(51) International Patent Classification:
A61K 31/522 (2006.01)

(21) International Application Number:
PCT/US2006/009390

(22) International Filing Date: 16 March 2006 (16.03.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/663,059 18 March 2005 (18.03.2005) US

(71) Applicant (for all designated States except US); UNIVERSITY OF ROCHESTER [US/US]; 601 Elmwood Avenue, Box OTT, Rochester, New York (US).

(72) Inventors; and


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

Published:
— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITIONS AND METHODS RELATED TO THE MANIPULATION OF ADENOSINE 2A RECEPTOR SIGNALING FOR THE TREATMENT OF HIV ASSOCIATED NEUROLOGICAL DAMAGE

(57) Abstract: Provided herein are compositions and methods relating to the treatment and prevention of HTLV-1 associated dementia (HAD) utilizing a modulator of adenosine receptor signaling.
ACKNOWLEDGMENTS

This work was with government support under National Institutes of Health PO1 AI050244 and 5T32DA007232. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Despite the efficacy of highly active antiretroviral therapy (HAART) in reducing viral burden, neurologic disease associated with HIV-1 infection of the central nervous system CNS has not decreased in prevalence. HIV-1 does not induce disease by direct infection of neurons, although extensive data suggest that intra-CNS viral burden correlates with both the severity of virally-induced neurologic disease, and with the generation of neurotoxic metabolites. Many of these molecules are capable of inducing neuronal apoptosis in vitro, but neuronal apoptosis in vivo does not correlate with CNS dysfunction. Thus, the mechanism of virally-induced neurologic disease is not known in the literature.

BRIEF SUMMARY OF THE INVENTION

Provided herein is a method of protecting a neuron from dysfunction induced by an HIV neurotoxin comprising contacting the cell with a modulator of adenosine receptor signaling.

Further provided is a method of treating or preventing HIV-1 associated dementia (HAD) in a subject in need of such treatment or prevention, comprising administering to the subject a therapeutically effective dose of a modulator of adenosine receptor signaling.

Further provided is a composition, comprising a modulator of adenosine receptor signaling and a molecule that inhibits mitochondrial hyperpolarization in a neural cell.

Additional advantages of the disclosed method and compositions will be set forth in part in the description which follows, and in part will be understood from the description, or may be learned by practice of the disclosed method and compositions. The advantages of the disclosed method and compositions will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following
means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the disclosed method and compositions and together with the description, serve to explain the principles of the disclosed method and compositions.

Figure 1 shows that an adenosine receptor 2A (A2AR) antagonist protects neurons against Tat-induced apoptosis. CGNs were exposed to HIV-1 Tat (Tat, 500 nM), or vehicle alone (NT), in the presence or absence of the A2A antagonist, ZM241385. After 48h, cultures were analyzed for the percentage of apoptotic cells using the TUNEL assay. Data represent mean ± SEM for one experiment that was performed in triplicate; data are representative of two independent experiments.

Figure 2 shows that ATL455, an A2AR antagonist protects neurons against Tat-induced apoptosis. CGNs were exposed to HIV-1 Tat (Tat, 500 nM), or vehicle alone (No Tat), in the presence or absence of the A2AR antagonists, ATL455 and ZM241385, or the A2AR agonists ATL313 and CGS21680. After 48h, cultures were analyzed for the percentage of apoptotic cells using the TUNEL assay. Data represent mean ± SEM for one experiment that was performed in triplicate; data are representative of two independent experiments.

Figure 3 shows that adenosine receptors control nitric oxide (NO) secretion induced by Tat. Human primary monocytes were treated with Tat in the presence or absence of the indicated adenosine receptor agonists for 8h, and nitric oxide levels in the culture medium of the cells were then quantitated using the Griess reaction (Active Motif). Data represent mean±SEM of three experiments.

Figure 4 shows that Tat-induced monocyte activation is opposed by the A2AR agonist, CGS21680. Human primary monocytes were treated with Tat (100 nM) in the presence or absence of the A2AR agonist, CGS21680 (CGS; 1μM) or the A2AR antagonist, ZM241385 (100 nM) for 4h. TNFα levels in culture supernatants were then quantitated by ELISA. Data represent mean±SEM of three replicates, from a single representative experiment.
Figure 5 shows Tat induces inflammatory gene expression by primary human monocytes. Primary monocytes were seeded in separate triplicate wells, and exposed independently to LPS (100 ng/ml), vehicle (NT) or Tat (100 nM) in the presence or absence of the A2AR agonist, CGS21680 (1 μM). After 4h, cells were harvested. (A) TNFα was measured in cell supernatants by ELISA. Mean data values (±S.D.) are shown for the triplicate wells. (B) The corresponding cells from each well in panel A were lysed and subjected to qRT-PCR analysis. Each cDNA sample was analyzed in quadruplicate. Assay results are presented as fold induction of gene expression, compared to untreated cells, after normalization to levels of GAPDH. Data represent mean ± SD for each individual experimental replicate/well.

Figure 6 shows that Tat-induced monocyte activation is opposed by the A2AR agonist, ATL313. Human primary monocytes were treated with Tat (100 nM) in the presence or absence of the A2AR agonist, ATL313 (1-4 nM) or the A2AR antagonist, ATL455 (1-4 nM) for 4h. TNFα levels in culture supernatants were then quantitated by ELISA. Data represent mean±SEM of three replicates, from a single representative experiment.

Figure 7 shows a dose response analysis for inhibition of Tat-induced monocyte activation by the A2AR agonist, ATL313. Human primary monocytes were treated with Tat (100 nM) in the presence or absence of the A2AR agonist, ATL313 (at the indicated doses) for 4h. TNFα levels in culture supernatants were then quantitated by ELISA. Data represent mean±SEM of three replicates, from a single representative experiment.

Figure 8 shows that Tat and PAF increase synaptic vesicular activity. Images of (A) Brightfield and (B) fluorescent FM1-43 labeling show that FM1-43 uptake results in labeling that is punctate and oriented primarily along neuronal processes. (C) Moreover, activity dependent FM1-43 release by KCl depolarization preferentially reduces the punctate vesicular labeling versus background signal. (D) In 14 and 14 day old primary neurons, 2.5 μg/ml Tat and 4.25 μg/ml Tat induces an increase in spontaneous activity dependent vesicular uptake, an effect that is both dose and culture age dependent. (E) However, no effect is seen with mutated Tat protein, suggesting that Tat’s effect is due to biologically specific activities of its functional region. (F) 464 nM PAF had an even greater effect on FM1-43 uptake at 24 hours.

