of a constitutive active form of FOXO1 in quiescent T lymphocytes blocks their proliferation induced by antigen (6). Moreover, mice with a disruption of the *foxo1* gene in T cells show an elevated number of activated T cells in lymphoid organs (7). Additionally to this, Trinité and colleagues have demonstrated that using AS1842856, a drug that inhibits FOXO1, in combination with IL-4, IL-7 or Dynabeads Human T-Activator CD3/CD28 but never alone, accelerates HIV-1 replication once applied on resting CD4+ T cells. As previously written above, those resting CD4+ T cells were up-front treated with IL-7 effective at enhancing HIV-1 proviral reactivation (Xiang Wang and colleagues, 2005) or IL-4, corresponding to a useful alternative to IL-7, or activated with Dynabeads Human T-Activator CD3/CD28.

Nevertheless, the impact of AS1842856 inhibitor has never been investigated taken alone.

SUMMARY OF THE INVENTION:

The current study sought to understand the cellular consequences of AS1842856 inhibitor on resting CD4+ T cells. As mentioned above, FOXO1 seems to be an important molecule to actively maintain human T lymphocytes in a quiescence state. Interfering with FOXO1 activity in the context of HIV-1 infection might thus represent a new and valuable approach that has not yet been explored to understand the molecular processes involved in the non-permissiveness of resting CD4+ T cells for HIV-1 replication, but also to reactivate latent proviral forms of the virus. A pharmacological and specific inhibitor of FOXO1, the AS1842856 compound, has been used both in vitro and in vivo to uncover the role of FOXO1

in various cellular models (14–20). Using this drug, the inventors were able to show that FOXO1 inhibition alone was sufficient to trigger a G0→G1 transition of human peripheral blood T cells (PBT) upstream of the R restriction point of the cell cycle leading to a reactivation of the latent T cells. This transition was characterized by a parallel increase in cell size, metabolic activity and RNA synthesis. The inventors also showed that FOXO1 inhibition was accompanied by the inactivation of the SAMHD1 HIV restriction factor together with a permissiveness of resting human CD4+ T cells to lentiviral infection. They finally demonstrated using HIV-1 latency models that the drug reactivates HIV-1 provirus. Taken together these results demonstrate that FOXO1 plays a major role in T lymphocyte/HIV-1 interaction, and that its inhibition with AS1842856 is a new potential clinical strategy for HIV 1 therapies seeking to the eradication of latent provirus reservoirs.

Thus, the present invention relates to a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs for use in the treatment of latent viruses' infection in a subject in need thereof. Particularly, the invention is described by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

A first aspect of the invention relates to a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs for use in the treatment of latent viruses' infection in a subject in need thereof.

In a particular embodiment, the invention relates to a FOXO1 inhibitor which reactivates latent viruses in CD4+ T cell reservoirs for use in the treatment of latent viruses' infection in a subject in need thereof.

In a particular embodiment, the invention relates to a FOXO1 inhibitor for reactivating latent viruses in host-cell reservoirs in a subject in need thereof. In particular embodiment, the host-cell reservoirs are CD4+ T cells reservoirs.

In a particular embodiment, the invention relates to a FOXO1 inhibitor for use in the treatment of latent viruses' infection in a subject in need thereof.

Said latent virus is selected from the group consisting of HIV-1 or HIV-2, FIV (Feline Immunodeficiency Virus), SIV (Simian Immunodeficiency Virus).

In a particular embodiment the latent virus is the HIV-1.

As used herein, HIV-1 refers to all of stages of infection corresponding to Acute Infection, Clinical Latency and Advanced Disease also called AIDS.

As used herein, CD4+ T cells reservoirs correspond to infected CD4+ cells that demonstrate an enough long time survival to revert back to a resting memory state, or quiescent

state, which is nonpermissive for viral gene expression. Those cells are found to be at a G0 stage, also known as resting phase in which they are characterized by the ability to re-enter the cell cycle in response to normal physiological stimuli.

As used herein, “FOXO1” belongs to “Forkhead Box class O” transcription factors which are known to be key molecules to regulate and maintain cell quiescence in various cell types. In unstimulated cells, these transcription factors are in the nucleus, unphosphorylated and active, thereby maintaining the transcription of numerous genes. They act as key regulators to coordinate signals delivered by growth factors to molecular events leading to cell growth and cell division. FOXO1 corresponds to the most abundant FOXO molecule present in T cells (Entrez Gene ID number: 2308).

As used herein “FOXO1 inhibitor” denotes an inhibitor which induces a transition from quiescence G0 to the G1 phase of the cell cycle, by this reversing HIV-1 latency in T lymphocytes. The use of the inhibitor of the present invention induces both bioenergetics and transcriptional activities of T cells, together with a significant increase of their cell size, but without any cell division. The FOXO1 inhibitor allows SAMHD1 phosphorylation. SAMHD1 is a cellular quiescence factor and a well-known restriction factor of HIV infection. This phosphorylation correlates with loss of its ability to restrict HIV. Moreover, the FOXO1 inhibitor of the present invention does not only orchestrate the pre-integrative but also post-integrative stages of the viral cycle. Indeed, inhibition of FOXO1 potentiates LTR activity.

In one embodiment, the inhibitor according to the invention may be a low molecular weight compound, e. g. a small organic molecule (natural or not).

The term "small organic molecule" refers to a molecule (natural or not) of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e. g., proteins, nucleic acids, etc.). Particular small organic molecules range in size up to about 10000 Da, more particularly up to 5000 Da, more particularly up to 2000 Da and most particularly up to about 1000 Da.

In a particular embodiment the FOXO1 inhibitor corresponds to the compound AS1842856.

The term “AS1842856” refers to cell-permeable inhibitor that blocks the transcription activity of FOXO1.

The present invention provides for an isolated single domain antibody, wherein said antibody inhibits FOXO1.

As used herein the term “single domain antibody” has its general meaning in the art and refers to the single heavy chain variable domain of antibodies of the type that can be found in

Camelid mammals which are naturally devoid of light chains. Such single domain antibody are also called VHH or “nanobody®”. For a general description of (single) domain antibodies, reference is also made to the prior art cited above, as well as to EP 0 368 684, Ward et al. (Nature 1989 Oct 12; 341 (6242): 544-6), Holt et al., Trends Biotechnol., 2003, 21(11):484-490; and WO 06/030220, WO 06/003388. The nanobody has a molecular weight approximately one-tenth that of a human IgG molecule, and the protein has a physical diameter of only a few nanometers. One consequence of the small size is the ability of camelid nanobodies to bind to antigenic sites that are functionally invisible to larger antibody proteins, i.e. , camelid nanobodies are useful as reagents to detect antigens that are otherwise cryptic using classical immunological techniques, and as possible therapeutic agents. Thus yet another consequence of small size is that a nanobody can inhibit as a result of binding to a specific site in a groove or narrow cleft of a target protein, and hence can serve in a capacity that more closely resembles the function of a classical low molecular weight drug than that of a classical antibody. The low molecular weight and compact size further result in nanobodies being extremely thermostable, stable to extreme pH and to proteolytic digestion, and poorly antigenic. Another consequence is that nanobodies readily move from the circulatory system into tissues, and even cross the blood-brain barrier and can treat disorders that affect nervous tissue. Nanobodies can further facilitated drug transport across the blood brain barrier. See U.S. patent application 20040161738 published August 19, 2004. These features combined with the low antigenicity to humans indicate great therapeutic potential. The amino acid sequence and structure of a single domain antibody can be considered to be comprised of four framework regions or "FRs" which are referred to in the art and herein as "Framework region 1" or "FRl "; as "Framework region 2" or "FR2"; as "Framework region 3 " or "FR3"; and as "Framework region 4" or “FR4” respectively; which framework regions are interrupted by three complementary determining regions or "CDRs", which are referred to in the art as "Complementarity Determining Region for "CDRl”; as "Complementarity Determining Region 2" or "CDR2” and as "Complementarity Determining Region 3" or "CDR3", respectively. Accordingly, the single domain antibody can be defined as an amino acid sequence with the general structure : FRl - CDRl - FR2 - CDR2 - FR3 - CDR3 - FR4 in which FRl to FR4 refer to framework regions 1 to 4 respectively, and in which CDRl to CDR3 refer to the complementarity determining regions 1 to 3. In the context of the invention, the amino acid residues of the single domain antibody are numbered according to the general numbering for VH domains given by the International ImMunoGeneTics information system aminoacid numbering (http://imgt.cines.fr/).