Figure 9 shows that Tat and PAF increase ROS in cortical neuronal cultures. Treatment of rat cortical neurons for 24 hours with Tat or PAF induced elevated levels of
ROS versus control, as measured with oxidizable dye indicator 5-(and-6)-chloromethyl-2',7'dichlorodihydrofluorescin diacetate, acetyl ester (CM-H2DCFDA) (DCF). This effect was greatest with 2.5 μg/ml Tat, resulting in a 110% increase in ROS production (*p < .0001 versus control), whereas 464 nM PAF resulted in a 50% increase in ROS production, and the H2O2 positive control induced a 90% increase.

Figure 10 shows that antioxidants ameliorate the Tat-induced rise in vesicular uptake of neurotransmitter. (A) The antioxidant TUDCA completely eliminated Tat’s effects on FMI-43 uptake for all doses of Tat.

Figure 11 shows that Tat and PAF cause a dose-dependent mitochondrial hyperpolarization in cortical neurons. (A) Treatment of rat cortical neurons with 100 ng/ml or 2.5 μg/ml Tat for 1, 4, 10, 24, 26, 36, or 48 hours resulted in a dose-dependent biphasic increase in mitochondrial membrane potential. At each dose, initial peaks were followed by periods of apparent mitochondrial membrane potential (Δψ_m) stabilization, followed by increased Δψ_m again at later time points, and the later increase in Δψ_m persisted until the end of the analysis (48 hours). A biologically inactive mutated Tat peptide (green) produced no effect. Curve fits are non-formulaic software interpolations of the data points. (B) Like Tat, its downstream mediator PAF also induced a dose-dependent rise in Δψ_m (*p < .002 or p < .0001 versus control, respectively) C) Effect of high dose FCCP (5 μM) on mitochondrial TMRM uptake at 1 hour is shown. This loss of TMRM signal with 5 μM FCCP (which would induce strong mitochondrial depolarization) helps confirmed that Tat was increases Δψ_m.

Figure 12 shows that neuronal ATP/ADP ratio increases then declines with chronic Tat exposure. Primary cortical neurons were treated for 1, 24, or 48 hours with 0 (control), 0.1, or 2.5 μg/ml Tat. ATP levels, as well as ATP/ADP ratios, were sharply increased by both doses of Tat at 1 hour, and by 2.5 μg/ml Tat at 24 hours. By 48 hours, ATP levels, while still elevated, had declined from peak levels at 24 hours, and ATP/ADP ratio had fallen to control level or below (i.e. ≤1.0). P-values shown are versus time matched control.

Figure 13 shows that the KATP channel antagonist Tolbutamide attenuates Tat-induced increases in Δψ_m and neuronal apoptosis. Co-treatment of 0.1 μg/ml or 2.5 μg/ml Tat treated cortical cultures with the mitochondrial KATP channel antagonist tolbutamide (100 μM) (A) completely prevented the Tat mediated rise in Δψ_m at 1 and 24 hours, and (B) partially attenuated Tat’s dose-dependent induction of apoptotic cell death over 24
hours. 100 μM tolbutamide alone had no significant effect on Δψm, and showed no toxicity.

Figure 14 shows that sublethal cPAF exposure leads to dendrite beading and disruption of spines. (A) cPAF causes dendrite beading and loss of dendritic spines. (A') Low-magnification images show minimal changes in dendrite morphology and total spine number in control cultures (left). In cPAF-treated cultures (right), dendrite beading (red arrows) is accompanied by spine loss and sprouting of filopodia (green arrowheads) but gross aspects of dendrite structure are preserved. (B) No cells developed dendritic swellings in control cultures, while 55±3% of cPAF-exposed cells beaded. (C) 43±5% of dendritic spines were lost during cPAF exposure, while total spine number was maintained in control cultures.

Figure 15 shows that cPAF promotes dendrite beading and failure of long-term potentiation in acute hippocampal slices. CA1 pyramidal neurons were exposed to 1μM cPAF or vehicle for 30-60min and simultaneously imaged and recorded by whole-cell patch clamp before and after delivery of a high-frequency stimulus (HFS: three 1s, 100Hz trains, every 20s) applied to Schaffer collateral axons via a bipolar stimulating electrode planted in the stratum radiatum. (A) Dendrite beading in a cPAF-exposed cell 45min after HFS, with no disruption of dendrite or spine morphology in control cells. (B) HFS elicited beading in half of the cells from cPAF-treated slices, and in none from control slices. (C) Excitatory synaptic transmission between Schaffer collateral axons and CA1 pyramidal neurons is strongly potentiated following HFS in control slices. In cPAF-treated slices, EPSPs in cells that did not develop dendrite beading underwent a smaller potentiation, while EPSPs in cells whose dendrites did bead were not potentiated at all. (EPSP traces at baseline and 40min from representative control cell and cPAF-treated beaded cell are at right.)

DETAILED DESCRIPTION OF THE INVENTION

The disclosed method and compositions may be understood more readily by reference to the following detailed description of particular embodiments and the Example included therein and to the Figures and their previous and following description.

Provided are methods and compositions for treating HIV-related neurological disorders by modulating adenosine receptor signaling. Thus, disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed method and compositions.
These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if an adenosine receptor antagonist is disclosed and discussed and a number of modifications that can be made to the an adenosine receptor antagonist are discussed, then each and every combination and permutation of the an adenosine receptor antagonist and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, is this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods, and that each such combination is specifically contemplated and should be considered disclosed.

It is understood that the disclosed method and compositions are not limited to the particular methodology, protocols, and reagents described as these can vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It is to be understood that the disclosed method and compositions are not limited to specific synthetic methods, specific analytical techniques, or to particular reagents unless otherwise specified, and, as such, can vary. It is also to be understood that the
terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a molecule" includes a plurality of such molecules, reference to "the molecule" is a reference to one or more molecules and equivalents thereof known to those skilled in the art, and so forth.

"Optional" or "optionally" means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, also specifically contemplated and considered disclosed is the range from the one particular value and/or to the other particular value unless the context specifically indicates otherwise. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another, specifically contemplated embodiment that should be considered disclosed unless the context specifically indicates otherwise. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint unless the context specifically indicates otherwise. Finally, it should be understood that all of the individual values and sub-ranges of values contained within an explicitly disclosed range are also specifically contemplated and should be considered disclosed unless the context specifically indicates otherwise. The foregoing applies regardless of whether in particular cases some or all of these embodiments are explicitly disclosed.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed method and compositions belong. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present method and compositions, the particularly useful methods, devices, and materials are as described. Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such disclosure by virtue of prior
invention. No admission is made that any reference constitutes prior art. The discussion of references states what their authors assert, and applicants reserve the right to challenge the accuracy and pertinence of the cited documents. It will be clearly understood that, although a number of publications are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

Throughout the description and claims of this specification, the word “comprise” and variations of the word, such as “comprising” and “comprises,” means “including but not limited to,” and is not intended to exclude, for example, other additives, components, integers or steps.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the method and compositions described herein. Such equivalents are intended to be encompassed by the following claims.