Camel Ig can be modified by genetic engineering to yield a small protein having high affinity for a target, resulting in a low molecular weight antibody-derived protein known as a "nanobody" or “VHH”. See U.S. patent number 5,759,808 issued June 2, 1998; see also Stijlemans, B. et al. , 2004 J Biol Chem 279: 1256-1261 ; Dumoulin, M. et a/. , 2003 Nature 424: 783-788; Pleschberger, M. et al. 2003 Bioconjugate Chem 14: 440- 448; Cortez-Retamozo, V. et al. 2002 Int J Cancer 89: 456-62; and Lauwereys, M. et al. 1998 EMBO J 17: 3512-3520. Engineered libraries of camelid antibodies and antibody fragments are commercially available, for example, from Ablynx, Ghent, Belgium. In certain embodiments herein, the camelid antibody or nanobody is naturally produced in the camelid animal, i.e., is produced by the camelid following immunization with [antigen] or a peptide fragment thereof, using techniques described herein for other antibodies. Alternatively, the [antigen]-binding camelid nanobody is engineered, i.e. , produced by selection for example from a library of phage displaying appropriately mutagenized camelid nanobody proteins using panning procedures with FOXO1 as a target.

In some embodiments, the single domain antibody is a “humanized” single domain antibody.

As used herein the term “humanized” refers to a single domain antibody of the invention wherein an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring VHH domain has been "humanized", i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring VHH sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional chain antibody from a human being. Methods for humanizing single domain antibodies are well known in the art. Typically, the humanizing substitutions should be chosen such that the resulting humanized single domain antibodies still retain the favorable properties of single domain antibodies of the invention. The one skilled in the art is able to determine and select suitable humanizing substitutions or suitable combinations of humanizing substitutions. For example, the single domain antibodies of the invention may be suitably humanized at any framework residue that the single domain antibodies remain soluble and do not significantly loss their affinity for FOXO1.

In another embodiment, the FOXO1 inhibitor according to the invention is an inhibitor of foxo1 gene expression.

Small inhibitory RNAs (siRNAs) can also function as inhibitors of foxo1 expression for use in the present invention. DHODH or Chk1 gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the

production of a small double stranded RNA, such that foxo1 gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see for example Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of foxo1 gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of DHODH or CHk1 mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of foxo1 gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and particularly cells expressing FOXO1. Particularly, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a particular type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Particular viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, 1990 and in Murry, 1991).

Particular viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple

series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUCl9, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, eye, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

For example, an shRNA used for the invention can have the following sequence: 5-GCCGGAGTTTAGCCAGTCCAA-3' (SEQ ID NO: 1).

In a particular embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter. The promoter may be specific for Muller glial cells, microglia cells, endothelial cells, pericyte cells and astrocytes For example, a specific expression in Muller glial cells may be obtained through the promoter of the glutamine synthetase gene is suitable. The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subjects at

risk of contracting the disease or suspected to have contracted the disease as well as subjects who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness (e.g., the pattern of dosing used during therapy). A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

As used herein, the term “subject” denotes a mammal, such as a rodent, a feline, a canine, and a primate. Particularly, the subject according to the invention is a human.

In a particular embodiment, the invention also relates to a method for treating latent viruses’ infection comprising administering to a subject in need thereof a FOXO1 inhibitor which reactive latent viruses in host-cell reservoirs.

The present invention also relates to a method for reactive latent viruses in host-cell reservoirs by administering to a subject in need thereof a FOXO1 inhibitor.

In a particular embodiment, the latent virus is the HIV-1.

In order to test the functionality of a putative FOXO1 inhibitor a gene reporter assay could be released as followed. HEK293 cells maintained in Dulbecco's modified Eagle's

medium (DMEM; Invitrogen) in 10% fetal bovine serum (FBS) were transfected using lipofectamin (Invitrogen). Transfection efficiency was controlled by including pGL4.75 in each transfection experiment. To ensure equal amounts of DNA, empty plasmids were included for each transfection. Cells were cultured in DMEM supplemented with 10% FBS for 5 h after transfection, after which medium was replaced with DMEM supplemented with 1% FBS with or without the addition of FOXO1 inhibitor AS1842856 at 500nM. Cells were then incubated a further 20 h. Luciferase activity was measured and normalized for R. reniformis luciferase activity for each sample.

Therapeutic composition

In another object, the invention relates to a therapeutic composition comprising a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs for use in the treatment of latent viruses' infection.

In a particular embodiment, the invention relates to a therapeutic composition comprising a FOXO1 inhibitor for use in the treatment of HIV-1, HIV-2, SIV and FIV in a subject in need thereof.

In a particular embodiment, the invention relates to a therapeutic composition comprising a FOXO1 inhibitor for use in the treatment of HIV-1 in a subject in need thereof.

In a particular embodiment, the invention also relates to a method for treating latent viruses' infection comprising administering to a subject in need thereof a therapeutically effective amount of a FOXO1 inhibitor.

In a particular embodiment, the invention also relates to a method for treating HIV-1, HIV-2, SIV and FIV comprising administering to a subject in need thereof a therapeutically effective amount of a FOXO1 inhibitor.

In a particular embodiment, the invention relates to a method for treating HIV-1 comprising administering to a subject in need thereof a therapeutically effective amount of a FOXO1 inhibitor.

Any therapeutic agent of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

As used herein, the term "therapeutically effective amount" or "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount of a FOXO1 inhibitor of the present invention may vary according to factors such as the disease state, age, sex, and weight of the

individual, and the ability of the antibody of the present invention to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the combination of a FOXO1 inhibitor of the present invention are outweighed by the therapeutically beneficial effects. The efficient dosages and dosage regimens for the combination of a FOXO1 inhibitor of the present invention depend on the disease or condition to be treated and may be determined by the persons skilled in the art. A physician having ordinary skill in the art may readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician could start doses of the oligomers of the present invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable dose of a composition of the present invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect according to a particular dosage regimen. Such an effective dose will generally depend upon the factors described above. For example, a therapeutically effective amount for therapeutic use may be measured by its ability to stabilize the progression of disease. Typically, the ability of a compound to inhibit FOXO1 may, for example, be evaluated in an animal model system predictive of efficacy to reverse latency for HIV-1 cure (e.g. simian immunodeficiency virus (SIV)/macaque model). Alternatively, this property of a composition may be evaluated by examining the ability of the compound to induce cytotoxicity by in vitro assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound may decrease latent reservoirs, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected. An exemplary, non-limiting range for a therapeutically effective amount of an antibody of the present invention is about 0.1-100 mg/kg, such as about 0.1-50 mg/kg, for example about 0.1-20 mg/kg, such as about 0.1-10 mg/kg, for instance about 0.5, about such as 0.3, about 1, about 3 mg/kg, about 5 mg/kg or about 8 mg/kg. An exemplary, non-limiting range for a therapeutically effective amount of an antibody of the present invention is 0.02-100 mg/kg, such as about 0.02-30 mg/kg, such as about 0.05-10 mg/kg or 0.1-3 mg/kg, for example about 0.5-2 mg/kg. Administration may e.g. be intravenous, intramuscular, intraperitoneal, or subcutaneous, and for instance administered proximal to the site of the target. Dosage regimens in the above methods of treatment and uses are adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be

proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. In some embodiments, the efficacy of the treatment is monitored during the therapy, e.g. at predefined points in time. In some embodiments, the efficacy may be monitored by visualization of the disease area, or by other diagnostic methods described further herein, e.g. by performing one or more PET-CT scans, for example using a labelled antibody of the present invention, fragment or mini-antibody derived from the antibody of the present invention. If desired, an effective daily dose of a pharmaceutical composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. In some embodiments, the oligomers of the present invention are administered by slow continuous infusion over a long period, such as more than 24 hours, in order to minimize any unwanted side effects. An effective dose of an antibody of the present invention may also be administered using a weekly, biweekly or triweekly dosing period. The dosing period may be restricted to, e.g., 8 weeks, 12 weeks or until clinical progression has been established. As non-limiting examples, treatment according to the present invention may be provided as a daily dosage of an antibody of the present invention in an amount of about 0.1-100 mg/kg, such as 0.2, 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, or alternatively, at least one of weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 after initiation of treatment, or any combination thereof, using single or divided doses every 24, 12, 8, 6, 4, or 2 hours, or any combination thereof.

The FOXO1 inhibitor of the invention may be used alone or in combination with any suitable agent.

Any therapeutic agent of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, parenteral, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

Particularly, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions. In particular, these may be in organic solvent such as DMSO, ethanol which upon addition, depending on the case, of sterilized water or physiological saline permit the constitution of injectable solutions.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently can be used.