Provided herein is a method of protecting a neuron from dysfunction induced by an HIV neurotoxin comprising contacting the cell with a modulator of adenosine receptor signaling. Non-limiting examples of neuronal dysfunction include alterations in mitochondrial function, neurotransmitter uptake and release, neuronal architecture, synaptic transmission, and neuronal cell death. Thus, in one aspect, an HIV neurotoxin can result in neuronal cell death. As used herein, neuronal cell death includes either apoptosis or necrosis of neurons that can occur as a result of exposure to neurotoxins associated with HIV.

As used herein, apoptosis refers to programmed cell death that is signaled by the nuclei when age or state of cell health and condition dictates. Apoptosis is an active process requiring metabolic activity by the dying cell, often characterized by cleavage of the DNA into fragments that give a so called laddering pattern on gels. Cells that die by apoptosis do not usually elicit the inflammatory responses that are associated with necrosis. As used herein, necrosis refers to cell death in response to a major insult, resulting in a loss of membrane integrity, swelling and rupture of the cell. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue.
Disclosed is a method of treating or preventing HIV-1 associated dementia (HAD) in a subject in need of such treatment or prevention, comprising administering to the subject a therapeutically effective dose of a modulator of adenosine receptor signaling. HIV associated dementia (HAD) is comprised of a spectrum of conditions from the mild HIV-1 minor cognitive-motor disorder (MCMD) to severe and debilitating AIDS dementia complex. Symptoms begin with motor slowing and may progress to severe loss of cognitive function, loss of bladder and bowel control, and paraparesis. A classification system has been formulated for HIV associated dementia, wherein subjects are classified as being Stage 0 (Normal), Stage 0.5 (Subclinical or Equivocal), Stage 1 (Mild), Stage 2 (Moderate), Stage 3 (Severe), or Stage 4 (End-Stage). Thus, the subject of the provided method can therefore be classified as Stage 0, Stage 0.5, Stage 1, Stage 2, Stage 3, or Stage 4.

By “treat” or “treatment” is meant a method of reducing the effects of a disease or condition. Treatment can also refer to a method of reducing the disease or condition itself rather than just the symptoms. The treatment can be any reduction from native levels and can be but is not limited to the complete ablation of the disease, condition, or the symptoms of the disease or condition. For example, a disclosed method for treatment of HAD is considered to be a treatment if there is a 10% reduction in one or more symptoms of the disease in a subject with the disease when compared to native levels in the same subject or control subjects. Thus, the reduction can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between as compared to native or control levels. For example, in the case of HAD, to treat HAD in a subject can comprise improving the disease classification. (e.g. from stage 3 to stage 2, from stage 2 to stage 1, from stage 1 to 0.5 or from stage 0.5 to 0).

As used throughout, “preventing” means to preclude, avert, obviate, forestall, stop, or hinder something from happening, especially by advance planning or action. For example, to prevent HAD in a subject is to stop or hinder the subject from advancing in disease classification (e.g. from stage 0 to stage 0.5, from stage 0.5 to stage 1, from stage 1 to stage 2, from stage 2 to stage 3, or from stage 3 to stage 4).

Microglia, macrophages and astrocytes are major HIV-1 targets in the brain, whereas HIV-1 infected neurons have been rarely observed. This indicates that indirect mechanisms may account for the severe neuronal damage observed in these patients. In addition to the production of cytokines, HIV-1 infected and/or functionally activated
mononuclear cells and astrocytes can produce a number of soluble mediators, including the structural and regulatory proteins gp120, Tat, and platelet activating factor (PAF), which can exert damaging effects on both developing and mature neural tissues.


Endogenous adenosine plays a pivotal role in the regulation of neural cell fate. The actions of adenosine are mediated by specific receptors located on cell membranes, which belong to the family of G protein-coupled receptors. Currently, four adenosine receptors have been cloned: A₁, A₂A, A₂B, and A₃. The disclosed modulator of adenosine receptor signaling can comprise any composition that will alter a biological property of either adenosine or adenosine receptors in a cell, such as for example their synthesis, degradation, translocation, binding, or phosphorylation, such that the alteration results in a net increase or decrease in adenosine receptor signaling in the cell. As a non-limiting example, the provided modulator can be a nucleic acid that alters expression of either adenosine or adenosine receptor in a cell, such as for example RNAi or antisense nucleic acids. As another example, the provided modulator can be a polypeptide that alters the binding of adenosine to adenosine receptors, such as for example soluble adenosine receptors, mutant adenosine ligands or antibodies specific for adenosine or adenosine receptors. As another example, the provided modulator can comprise informational molecules that modulate adenosine receptor expression (such as short-interfering RNAs or peptide nucleic acids) or molecules that may regulate downstream signaling events that may occur as a result of adenosine receptor stimulation.

Thus, the provided modulator of adenosine receptor signaling can be a small molecule comprising a modified adenosine (6-amino-9-beta-D-ribofuranosyl-9-H-purine). Modifications that can be made to adenosine are well known in the art. These
modifications include those that result in adenosine receptor agonists and antagonists. These agonists and antagonists can be either receptor selective or non-selective. Provided herein is the use of these adenosine receptor agonists and antagonists in the treatment of HAD.

The modulator of the present method can be an adenosine 1 receptor (A₁R) antagonist. The modulator can be an adenosine 2A receptor (A₂AR) antagonist. The modulator can be an adenosine 2B receptor (A₂BR) antagonist. The modulator can be an adenosine 3 receptor (A₃R) antagonist. Thus, the modulator can be any adenosine receptor selective antagonist, whether known in the art or later developed. Non-limiting examples of A₂AR selective antagonists include ATL455, ZM241385, KW-6002 (istradefylline), SCH 58261, and the pharmaceutically acceptable salts thereof.


KW-6002 (istradefylline) is (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione. KW-6002 has been evaluated humans as a treatment for Parkinson’s disease (W. Bara-Jimenez, MD, A. Sherzai, MD, T. Dimitrova, MD, A. Favit, MD, F. Bibbiani, MD, M. Gillespie, NP, M.J. Morris, MRCPsych, M.M. Mouradian, MD and T.N. Chase, MD Adenosine A₂A receptor antagonist treatment of Parkinson’s disease. Neurology. 2003 Aug 12;61(3):293-6).

SCH 58261 is 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine.

These modifications to adenosine to produce antagonists are exemplary and provide guidance to and description for other antagonistic adenosine modifications.