In each of the embodiments of the treatment methods described herein, the FOXO1 inhibitor is delivered in a manner consistent with conventional methodologies associated with management of the disease or disorder for which treatment is sought. In accordance with the disclosure herein, an effective amount of the antibody or antibody-drug conjugate is administered to a patient in need of such treatment for a time and under conditions sufficient to prevent or treat the disease or disorder.

The present invention is also provided for therapeutic applications where the FOXO1 inhibitor of the present invention may be used in combination with at least one further therapeutic agent, e.g. antiretroviral therapy also named highly active antiretroviral therapy (HAART). Such administration may be simultaneous, separate or sequential. For simultaneous administration the agents may be administered as one composition or as separate compositions, as appropriate.

As used herein, the terms “combined treatment”, “combined therapy” or “therapy combination” refer to a treatment that uses more than one medication. The combined therapy may be dual therapy or bi-therapy.

As used herein, the term “administration simultaneously” refers to administration of 2 active ingredients by the same route and at the same time or at substantially the same time. The term “administration separately” refers to an administration of 2 active ingredients at the same time or at substantially the same time by different routes. The term “administration

sequentially" refers to an administration of 2 active ingredients at different times, the administration route being identical or different.

The further therapeutic agent is typically relevant for the disorder to be treated.

Thus, the invention relates to i) a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs and ii) a further therapeutic agent as combined preparation for use in the treatment of latent viruses' infection in a subject in need thereof.

In other words, the invention relates to a method for treating latent viruses' infection comprising administering to a subject in need thereof a FOXO1 inhibitor which reactivates latent viruses in host-cell reservoirs in combination with further therapeutic agent.

In a particular embodiment, the invention relates to i) a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs and ii) a further therapeutic agent as combined preparation for use in the treatment HIV-1, HIV-2, SIV and FIV in a subject in need thereof

In another object, the invention relates to a therapeutic composition comprising a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs and a further therapeutic agent for use in the treatment of latent viruses' infection.

In some embodiment, the further therapeutic agent is antiretroviral agent and/or latency-reversing agent.

As used herein, the term "anti-retroviral agents" or "HARRT" refers to any compound, natural or synthetic, used for treating viral infections and include reverse transcriptase inhibitors (NRTIs) such as iamivudine, zidovudine, abacavir, stavudine, emtricitabine, tenofovir disoproxil and tenofovir alafenamide; non-nucleoside reverse transcriptase inhibitor (NNRTI) such as nevirapine, efavirenz, rilpivirine and doravirine; integrase inhibitors (INSTI) such as elvitegravir, dolutegravir, raltegravir and bictegravir; protease inhibitors such as iopinavir, atazanavir and darunavir; and pharmacokinetics enhancer such as ritonavir and cobicistat, l.

As used herein, the term "latency-reversing agents (LRA)" refers to compounds able to awake the latent virus from its dormant state with the purpose of making infected cells visible to the immune system. Example of LRA includes benzotriazoles; PKC agonist such as ingenol and bryostatin-1; bromodomain inhibitors and histone deacetylase inhibitors such as SAHA and Romidepsin.

Current HAART options are combinations (or "cocktails") consisting of at least three medications belonging to at least two types, or "classes," of antiretroviral agents. Initially treatment is typically a non-nucleoside reverse transcriptase inhibitor (NNRTI) plus two

nucleoside analog reverse transcriptase inhibitors (NRTIs). Typical NRTIs include: zidovudine (AZT) or tenofovir (TDF) and lamivudine (3TC) or emtricitabine (FTC). Combinations of agents which include protease inhibitors (PI) are used if the above regimen loses effectiveness.

In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of the FOXO1 inhibitor into host cells. The formation and use of liposomes and/or nanoparticles are known to those of skill in the art.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) are generally designed using polymers able to be degraded in vivo. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be are easily made.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. AS1842856 allows HIV-1 infection of human resting T cells.

AS1842856-treated resting T cells (500nM during 7 days) were infected with the HIV-1 strain NL4.3. After 3 days of infection, GAG expression was measured by FACS using a GAG-specific Ab (Mean results +/- SE with cells from 3 different donors).

Figure 2: AS1842856 induces a substantial T-cell metabolism increase

A. PBT were cultured with or without 500nM of AS1842856 for 7 days and their cell size (FSC) analyzed by FACS. **B.** Glucose uptake of T-cells treated or not with AS1842856 (500nM) for 7 days was measured by FACS after labelling with 2-NBDG. Mean results +/- SE with T cells from four independent donors **C.** High-resolution respirometry of living T-cells (25.106/ml) treated or not with AS1842856 (500nM) for 7 days was measured using an

Oxygraph-2k instrument (Oroboros Instrument). Routine respiration (respiratory steady state, left panel) was first measured, followed by the addition of Oligomycin (1µM) to inhibit ATP synthase, reducing respiration to a baseline leak state. Successive CCCP (carbonyl cyanide m-chlorophenyl hydrazine) titrations were then used to stimulate respiration to the non-coupled state of electron transfer capacity, giving the maximum respiratory capacity (left panel). Mean results +/- SE (normalized to 10.10^6 cells/ml) with T cells from 5 independent donors are shown.

Figure 3: AS1842856 is a potent activator of HIV-1 LTR in human T cell.

PBT were stimulated for 3 days with anti-CD3/CD28 beads and then infected with a pseudotyped HIV-1 retrovirus encoding GFP. Three days after infection, AS1842856 (500nM) was added to the culture. GFP expression levels were measured by flow cytometry three days after AS1842856 addition. % of GFP positive cells (left panel) and mean GFP expression measured in the GFP-positive gated cell population (right panel) of 4 different donors are shown.

Figure 4: AS1842856 allows HIV-1 latent provirus reactivation *in vitro*.

J-Lat A1 cells were incubated with different concentrations 400nM of AS1842856 and GFP expression measured by FACS after 2 days of culture. % of GFP-positive cells (left panel) and mean GFP intensity (MFI) in the GFP-positive gated cell population (right panel) after a 2 day-treatment. Mean results +/- SE from 5 independent experiments.

Figure 5: AS1842856 allows HIV-1 latent provirus reactivation *ex vivo*.

A. Simian PBT were cultured in the presence or absence of AS1842856 (500nM) for 7 days. RNA and DNA cell content was measured by FACS on the gated cells after acridine orange staining (mean results +/- SE with cells from 3 independent animals). **B.** CD4+ T cells from 4 animals infected with SIV mac251, and treated for 6 months with antiretroviral agents (Tenofovir, Emtricitabine, Dolutegravir) were cultured for two days with or without, AS1842856 or anti-CD3/CD28 coated beads, used as positive control. Heterologous simian splenocytes from non-infected animals stimulated for two days with anti-CD3/CD28 were added to the culture for nine days. At the end of the culture period, measurements by quantitative PCR of GAG sequence in genomic DNA were performed.

Figure 6: Synergic effect of AS1842856 with LRA to reactivate HIV-1 latent forms.

J-Lat A2 cells were incubated with various concentrations of AS1842856 and a various concentrations of SAHA (A) or Romidepsin (B). Percentage of GFP positive cells (left panel) and mean GFP intensity in the GFP-positive was measured by FACS after 2 days of culture.

EXAMPLE:

Material & Methods

Cells.

Human PBT were purified from the blood of healthy donors as described (Froehlich et al 2016 oncotarget). JLAT and HEK293T cells were cultivated in complete RPMI medium. Where indicated, anti- CD3/anti-CD28-coated Dynabeads (1 beads for 5 cells, Invitrogen), IL-2 (20 U/ml, R&D Systems) were added to the culture medium.

Oroboros measurements of O2 consumption.

O2 concentration and consumption by T cells was measured by a high-resolution respirometer (Oroboros Oxygraph-2k). Both electrodes were calibrated at 37°C and 100% oxygen before adding 2.5 ml of cells (2x 107 cells/ml) to each chamber. After stabilization of the basal respiratory rate (i.e. in the absence of any exogenous agent) oligomycin (1µM final) and then successive additions of CCCP (1µM final) at intervals of 300 sec were added to reach the optimal concentration causing a maximal uncoupled respiratory rate.

Western blot analysis.

Protein expression levels were analyzed by Western blot as described. Blotting antibodies used were anti-SAMHD1 (cell signaling), anti-SAMHD1P Thr592 (cell signaling), anti-CDK2 (Santa Cruz), anti-p27 (BD Biosciences), anti-RBP Ser807/811 (cell signaling) followed by goat-anti-mouse- or goat-anti-rabbit-HRP (Jackson ImmunoReseach) incubation and ECL revelation.

Flow cytometry.