The provided modulator can be an adenosine 1 receptor (A₁R) agonist. The modulator can be an adenosine 2A receptor (A₂AR) agonist. The modulator can be an adenosine 2B receptor (A₂BR) agonist. The modulator can be an adenosine 3 receptor (A₃R) agonist, such as for example CF101 (Aderis Pharmaceuticals). Thus, the provided modulator can be any adenosine receptor selective agonist, whether known in the art or
later developed. Non-limiting examples of A2αR selective agonist include ATL146e, ATL313, PJ-1165, Binodenoson (MRE-0470), MRE-0094, CGS21680, and the pharmaceutically acceptable salts thereof.


These modifications to adenosine to produce agonists are exemplary and provide guidance to and description for other agonistic adenosine modifications.

Any of the compounds described herein can be the pharmaceutically-acceptable salt thereof. In one aspect, pharmaceutically-acceptable salts are prepared by treating the free acid with an appropriate amount of a pharmaceutically-acceptable base. For example, one or more hydrogen atoms of the SO₃H group can be removed with a base.

Representative pharmaceutically-acceptable bases are ammonium hydroxide, sodium hydroxide, potassium hydroxide, lithium hydroxide, calcium hydroxide, magnesium hydroxide, ferrous hydroxide, zinc hydroxide, copper hydroxide, aluminum hydroxide, ferric hydroxide, isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-dimethylaminoethanol, 2-diethylaminoethanol, lysine, arginine, histidine, and the like.

In another aspect, if the compound possesses a basic group, it can be protonated with an acid such as, for example, HCl or H₂SO₄, to produce the cationic salt.
example, the techniques disclosed in U.S. Patent No. 5,436,229 for producing the sulfate salts of argininal aldehydes, which is incorporated by reference in its entirety, can be used herein. In one aspect, the reaction of the compound with the acid or base is conducted in water, alone or in combination with an inert, water-miscible organic solvent, at a temperature of from about 0°C to about 100°C such as at room temperature. In certain aspects where applicable, the molar ratio of the compounds described herein to base used are chosen to provide the ratio desired for any particular salts. For preparing, for example, the ammonium salts of the free acid starting material, the starting material can be treated with approximately one equivalent of pharmaceutically-acceptable base to yield a neutral salt.

It is contemplated that the pharmaceutically-acceptable salts of the compounds described herein can be used as prodrugs or precursors to the active compound prior to the administration. For example, if the active compound is unstable, it can be prepared as its salt form in order to increase stability in dry form (e.g., powder).

The severity of dementia in persons with HIV-1 associated neurologic disease is strongly correlated with the number of macrophages and microglia within the basal ganglia and frontal lobes [Glass, J. D., et al. 1995. Ann Neurol 38:755-762]. Thus, the activation of microglia and brain macrophages plays a crucial role in the induction of neuronal dysfunction and damage. Thus, the herein disclosed agonists of adenosine receptor signaling can inhibit HAD in a subject in part by inhibiting the recruitment of monocytes to the CNS.

The disclosed method can be further combined with other therapeutic approaches for the treatment of HIV-1 infection or HAD. Thus, the disclosed method can further comprise administering to the subject an antiretroviral compound. Antiretroviral drugs inhibit the reproduction of retroviruses such as HIV. Antiretroviral agents are virustatic agents which block steps in the replication of the virus. The drugs are not curative; however continued use of drugs, particularly in multi-drug regimens, can significantly slow disease progression. There are three main types of antiretroviral drugs, although only two steps in the viral replication process are blocked. Nucleoside analogs, or nucleoside reverse transcriptase inhibitors (NRTIs), act by inhibiting the enzyme reverse transcriptase. Because a retrovirus is composed of RNA, the virus must make a DNA strand in order to replicate itself. Reverse transcriptase is an enzyme that is essential to making the DNA copy. The nucleoside reverse transcriptase inhibitors are incorporated
into the DNA strand. This is a faulty DNA molecule that is incapable of reproducing. The non-nucleoside reverse transcriptase inhibitors (NNRTIs) act by binding directly to the reverse transcriptase molecule, inhibiting its activity. Protease inhibitors act on the enzyme protease, which is essential for the virus to break down the proteins in infected cells.

Without this essential step, the virus produces immature copies of itself, which are non-infectious. A fourth class of drugs called fusion inhibitors block HIV from fusing with healthy cells.

Thus, the antiretroviral compound can comprise one or more molecules selected from the group consisting of protease inhibitors [PI], fusion inhibitors, nucleoside reverse transcriptase inhibitors [NRTI], and non-nucleoside reverse transcriptase inhibitors [NNRTI].

Thus, the antiretroviral compound of the provided method can be a PI, such as a PI selected from the group consisting of Indinavir, Amprenavir, Nelfinavir, Saquinavir, Fosamprenavir, Lopinavir, Ritonavir, and Atazanavir, or any combinations thereof.

Thus, the antiretroviral compound of the provided method can be a fusion inhibitor, such as for example Enfuvirtide.

Thus, the antiretroviral compound of the provided method can be a NRTI, such as a NRTI selected from the group consisting of Abacavir, Stavudine, Didanosine, Lamivudine, Zidovudine, Zalcitabine, Tenofovir, and Emtricitabine, or any combinations thereof.

Thus, the antiretroviral compound of the provided method can be a NNRTI, such as a NNRTI selected from the group consisting of Efavirenz, Nevirapine, and Delavirdine.

The disclosed method can further comprise administering to the subject an inhibitor of mitochondrial hyperpolarization. As used herein, mitochondrial hyperpolarization (MHP) refers to an elevation in the mitochondrial transmembrane potential, ΔΨ_m (delta psi), i.e., negative inside and positive outside). The ΔΨ_m is the result of an electrochemical gradient maintained by two transport systems – the electron transport chain and the F_0F_1-ATPase complex. For a review, see Perl et al. 2004 Trends in Immunol. 25:360-367. Briefly, the electron transport chain catalyzes the flow of electrons from NADH to molecular oxygen and the translocation of protons across the inner mitochondrial membrane, thus creating a voltage gradient with negative charges inside the mitochondrial matrix. F_0F_1-ATPase utilizes the extruded proton to synthesize ATP. MHP leads to uncoupling of oxidative phosphorylation, which disrupts ΔΨ_m and damages
integrity of the inner mitochondrial membrane. Disruption of ΔΨm has been proposed as the point of no return in cell death signaling. This releases cytochrome c and other cell-death-inducing factors from mitochondria into the cytosol. Thus, the inhibitor of the present method can be a F0F1-ATPase agonists.

KATP channels participate in controlling plasma and mitochondrial membrane polarity, by controlling K⁺ efflux at the plasma membrane, and K⁺/H⁺ exchange at the mitochondrial membrane. As such, both plasma membrane and mitochondrial membrane KATP channels can effect mitochondrial polarization. Thus, the inhibitor of the present method can be a KATP channel antagonist. The KATP channel antagonist can be selected from the group consisting of Tolbutamide, hydroxydecanoic acid (5-HD), glibenclamide (glyburide), and meglitinide analog (e.g. Repaglinide, A-4166).

The inhibitor of the present method can be an electron transport inhibitor. The electron transport chain (ETC) is the biomolecular machinery present in mitochondria that couples the flow of electrons to proton pumps in order to convert energy from sugar to ATP. The electron transport chain couples the transfer of an electron from NADH (nicotinamide adenine dinucleotide) to molecular oxygen (O2) with the pumping of protons (H⁺) across a membrane. The charge gradient that results across the membrane serves as a battery to drive ATP Synthase. The electron transport chain is made up of several integral membrane complexes: NADH dehydrogenase (complex I), Coenzyme Q -cytochrome c reductase (complex III), and Cytochrome c oxidase (complex IV). Succinate -Coenzyme Q reductase (Complex II) connects the Krebs cycle directly to the electron transport chain.

Thus, the inhibitor of the provided method can be an inhibitor of any component of the ETC. Thus, the inhibitor can be an inhibitor of complex I, II, III, or IV. For example, diphenylene iodonium (DPI) and rotenone are specific inhibitors of complex I, succinate-q reductase (TTFA) is an inhibitor of complex II, antimycin A and myxothiazole are inhibitors of complex III, and potassium cyanide (KCN) is an inhibitor of complex IV.

Thus, the inhibitor of the provided method can be selected from the group consisting of diphenylene iodonium (DPI), rotenone, antimycin, myxothiazole, succinate-q reductase (TTFA), and potassium cyanide (KCN).

The inhibitor of the present method can be an uncoupler. As used herein an "uncoupler" is a substance that allows oxidation in mitochondria to proceed without the usual concomitant phosphorylation to produce ATP; these substances thus "uncouple"
oxidation and phosphorylation. As an example, Trifluorocarbonylcyanide Phenylhydrazone (FCCP) is a chemical uncoupler of electron transport and oxidative phosphorylation. FCCP permeabilizes the inner mitochondrial membrane to protons, destroying the proton gradient and, in doing so, uncouples the electron transport system from the oxidative phosphorylation system. In this situation, electrons continue to pass through the electron transport system and reduce oxygen to water, but ATP is not synthesized in the process.

The uncoupler of the present method can agonize, antagonize or modulate the expression of endogenous mitochondrial uncoupling proteins (UCPs). As a non-limiting example, the uncoupler of the present method can be the beta-adrenergic agonist CL-316,243 (disodium (R,R)-5-(2-((2-(3-chlorophenyl)-2-hydroxyethyl)-amino)propyl)-1,3-benzodioxole-2,3-dicarboxylate) (Yoshida et. al., Am J Physiol. 1998. 274(3 Pt 1): p. E469-75).

The uncoupler of the present method can be a protonophore. Thus, the inhibitor of the present method can be a protonophore. As used herein, a "protonophore" is a molecule that allows protons to cross lipid bilayers. The protonophore can be FCCP. The protonophore can also be 2,4,-dinitrophenol (DNP). The protonophore can be also m-chlorophenylhydrazone (CCCP). The protonophore can also be pentachlorophenol (PCP).

The disclosed method can further comprise contacting the cell with an antioxidant. Generally, antioxidants are compounds that react with, and typically get consumed by, oxygen. Since antioxidants typically react with oxygen, antioxidants also typically react with the free radical generators, and free radicals. ("The Antioxidants--The Nutrients that Guard Your Body" by Richard A. Passwater, Ph. D., 1985, Keats Publishing Inc., which is herein incorporated by reference at least for material related to antioxidants). The herein disclosed antioxidant can be any antioxidant, and a non-limiting list would included but not be limited to, non-flavonoid antioxidants and nutrients that can directly scavenge free radicals including multi-carotenes, beta-carotenes, alpha-carotenes, gamma-carotenes, lycopene, lutein and zeaxthins, selenium, Vitamin E, including alpha-, beta- and gamma-tocopherol, particularly a-tocopherol, etc., vitamin E succinate, and trolox (a soluble Vitamin E analog) Vitamin C (ascorbic acid) and Niacin (Vitamin B3, nicotinic acid and nicotinamide), Vitamin A, 13-cis retinoic acid, , N-acetyl-L-cysteine (NAC), sodium ascorbate, pyrrolidin-ethidio-carbamate, and coenzyme Q10; enzymes which catalyze the destruction of free radicals including peroxidases such as glutathione peroxidase (GSHPX)
which acts on H$_2$O$_2$ and such as organic peroxides, including catalase (CAT) which acts on H$_2$O$_2$, superoxide dismutase (SOD) which disproportionates O$_2$H$_2$O$_2$; glutathione transferase (GSHTx), glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PD), and mimetics, analogs and polymers thereof (analog and polymers of antioxidant enzymes, such as SOD, are described in, for example, U.S. patent Ser. No. 5,171,680 which is incorporated herein by reference for material at least related to antioxidants and antioxidant enzymes); glutathione; ceruloplasmin; cysteine, and cysteamine (beta-mercaptoethylamine) and flavonoids and flavinoid like molecules like folic acid and folate. A review of antioxidant enzymes and mimetics thereof and antioxidant nutrients can be found in Kumar et al, Pharmac. Ther. Vol 39: 301, 1988 and Machlin L. J. and Bendich, F.A.S.E.B. Journal Vol.1:441-445, 1987 which are incorporated herein by reference for material related to antioxidants.

Thus, the disclosed method can further comprise contacting the cell with an antioxidant selected from the group consisting of tauroursodeoxycholic acid (TUDCA), N-acetylcysteine (NAC) (600-800 mg/day), Mito-Coenzyme Q10 (Mito-CoQ) (300-400 mg/day), Mito-VitaminE (Mito-E) (100 – 1000 mg/day), Coenzyme Q10 (300-400 mg/day), and idebenone (60 - 120 mg/day).

N-acetylcysteine (NAC) is used to replenish Glutathione (GSH) that has been depleted in HIV-infected individuals by acetaminophen overdose. (De Rosa SC, Zaretsky MD, Dubs JG, Roederer M, Anderson M, Green A, Mitra D, Watanabe N, Nakamura H, Tjioe I, Deresinski SC, Moore WA, Ela SW, Parks D, Herzenberg LA, Herzenberg LA. N-acetylcysteine replenishes glutathione in HIV infection. European Journal of Clinical Investigation, 30(10):915). Thus, in one embodiment of the provided invention, NAC is not used to replenish Glutathione (GSH) in HIV-infected subjects. In another embodiment of the method NAC is not used to treat HAD.