The antibodies for flow cytometric analysis: anti-CD4 and anti-CD8 were from BD biosciences, anti-CD62L (MEL14) and anti-CD45RA were from eBioscience, anti-CD71 was from Pharmingen, anti-CD98 was from Miltenyi and anti-GAG (clone KC57) from Beckman Coulter). For staining with GAG and SAMHD1P staining, cells were first fixed with 4% paraformaldehyde (PFA), then permeabilized in a buffer containing PBS, 1% BSA, 0.1% Triton X-100. For acridine orange staining, 106 cells were washed with PBS-2% FCS at 4°C and labeled with 0,4ml of solution A (Triton X100 0.1%, HCL 0.1 mM, NaCl 150 mM), 1.2 ml of solution B (critic acid 0.1M, Na2HPO4 0.2M, NaCl 150mM, EDTA 1mM) and 0.6 ml of acridine orange (1µg/ml, TermoFischer) and directly analyzed by flow cytometry. For glucose uptake measurements, PBT treated or not during 7 days with AS1842856 (500 nM) were washed twice with PBS and incubated for 45 min at 37°C with PBS, Hepes 10 mM. 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose; Sigma), a fluorescent glucose analog (final concentration of 25µM), was then added and cells maintained for an additional incubation time of 30 min at 37°C. After two PBS washes, cell fluorescence was

analyzed by FACS. Immuno-stained samples were run on BD FACS Calibur and analyzed using FlowJo software.

ImageStream flow cytometry.

At the end of the culture, cells were washed once in cold PBS and fixed for 20 minutes on ice in cytofix/cytoperm (BD Bioscience) solution. Cells were then staining with anti NFAT (D43B1) (Cell signaling), and finally with anti-rabbit Alexa-488 (Cell signaling). DAPI (Sigma D21490) (5 nM) was added to stain the nucleus immediately before analyses. Flow cytometry was performed on an ImageStreamX MKII high-speed imaging flow cytometer (Amnis Corporation) and analyzed with aIDEAS Analysis Software (Amnis Corporation).

Calcium Measurements.

T cells were incubated for 20 min at 37°C with 1.5 μM Fura-2/AM (Molecular Probes). Experiments were performed at 37°C in mammalian saline buffer (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 20 mM HEPES, 11 mM glucose). Calcium measurements by spectrofluorimetry were performed as previously described (42) with a Cary Eclipse spectrofluorimeter (Varian) (excitation: 340 and 380 nm; emission: 510 nm).

Viral production, titration, and infection.

For the production of GFP viral particles, HEK293T were transfected with psPAX2 lentiviral packaging plasmid along with the plasmid encoding VSV-G and HIV-1 LTR-GFP (24). The titer of the virus stock was measured by flow cytometry analysis of GFP, 3 days after infection of Jurkat cells. Replication-competent HIV-1 NL4.3 strains, were produced in HEK293T cells by cotransfection of the proviral plasmid in combination with pVSVg using the calcium phosphate precipitation technique as described previously (43). The amounts of CAp24 produced were determined by enzyme-linked immunosorbent assay (ELISA; Innogenetics). 106 primary cells were infected using 250 ng of CAp24 for 3 to 7 days.

Detection of SIV viral DNA.

Cells were first purified by Ficoll-Hypaque gradient centrifugation, then resting CD4+ T cells were isolated using resting CD4+ kit (StemCell). After two days of culture, with AS1842856 or anti-CD3/CD28 beads, 3x106 resting CD4+ T cells were cocultivated with 106 activated heterologous simian splenocytes for nine days. For SIV DNA quantifications, cells were lysed in Tween-20 (0.05%), Nonidet P-40 (0.05%), and proteinase K (100 μg/mL) for 30 min at 56°C, followed by 15 min at 98°C. Gag sequences were amplified together with the rhesus macaque CD3γ chain in triplicate using the "outer" 3′/5′ primer pairs by 15 min of denaturation at 95°C, followed by 22 cycles of 30 s at 95°C, 30 s at 60°C, and 3 min at 72°C. SIV Gag and CD3γ were quantified within each of the PCR products in LightCycler®

experiments performed on 1/280th of the PCR products; "inner" 3′/5′ primer pairs and the LightCycler®480 SYBR Green I Master Mix (Roche Diagnostics, Meylan, France) were used. The PCR cycling program consisted of 10 min of initial denaturation at 95°C, 40 cycles of 10 s at 95°C, 6 s at 64°C, and 15 s at 72°C. Fluorescence measurements were performed at the end of the elongation steps. Plasmids containing one copy of both the CD3γ and SIV Gag amplicons were used to generate standard curves. Quantifications were performed in independent experiments using the same first-round serial dilution standard curve. Quantifications were made in triplicate for all samples studied. The results were expressed as the absolute number of SIV copies per 105 cells.

Statistical Analysis.

Means +/- SE are shown when indicated. Statistically significant differences between groups were assessed with an unpaired Student's t test. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Results

FOXO1 inhibition alone by AS1842856 allows HIV-1 infection of resting T cells.

We first determine whether the sole FOXO1 inhibition with AS1842856 allowed the infection of resting T cells by HIV-1 in the absence of any additional treatment. For this aim, peripheral blood human T cells (PBT) were cultured with or without AS1842856 and then brought into contact with a VSV-G non-replicative lentiviral vector expressing GFP under LTR control. Three days later, the percentage of GFP-positive cells was analyzed by flow cytometry. As a positive control, we looked also at the infection of PBT cells stimulated with anti-CD3/CD28 beads (data not shown). FACS dot plot analyses of the results obtained with a representative donor, as well as mean results from five donors are not shown here. The percentage of GFP-positive cells was strongly increased in CD3/CD28 activated T cells. Mainly, we also observed a marked increase of GFP positive cells after FOXO1 inhibition. Because the use of V-SVG envelope to infect resting T cells can introduce a bias in our results (16), we checked the capacity of AS1842856-treated resting T cells to be infected with a bona fida HIV 1 strain, NL4.3. Three days after infection, intracellular expression of the GAG precursor was measured by flow cytometry. As shown in Figure 1 the number of GAG+ cells was significantly higher with AS1842856. It should be noted that the percentage of CD4+ T cells after 3 days of infection with NL4.3 viruses was comparable between AS1842856-treated and untreated cells. Thus the sole FOXO1 inhibition, in the absence of any other stimulation allowed infection of resting T cells by HIV-1.

Inhibition of FOXO1 activity increases T cell metabolism.

Retrovirus replication is highly dependent of the metabolic activity of the cellular host machinery (17, 18). We therefore hypothesized that this susceptibility to HIV-1 infection of FOXO1-inhibited resting T cells could be due to an increased cell metabolism. Cell size variation is often linked to metabolism rate. As shown in Figure 2A, after 7 days of culture with 500 nM of AS1842856, T cells exhibited a substantial size increase. Time-course analyses showed a continuous rise of the cell size, usually maximum after 7 days of culture and for drug concentrations between 200 nM and 500 nM (data not shown). Importantly, no associated toxicity of the drug was observed (data not shown). Parallel labelling of CD4+ and CD8+ T-cells and of their naïve and memory sub-populations showed that this cell size increase was very similar in both CD4+ and CD8+ T-cell subsets (data not shown). These results also indicated that within these two subsets both naïve and memory T cells were affected.

As increased cell metabolism is often associated with glucose consumption, we analyzed the glucose uptake in T cells treated or not with AS1842856. As shown in Figure 2B, FOXO1 inhibition induced a significant increase of glucose uptake. We also checked the consequences of AS1842856 treatment on mitochondrial respiration, another cell function associated with metabolism increase. We therefore performed high-resolution respirometry experiments of PBT treated or not with AS1842856 (Figure 2C). Results showed that respiration at the steady state was increased by AS1842856. Using oligomycin, an inhibitor of ATP synthase reducing respiration to the leak state, followed by successive addition of CCCP (carbonyl cyanide m-chlorophenyl hydrazone) to stimulate respiration to the non-coupled state of the electron transfer capacity, we also observed that the maximum respiratory capacity was strongly increased by the drug. Finally, we investigated the effect of AS1842856 on the expression of the receptor of transferrin (CD71) and the heavy chain of the system L amino-acid transporter (CD98). These cell-surface markers are known to be associated with an increased metabolic status in T lymphocytes (19–22). Mirroring the glucose uptake and mitochondrial respiration, we observed a significant increase of this two receptors on T cells treated with AS1842856. Both CD4+ and CD8+ T-cells and their naïve and memory subsets were affected (data not shown). These results demonstrated that FOXO1 inhibition by AS1842856 is self-sufficient to trigger an increase of the cellular metabolic activity of resting human T lymphocytes.