Coenzyme Q10 has been used to treat patients having the AIDS related complex. (Folkes K, Hanioka T, Xia LJ, McRae JT Jr, Langsjoen P. Coenzyme Q10 increases T4/T8 ratios of lymphocytes in ordinary subjects and relevance to patients having the AIDS related complex. Biochem Biophys Res Commun. 1991 Apr 30;176(2):786-91.) Bile acids such as TUDCA lead to a significant improvement in serum transaminase activities in subjects with hepatitis B and C. (Chen W, Liu J, Gluud C. Bile acids for viral hepatitis. Cochrane Database Syst Rev. 2003;(2):CD003181.) Thus, in one embodiment of the provided invention, Coenzyme Q10 is not used to treat patients having the AIDS.
related complex. In another embodiment of the method Coenzyme Q10 is not used to treat HAD.

Idebenone has been used to treat subjects with senile cognitive decline (Bergamasco B, Villardita C, Coppi R. Effects of idebenone in elderly subjects with cognitive decline. Results of a multicentre clinical trial. Arch Gerontol Geriatr. 1992 Nov-Dec;15(3):279-86.). Thus, in one embodiment of the provided invention, Idebenone is not used to treat subjects with senile cognitive decline. In another embodiment of the method Idebenone is not used to treat HAD.

The disclosed method can further comprise administering to the subject a neurotoxin inhibitor. The inhibitor can be a TNFα inhibitor, including TNFα-inhibitory monoclonal antibodies (e.g., etanercept), phosphodiesterase (PDE)-4 inhibitors (such as IC485, which can reduce TNFα production), thalidomide and other agents.

Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the C_2 domain, the C_3 domain and hinge region, but not the C_1 domain of IgG1. Etanercept is produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system. It consists of 934 amino acids and has an apparent molecular weight of approximately 150 kilodaltons. Etanercept has been evaluated in HIV-infected subjects receiving highly active antiretroviral therapy (HAART) (Sha BE, Valdez H, Gelman RS, Landay AL, Agosti J, Mitsuyasu R, Pollard RB, Mildvan D, Namkung A, Ogata-Arakaki DM, Fox L, Estep S, Erice A, Kilgo P, Walker RE, Bancroft L, Lederman MM. Effect of etanercept (Enbrel) on interleukin 6, tumor necrosis factor alpha, and markers of immune activation in HIV-infected subjects receiving interleukin 2. AIDS Res Hum Retroviruses. 2002 Jun 10;18(9):661-5).

IC485 is an orally administered, small molecule inhibitor of PDE4. Inhibition of PDE4 leads to an increase in the second messenger, cAMP, within cells. This inhibition may in turn reduce the cell's production of tumor necrosis factor alpha (TNF-alpha) and a variety of other inflammatory mediators. IC485 is being evaluated in patients with chronic obstructive pulmonary disease.

The inhibitor can be a PAF receptor antagonist (such as lexipafant, WEB2086, WEB2170, BN-52021 or PMS-601), a PAF degrading-enzyme such as PAF-
acylhydrolase (PAF-AH), or a molecule that regulates the expression of PAF-AH (such as pioglitazone and other PPAR-gamma inhibitors).


Phosphatidylecholines (1-O-alkoxy-2-amino-2-desoxy-phosphocholines and 1-pyrene-labeled analogs) were synthesized and used to examine interactions with recombinant human PAF-AH (Deigner HP, Kinscherf R, Claus R, Fynys B, Blencowe C, Hermetter A. Novel reversible, irreversible and fluorescent inhibitors of platelet-activating factor acetylhydrolase as mechanistic probes. Atherosclerosis. 1999 May;144(1):79-90).

The disclosed method can further comprise administering to the subject an inhibitor of GSK-3β. The inhibitor can be valproate or lithium.

Valproate has been administered to HIV-infected patients receiving efavirenz or lopinavir (DiCenzo R, Peterson D, Cruttenden K, Morse G, Riggs G, Gelbard H, Schifitto


Oct;10(5):284-92). Thus, the microglial deactivator can be minocycline. A typical dosage of minocyclin comprises 200 mg/day.

Other microglial deactivators that can be used in the present methods include PDE4 inhibitors (described above).

The disclosed method can further comprise administering to the subject an inhibitor of glutamate damage. The inhibitor can be a beta-lactam antibiotic such as for example ceftriaxone, which can have direct effects on glutamate transporter expression.

When delivered to animals, the beta-lactam ceftriaxone increases both brain expression of GLT1 that inactivates synaptic glutamate (Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature. 2005 Jan 6;433(7021):73-7) A typical dosage of cephtriaxone is 50 mg/kg/day.

A dose-dependent inhibition of high affinity glutamate uptake sites is observed after addition of exogenous recombinant human TNFα to human fetal astrocytes (PHFAs) (Fine SM, Angel RA, Perry SW, Epstein LG, Rothstein JD, Dewhurst S, Gelbard HA. Tumor necrosis factor alpha inhibits glutamate uptake by primary human astrocytes. Implications for pathogenesis of HIV-1 dementia. J Biol Chem. 1996 Jun 28;271(26):15303-6). Thus, the inhibitor of glutamate damage can be a TNFα inhibitor or a microglial deactivator (described above), which can have indirect effects on glutamate transporters.

Disclosed herein is a composition, comprising a modulator of adenosine receptor signaling and a molecule that inhibits mitochondrial hyperpolarization in a neural cell. The modulator of the provided composition can be any adenosine receptor signaling modulator provided herein. As an example, the composition can comprise the A₂AR antagonist ATL455 or ZM241685. The molecule of the disclosed composition that inhibits mitochondrial hyperpolarization in a neural cell can be any such molecule provided herein. Thus, the provided composition can comprise one or more of a KATP antagonist, an inhibitor of electron transport, a protonophore, or an antioxidant. The provided composition can further comprise an antiretroviral compound. The antiretroviral compound can be any antiretroviral compound as disclosed herein, such as one or more molecules selected from the group consisting of protease inhibitors [PI], nucleoside reverse transcriptase inhibitors [NRTI], and non-nucleoside reverse transcriptase inhibitors.
The provided composition can further comprise a neurotoxin inhibitor. The provided composition can further comprise an inhibitor of GSK-3β. The provided composition can further comprise a compound that enhances CNS uptake. The provided composition can further comprise a microglial deactivator. The provided composition can further comprise an inhibitor of glutamate damage.

The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose can be divided into multiple doses for purposes of administration. Consequently, single dose compositions can contain such amounts or submultiples thereof to make up the daily dose.

The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. For example, the disclosed A2A R antagonists can be administered at published dosages, such as those approved for human use.

A typical daily dosage of the disclosed modulators of adenosine receptor signaling used alone can range from about 0.05 to 5 mg/kg of body weight or more per day, depending on the factors mentioned above. In one aspect, the disclosed A2A R antagonists (e.g. ATL455, KW6002 and ZM241685) can be administered at doses ranging from 0.3 to 3 mg/kg of body weight per day; KW6002 can be administered to humans at doses up to 40 mg/day. In another aspect, the disclosed A2A R agonists (e.g. ATL146e, ATL313 and CGS21680) can be administered at from 0.05 to 50 mg/kg of body weight per day.

A typical daily dosage of the disclosed inhibitors of hyperpolarization used alone can range from about .001 mg/kg to up to 50 mg/kg of body weight or more per day, depending on the factors mentioned above. In one aspect, the disclosed KATP channel
antagonists can be administered at from .02 mg/kg to about 30 mg/kg of body weight per day. As non-limiting examples, Tolbutamide can be administered at from about 0.25 to 3 g/day; glibenclamide (glyburide) can be administered at from about 1.25 to 20 mg/day; and meglitinide analog (e.g. Repaglinide, A-4166) can be administered at from about 0.5 to 4 mg/day.

In another aspect, the disclosed inhibitors of the ECC (e.g., DPI, rotenone, antimycin, myxothiazole, TTFA, and KCN can be administered at from .001 mg/kg to 1 mg/kg of body weight per day. In another aspect, the disclosed protonophore (e.g., FCCP, DNP, CCCP, PCP) can be administered at from .001 mg/kg to 1 mg/kg of body weight per day. In one aspect, the disclosed beta-adrenergic agonist CL-316,243 can be administered at 0.01 to up to 1 mg/kg, including 0.1 mg/kg, of body weight or more per day.

In another aspect, the disclosed antioxidants can be administered at from 1 mg/day to 1000 mg/day. As non-limiting examples, N-acetylcysteine (NAC) can be administered at from about 600 mg/day to 800 mg/day; Mito-Coenzyme Q10 (Mito-CoQ) can be administered at from about 300 mg/day to 400 mg/day; Mito-VitaminE (Mito-E) can be administered from about 100 to 1000 mg/day); Coenzyme Q10 can be administered from about 300 mg/day to 400 mg/day; and idebenone can be administered at from about 60 mg/day to 120 mg/day.

The compositions can also be administered in vivo in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material can be administered to a subject, along with the nucleic acid or vector, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

Suitable carriers and their formulations are described in Remington: The Science and Practice of Pharmacy (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained
release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositions can be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled in the art.

Pharmaceutical compositions can include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions can also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

The pharmaceutical composition can be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration can be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. Thus, the disclosed compositions can be administered intracranially intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives can also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration can include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

Some of the compositions can be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

The compositions may be administered orally or parenterally (e.g., intravenously, intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, intracranially, topically or the like, including topical intranasal administration or administration by inhalant. As used herein, "intracranial administration" means the direct delivery of substances to the brain including, for example, intrathecal, intracisternal, intraventricular or trans-sphenoidal delivery via catheter or needle. As used herein, "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying
mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation. The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

Parenteral administration of the composition, if used, is generally characterized by injection. Injectable can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These can be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., Bioconjugate Chem., 2:447-451, (1991); Bagshawe, K.D., Br. J. Cancer, 60:275-281, (1989); Bagshawe, et al., Br. J. Cancer, 58:700-703, (1988); Senter, et al., Bioconjugate Chem., 4:3-9, (1993); Battelli, et al., Cancer Immunol. Immunother., 35:421-425, (1992); Pietersz and McKenzie, Immunolog. Reviews, 129:57-80, (1992); and Roffler, et al., Biochem. Pharmacol, 42:2062-2065, (1991)). Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells in vivo. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., Cancer Research, 49:6214-6220, (1989); and Litzinger and Huang, Biochimica et Biophysica Acta, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated
vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, DNA and Cell Biology 10:6, 399-409 (1991)).

The following examples are set forth below to illustrate the methods and results according to the present invention. These examples are not intended to be inclusive of all aspects of the present invention, but rather to illustrate representative methods and results. These examples are not intended to exclude equivalents and variations of the present invention which are apparent to one skilled in the art.

**Examples**

**Example 1: Adenosine 2A (A<sub>2A</sub>) receptor antagonist protects against Tat-induced neuronal apoptosis.**

Primary rat cerebellar granule neurons (CGNs) were exposed to a neurotoxic concentration of HIV-1 Tat, in the presence or absence of an A<sub>2A</sub>R antagonist (ZM241385). The A<sub>2A</sub>R antagonist was able to protect neurons against the otherwise lethal effects of exposure to HIV-1 Tat (Figure 1). These results demonstrate that A<sub>2A</sub>R antagonists can have therapeutic potential in the context of HAD. It is important to note that CGNs express adenosine A<sub>2A</sub> receptors [Vacas, J., et al. 2003. Brain Res 992:272-280].

Primary cultures of rat cerebellar granule neurons (CGNs) were exposed to HIV-Tat (500mM) alone or together with increasing doses of the A<sub>2A</sub>R antagonist ATL-455 or A<sub>2A</sub> agonists ATL313 or CGS21680. Cells are treated with the test compounds for defined time intervals of up to 48 hours (1, 4, 8, 24, and 48 hours). The A<sub>2A</sub>R antagonist ATL455 protected neurons against Tat-induced apoptosis (Fig. 2). Further, exposure of neurons to an A<sub>2A</sub>R agonists (ATL313 and CGS21680) did not result in an increase in cell death in the presence of Tat (Fig. 2).

RNAi can also be used to suppress expression of A<sub>2A</sub>R and mimic the effects of A<sub>2A</sub>R antagonist treatment. A<sub>2A</sub>R expression is inhibited using lentivirus vectors that
encode A2AR-specific shRNA. CGN cultures are transduced with viral vectors encoding shRNA targeted to A2AR, or scrambled shRNA sequences, or nothing (GFP only). For example, siRNA has been used to modify the levels of A2AR and A2BR in cardiac fibroblasts (Chen Y, Epperson S, Maksudova L, Ito B, Suarez J, Dillmann W, Villarreal F. Functional effects of enhancing or silencing adenosine A2b receptors in cardiac fibroblasts. Am J Physiol Heart Circ Physiol. 2004 Dec;287(6):H2478-86. Epub 2004 Jul 29).