Inhibition of FOXO1 induces a transition from quiescence (G0) to the G1 phase of the cell cycle.

The increase of cell size and number of organelles (such as mitochondria), as well as accumulation of nutrients, are hallmarks of the transition from the G0 to the G1 phase of the

cell cycle that are required to prepare the subsequent phases leading to mitosis (23). We therefore investigated the cell cycle status of PBT treated with AS1842856 using acridine orange staining, an intercalant dye that allows a parallel and simultaneous labelling of both RNA and DNA. As a positive control of cells increasing both their RNA and DNA contents, we used untreated T cells activated during 3 days with anti-CD3/CD28 beads. The results showed that AS1842856 markedly increased cellular RNA levels without any significant change of the DNA content, as well as CD3/CD28 beads increased both (data not shown). These results demonstrate that AS1842856-treated PBT show characteristic features of cells undergoing a true G0→G1 cell cycle progression, but without any cell division.

To further investigate this process, we checked whether expression and/or phosphorylation of several key molecular markers involved in cell progression along the G1 phase of the cell cycle were changed after AS1842856 treatment. A strong decrease of p27 expression, paralleled by an up-regulation of CDK2, were found, and levels of Rb phosphorylation were also increased (data not shown). Furthermore, we also observed the phosphorylation (i.e. the inactivation) of SAMHD1, a well-known quiescence and HIV 1 restriction factor in T cells. So, the sole FOXO1 inhibition induced not only an increase of T cell metabolism but also the G0→G1 transition associated with SAMHD1 phosphorylation that could explain the permissivity of AS1842856 treated resting T cells that we observed (data not shown).

AS1842856 potentiates LTR activity and reactivates latent forms of HIV 1.

Because HIV-1 replication in resting T cells is limited by the transcriptional activity of the viral LTR, we have also determined the consequences of FOXO1 inhibition on LTR activity (i.e. at the post-integrative level). For this purpose, PBT were stimulated with anti-CD3/CD28 beads and next infected with the previously used VSV-G non-replicative lentiviral vector expressing GFP. Then, the cells were incubated with or without AS1842856 for two days, and GFP expression levels measured by flow cytometry to see whether FOXO1 inhibition by the drug could activate the LTR integrated in the host cell genome (Figure 3). As the LTR activity is mainly controlled by NFAT and NF-κB, whose transcriptional activity is dependent on T cells activation, we explored their activity in PBT after AS1842856 treatment. No difference was detected in the activation of the NF-κB pathway, as revealed by the absence of the degradation of the NF-κB inhibitor IκBα (data not shown). In contrast, we observed a clear nuclear translocation of NFAT in AS1842856-treated cells (data not shown). In this experiment, ionomycin was used as a positive control increasing intracellular calcium and triggering NFAT activation. Interestingly enough, FOXO1 inhibition was found to even potentiate the effect of

ionomycin in this assay. We also found in parallel experiments that the AS1842856 drug increased the steady-state level of intracellular calcium in unstimulated T cells (data not shown) as well as the calcium response induced by ionomycin (data not shown), illustrating functionally the potentiation of NFAT pathway resulting of FOXO1 inhibition.

Next, we used the J-Lat cell model in order to confirm the consequences of FOXO1 inhibition on LTR activity, and mainly to explore the ability of AS1842856 to reactivate latent forms of HIV 1. J-Lat cells are Jurkat cells containing an integrated silent form of a minimal HIV-1 provirus encoding GFP (LTR-Tat-IRES-GFP), and in which GFP can be used as a fluorescent read-out of the reactivation of the latent provirus (24). As in Jurkat cells FOXO1 is mostly inactive, due to a defect of the lipid phosphatase PTEN, we first checked the FOXO1 transcriptional activity in different J-Lat clones by analyzing expression of CD62L, a well-known target of FOXO1. The A1 J-Lat clone expressing large amounts of CD62L, we selected it. After a 3 day treatment of J-Lat A1 clone with different concentrations of AS1842856, we observed a strong increase of the percentage of GFP-positive cells, as well as an increase of the mean of GFP expression (Figure 4), indicative of a reactivation of the LTR. These results were confirmed in the J-Lat cell clone A7 (data not shown). Interestingly, we observed that in this clone, which expresses low levels of CD62L, the level of virus reactivation induced by AS1842856 is only moderate (data not shown).

To strengthen these observations, we measured reactivation induced by a non-pharmacological approach by knocking-down FOXO1 expression in the J-Lat A1 clone using a FOXO1-specific shRNA construct (SEQ ID NO:1). These cells showed an increase percentage of GFP-positive cells, as compared to cells in which a control shRNA had been used (data not shown).

We also investigated whether these findings could be extended to primary T cells. For this purpose, we set up an experimental model using PBT activated with anti-CD3/anti-CD28-coated beads, then infected with a pseudo typed retrovirus encoding GFP in the absence of TAT. The cells were then maintained in culture with interleukin 2 (IL 2) for several weeks. The percentage of GFP-positive cells continuously decreased over time (data not shown), due to a gradual silencing of LTR activity, as reported previously (25). Cells were then treated with AS1842856 (500nM) or anti-CD3/anti-CD28-coated beads as a positive control, and latency reversion was assessed by measuring GFP fluorescence after 3 days of reactivation. AS1842856 treatment was found to increase the number of GFP-positive cells (data not shown). Repeating these experiments with 4 donors, we observed that, although lower than the reactivation induced by anti-CD3/anti-CD28 beads, a significant increase of virus reactivation was always found

with AS1842856 (data not shown). These results demonstrate that inhibiting FOXO1 with AS1842856 could reverse HIV-1 latency in human T lymphocytes.

In order to confirm this result in a model more relevant to physiopathology, we tested the capacity of AS1842856 to reactivate the latent proviral forms in a simian model. For this purpose, we have first verified that AS1842856 was able to induce the G0→G1 transition of simian T cells, as described for human T cells. As shown Figure 5A, after 7 days of culture AS1842856 induced an increase of transcriptional activity of simian T cells. Next, we purified CD4+ T cells from macaques that had been infected by SIV mac251, and then treated for 6 months with Tenofovir®, Emtricitabine® and Dolutegravir®. Cells were then cultured with AS1842856, or anti-CD3-CD28 coated beads as a positive control. Two days later activated splenocytes from non-infected macaques were added to the culture. Nine days later, DNA was extracted and the presence of viral DNA was analyzed by quantitative PCR. As shown in figure 5B, the sole inhibition of FOXO1 induced by AS1842856 led to latent proviruses recurrence in three out four animals in a manner comparable to the positive control. The absence of reactivation in the presence of AS1842856 observed for the fourth animal was observed not only after AS1842856 treatment but also with anti-CD3/CD28 coated beads, suggesting an individual response defect. These results demonstrated that inhibiting FOXO1 with AS1842856 could reverse HIV-1 latency established in vivo.

AS1842856 reactivates latent SIVmac in T cells from non-human primates under cART treatment.

In order to confirm this result in a model more relevant to pathophysiology, we investigated whether AS1842856 could reactivate latent SIVmac in CD4+ T cells from non-human primates under cART treatment. For this aim, we used rhesus macaques that had been previously infected by SIV mac251, and treated for 6 months with a triple antiretroviral therapy combining Tenofovir, Emtricitabine and Dolutegravir to induce latency. We first controlled that, as in human T cells, AS1842856 was able to induce the G0→G1 transition of T cells purified from the blood of healthy macaques (data not shown). Next, CD4+ T cells from the blood of the infected macaques were purified and cultured with AS1842856, anti-CD3-CD28 coated beads as a positive control, or vehicle only. Two days later, to amplify infectious viruses produced by CD4+ T cells, activated splenocytes from non-infected macaques were added. Nine days later genomic DNA was extracted and analyzed for the presence of viral GAG by quantitative PCR. GAG was undetectable in cells treated with vehicle only. In contrast, inhibition of FOXO1 by AS1842856 led to latent proviruses recurrence in three out of four animals in a manner comparable to the positive control. The absence of reactivation in the

presence of AS1842856 observed for the fourth animal was observed not only after AS1842856 treatment but also with anti-CD3/CD28, suggesting an individual response defect. To evaluate virus production obtained in these conditions, ultracentrifugated supernatants were used to infect freshly activated splenocytes from non-infected macaques. Five days post infection, substantial infection levels were obtained with supernatants obtained from macaques under cART treatment having shown a viral reactivation after AS1842856 treatment. These results demonstrate that inhibiting FOXO1 with AS1842856 reverses in vivo-induced retroviral latency leading to the production of infectious retroviral particles.

Synergic effect of AS1842856 with LRA to reactivate HIV-1 latent forms.

Numerous factors participate in the HIV-1 genome silencing and a combination of at least two LRAs is often needed to achieve the efficient activation of the silent virus. Histone deacetylase inhibitors (HDAi), by their of chromatin organization's role, is considering as LRA and were used in clinical trials. In this context, we tested the possible synergy between AS1842856 and two HDAi: SAHA and Romidepsin a more recent generation of HDAi. As shown in figure 6A and 6B, we can be observed in a JLAT model that the presence of AS1842856 strongly increase the percentage as well as the mean of GFP positive cells.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs for use in the treatment of latent viruses' infection in a subject in need thereof.

2. The FOXO1 inhibitor according to claim 1 wherein FOXO1 inhibitor reactivates latent viruses in CD4+ T cell reservoirs for use in the treatment of latent viruses in a subject in need thereof.

3. The FOXO1 inhibitor for use according to claims 1 and 2 wherein said latent virus is selected from the group consisting of HIV-1, HIV-2, SIV and FIV.

4. The FOXO1 inhibitor for use according to claims 1 to 3 wherein said latent virus is HIV-1.

5. The FOXO1 inhibitor for use according to claims 1 to 4 wherein FOXO1 inhibitor is a small organic molecule.

6. The FOXO1 inhibitor for use according to claims 1 to 5 wherein FOXO1 inhibitor is the AS1842856 drug.

7. A therapeutic composition comprising a FOXO1 inhibitor reactivating latent viruses in host-cell reservoirs for use in the treatment of latent viruses' infection.

8. A method for treating latent viruses' infection comprising administering to a subject in need thereof a FOXO1 inhibitor which reactivates latent viruses in host-cell reservoirs.

9. The FOXO1 inhibitor for use according to any one of claims 1 to 6, or the therapeutic composition according to claim 7, or the method for treating according to claim 8 wherein said FOXO1 inhibitor is administered orally, subcutaneously or by the intravenous route.

10. The FOXO1 inhibitor for use according to any one of claims 1 to 6, or the therapeutic composition according to claim 7, or the method for treating according to claim 8 wherein said FOXO1 inhibitor is administered in combination with antiretroviral agent and/or latency-reversing agent.

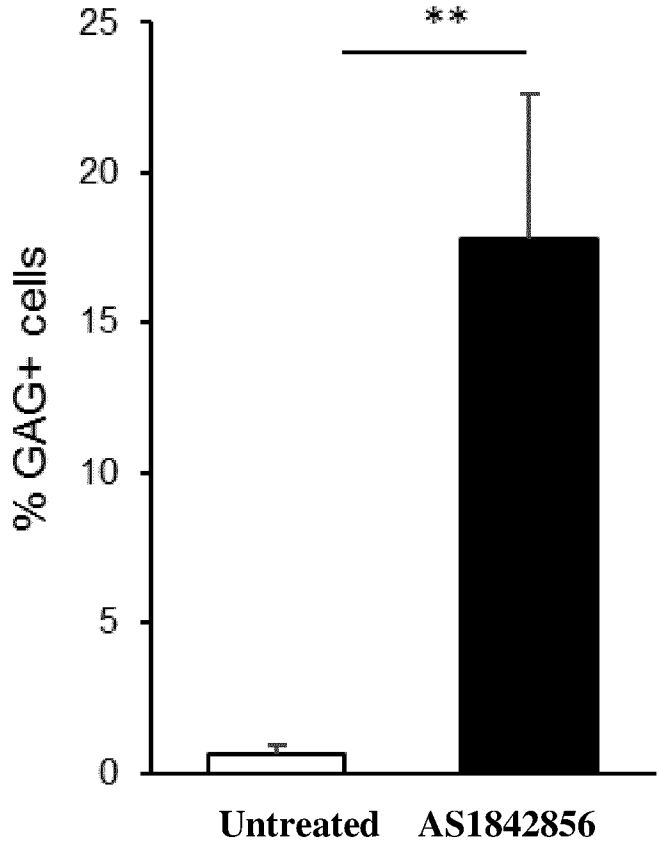


Figure 1

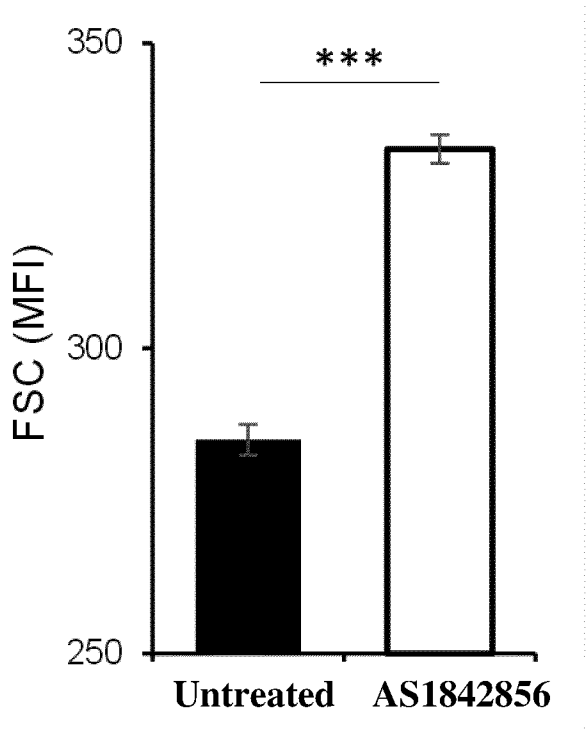


Figure 2A

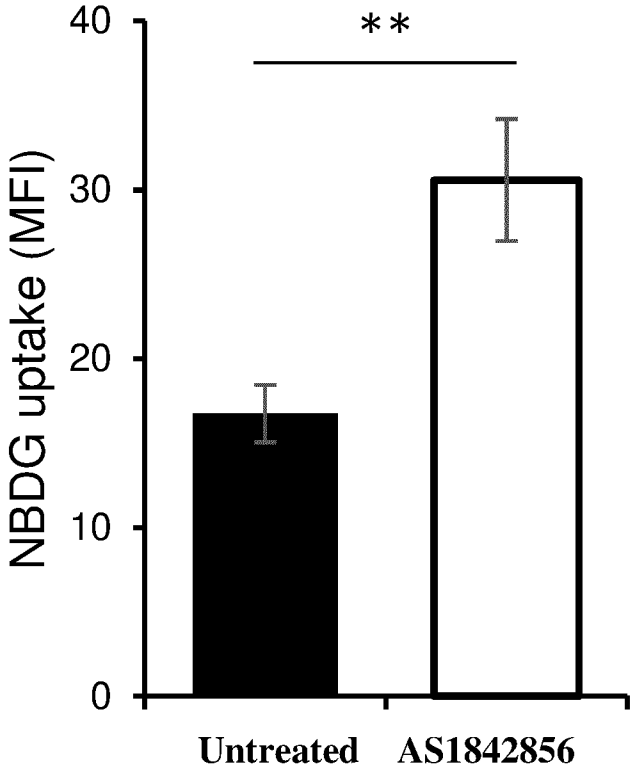


Figure 2B

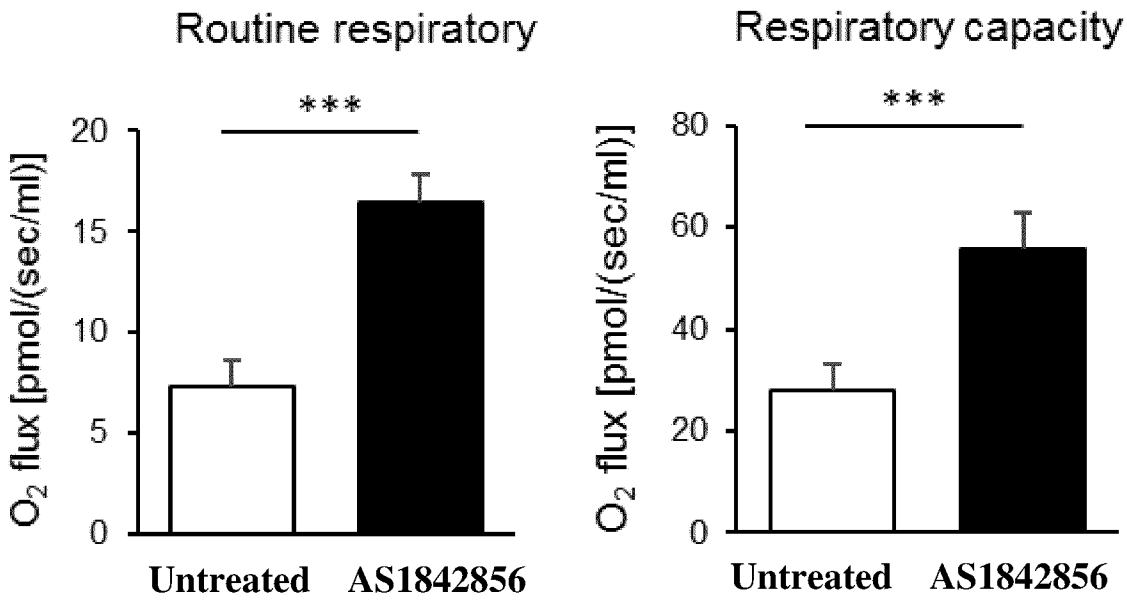


Figure 2C

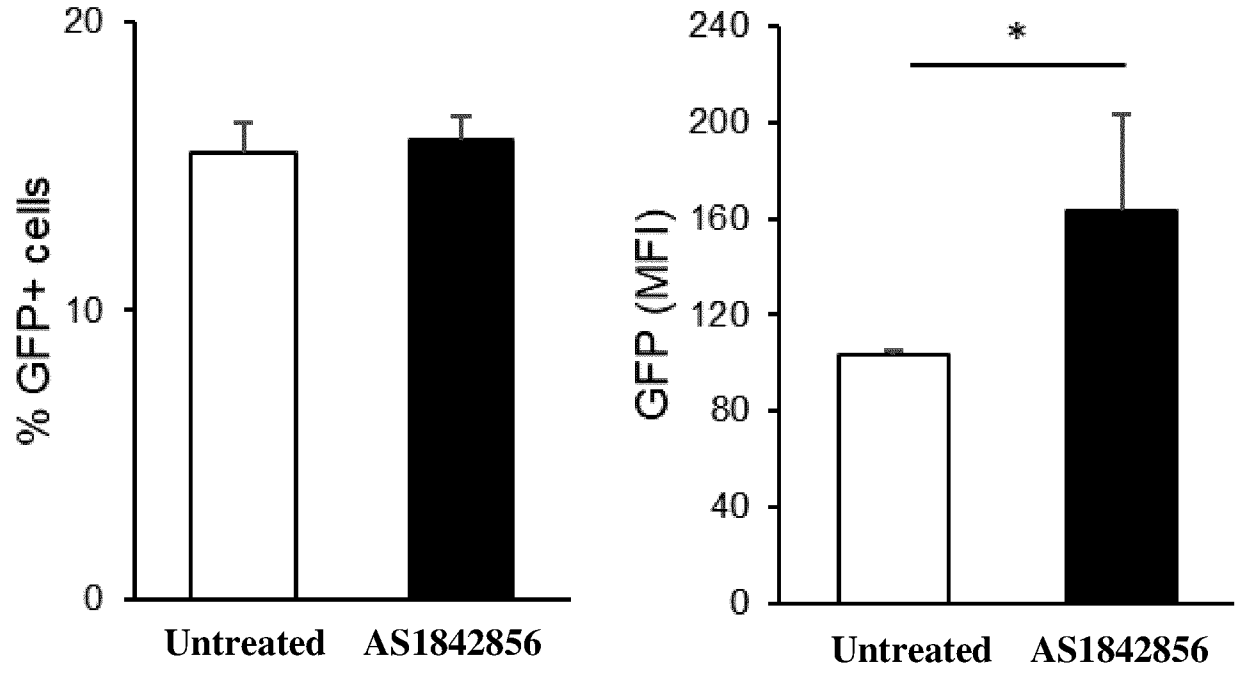


Figure 3

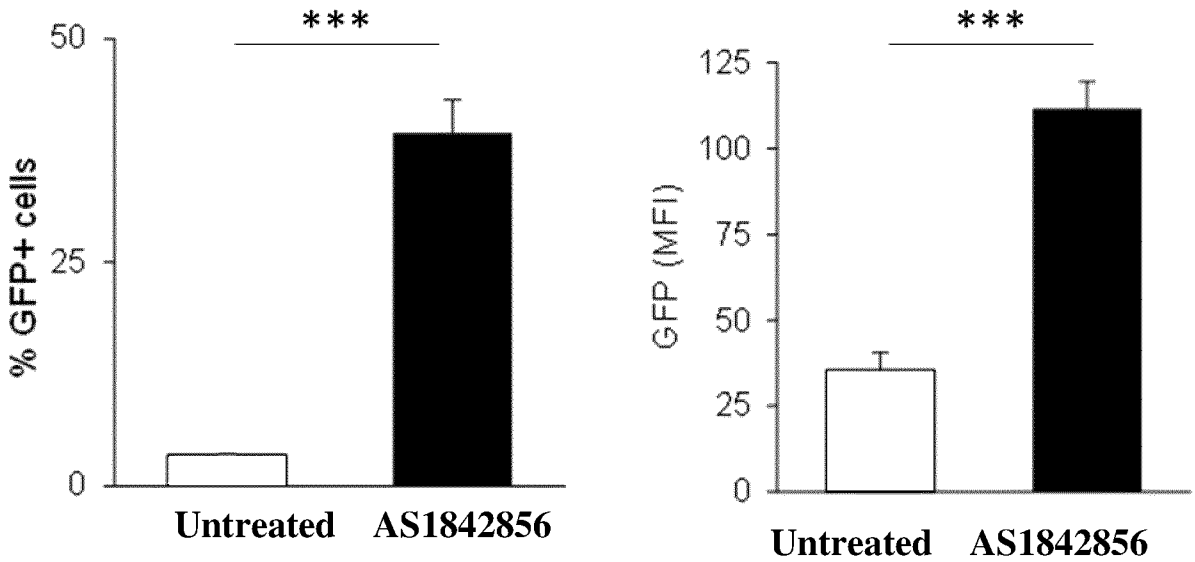


Figure 4

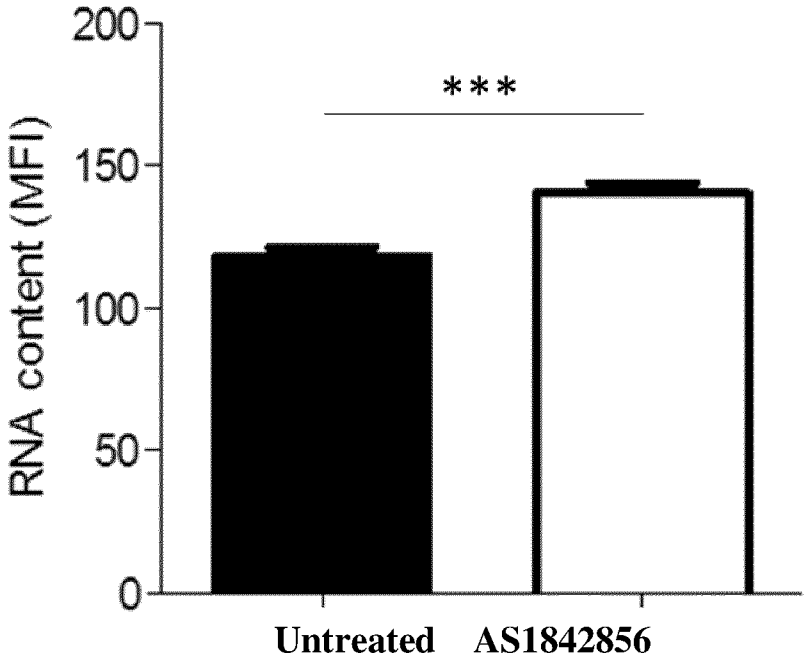


Figure 5A

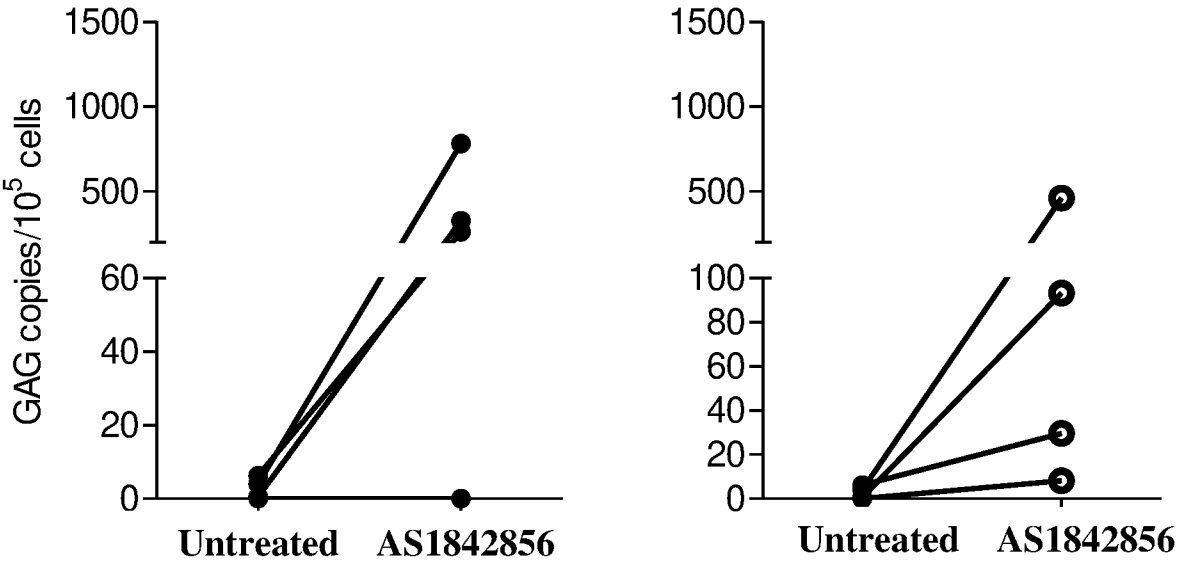


Figure 5B

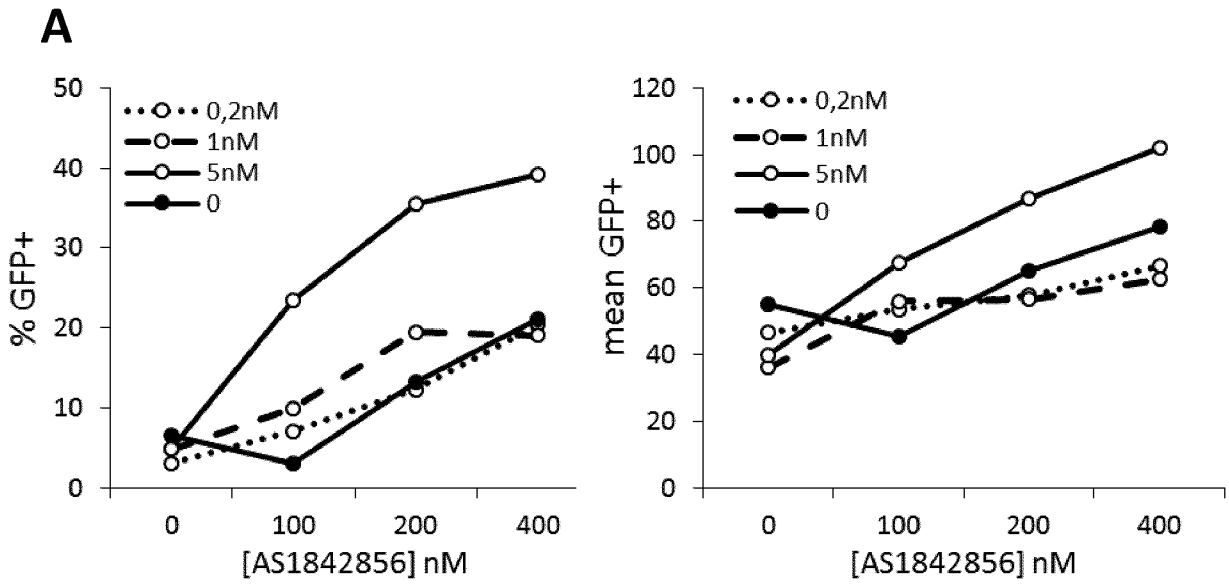


Figure 6A

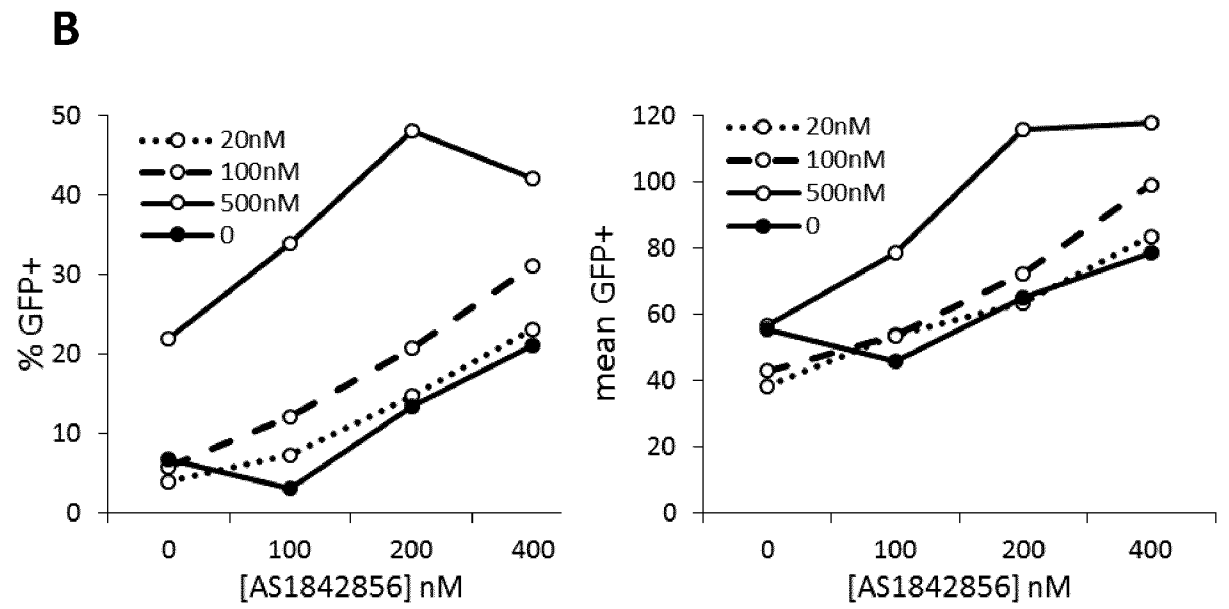


Figure 6B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/054432

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4704 A61P31/12
ADD.

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

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 practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 3 388 084 A1 (UNIV KYOTO [JP]) 17 October 2018 (2018-10-17) claims 1,6,13 paragraph [0034] -----	1-9
X	GAO RUOYUN ET AL: "FoxO1 Suppresses Kaposi's Sarcoma-Associated Herpesvirus Lytic Replication and Controls Viral Latency", JOURNAL OF VIROLOGY (ONLINE), AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 93, no. 3, 1 February 2019 (2019-02-01), pages e01681-18, 1, XP009515124, ISSN: 1098-5514, DOI: 10.1128/JVI.01681-18 page 9, paragraph 1 - paragraph 2 page 2, paragraph 1 - paragraph 2 ----- -/--	1,5-9

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

[X] See patent family annex.

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

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"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

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"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

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Date of the actual completion of the international search	Date of mailing of the international search report
18 May 2020	09/06/2020

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	Bonzano, Camilla

3

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/054432

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OTEIZA ALEXANDRA ET AL: "FoxO4 negatively controls Tat-mediated HIV-1 transcription through the post-transcriptional suppression of Tat encoding mRNA", JOURNAL OF GENERAL VIROLOGY, vol. 98, no. 7, July 2017 (2017-07), pages 1864-1878, XP009515128, page 1865, column 1, paragraph 1 - column 2, paragraph 1	1-9
X,P	ROUX ARTHUR ET AL: "FOXO1 transcription factor plays a key role in T cell-HIV-1 interaction", PLOS PATHOGENS, vol. 15, no. 5, May 2019 (2019-05), XP009515129, page 14, paragraph 3	1
X	ZACK JEROME A ET AL: "Quiescent CD4(+) T Cells Inhibit Multiple Stages of HIV Infection", TUMOR DORMANCY, QUIESCENCE, AND SENESCENCE, VOL 2: AGING, CANCER, AND NONCANCER PATHOLOGIES SPRINGER, PO BOX 17, 3300 AA DORDRECHT, NETHERLANDS, 2014, pages 253-262, XP009515127, page 254, column 1, paragraph 2 - column 2, paragraph 2	1-10

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/054432

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 3388084 A1	17-10-2018	AU 2016366250 A1	14-06-2018
		BR 112018011131 A2	21-11-2018
		CA 3007613 A1	15-06-2017
		CN 108601840 A	28-09-2018
		EP 3388084 A1	17-10-2018
		JP WO2017099034 A1	01-11-2018
		KR 20180093990 A	22-08-2018
		SG 11201804473R A	28-06-2018
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		WO 2017099034 A1	15-06-2017