A2AR-specific antibodies (Alpha Diagnostics International) are used to perform both immunoblot and immunofluorescence assays to examine knockdown of A2AR. A knockdown of 80% or greater is followed by the treatment of the transduced neurons with candidate HIV-1 neurotoxins (or mock-treated, for controls).

The signal transduction pathways triggered by activation of A2A receptors are still not completely understood. A2A receptors are generally accepted to couple to the Gs-adenylate cyclase (AC)-protein kinase A (PKA) pathway [Fredholm, B. B., et al. 2001. Pharmacol Rev 53:527-552], but can also couple to pathways involving G-proteins other than Gs (Go, Ga15/16, Gi/o) or to cAMP PKA independent signal transduction pathways. Thus, to elucidating the signalling pathway(s) triggered by A2A receptor antagonists, neurons are treated as described above, and the activity of cellular kinases such as PKA, ERK, AKT, JNK, and GSK-3β are evaluated.

The activation of transcription factors, e.g., AP-1, CREB, and NF-kB, is evaluated, as these factors represent potential endpoint targets of the signaling pathways initiated by A2AR). Transcription factor activity is assessed using assays of protein nuclear translocation, nuclear DNA binding activity and transient transcriptional reporter assays. Antagonism of A2AR signaling is expected to have a substantial effect on the activation of these transcription factors.

**Example 2: A2AR agonists prevent HIV-1 induced monocyte activation.**

Microglial and macrophage activation contributes to neuronal damage in HIV-1 infected individuals [Poluektova, L., et al. 2004. J Immunol 172:7610-7617], and is associated with increased expression of inducible nitric oxide synthase (iNOS) and production of NO.

HIV-1 Tat produced a dose-dependent increase in iNOS expression in human primary monocytes, which was maximal at 100 nM. The effect of adenosine receptor activation on Tat-induced upregulation of iNOS expression and NO release was then
determined. Tat-treatment resulted in a 4-fold increase in iNOS expression by primary monocytes and a similar elevation in NO release (Fig. 3). Co-treatment of cells with the A$_{2A}$ adenosine receptor-selective agonist CGS21680 or the nonselective A$_1$ and A$_3$ adenosine receptor agonist IB-MECA resulted in a roughly 50% inhibition of Tat-induced iNOS expression and a complete abrogation of Tat’s effect on NO secretion (Fig. 3). In contrast, incubation of cells with 1 μM R-PIA (an adenosine A$_1$ receptor agonist) had no effect on Tat-induced iNOS expression or NO release (Fig. 3), implicating the A$_{2A}$ and A$_3$ receptor subtypes in this anti-inflammatory effect. ZM241385 (an A$_{2A}$R antagonist) or MRS1220 (an A$_3$R antagonist) could prevent the anti-inflammatory effects of CGS21680 or IB-MECA, respectively.

The effect of an A$_{2A}$R agonist on TNFα release by primary human monocytes was also determined. Monocytes were stimulated with HIV-1 Tat, in the presence or absence of the A$_{2A}$R agonist CGS21680 or the A$_{2A}$R antagonist ZM251385 and TNFα release was then measured in cell culture supernatants using an ELISA assay. The results (Fig. 4) show that the A$_{2A}$R agonist abrogated the Tat-mediated increase in monocyte-derived TNFα production.

Quantitative real-time PCR analysis was used to measure TNFα message levels in Tat-treated monocytes. Primary human monocytes were prepared using CD14$^+$ immunomagnetic selection, and then exposed to LPS or to HIV-1 Tat for 4 hours, in the presence or absence of the A$_{2A}$R agonist CGS21680. Culture supernatants were tested for TNFα levels by ELISA assay (Fig. 5A), and RNA was extracted from cell pellets for quantitative reverse-transcription PCR (qRTPCR) analysis (Fig. 5B).

Consistent with the results in Figure 4, the A$_{2A}$R agonist CGS21680 strongly suppressed TNFα release in Tat-exposed monocytes (Fig. 5B). Moreover, a strong concordance was observed between the TNFα ELISA (Fig. 5A) and the qRTPCR analysis (Fig. 5B). Notably, the A$_{2A}$R agonist CGS21680 strongly suppressed TNFα release (and TNFα transcription) in Tat-exposed monocytes, but had little effect on IL1β mRNA levels in these cells (Fig. 5B).

A second A$_{2A}$R agonist ATL313 demonstrated very similar results to those shown in Figure 4 (see Fig 6 & 7). It was determined that exposure of monocytes to an A$_{2A}$R antagonist did not result in an increase in cellular activation in the presence of Tat (see data for ATL455; Fig. 6) and that the approximate IC$_{50}$ for ATL313’s inhibitory effect on Tat-stimulated TNFα release in monocytes is < 1nM. This is consistent with the known
receptor-binding properties of this compound (see Table 1). Collectively, the results shown in Figures 6 and 7 confirm and extend the findings in Figures 4 and 5, using a commercially relevant molecule (ATL313).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>A1</th>
<th>A2A</th>
<th>A2B</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL455</td>
<td>23</td>
<td>1.6</td>
<td>155</td>
<td>1000</td>
</tr>
<tr>
<td>ATL313</td>
<td>52</td>
<td>0.6</td>
<td>&gt;1000</td>
<td>320</td>
</tr>
</tbody>
</table>

Data represent Ki (nM), versus recombinant versions of the indicated human adenosine receptor subtypes.

Example 3: Bioenergetic defects in neurons exposed to HIV-1 neurotoxins

Cortical neuronal cultures were treated with Tat or cPAF for varying periods of time as an in vitro model of pre-synaptic nerve terminal function, using the lipophilic, fluorescent styryl dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43), which binds to synaptic vesicle membranes and is taken up into nerve terminals during normal activity (i.e., synaptic vesicle recycling). Fluorescent signal can be quantified to number of neurons, and after chemical depolarization with high concentrations of KCl, fluorescent signal is abolished, giving an index of nerve terminal activity in real-time. This model was used to demonstrate that both Tat and PAF increased vesicular uptake (of neurotransmitter) in rodent cortical cultures (Figure 8). The staining seen with FM1-43 was punctate and largely oriented along neuronal processes (Figure 8A, B), and released in a quantal fashion with a depolarizing concentration of KCl, all suggestive of synaptic vesicle recycling (Figure 8C). Interestingly, Tat caused a dose-dependent increase in FM1-43 uptake that was even more pronounced in aged cultures (Figure 8D), perhaps due to incomplete maturation of synaptic receptors in the younger cultures [Gelbard, H.A., et al., J Virol, 1994. 68(7): p. 4628-35]. However, an inactive mutant Tat protein had no effect on FM1-43 uptake, indicating the effect was a biologically specific effect of Tat (Figure 8E). PAF caused an even more robust increase in FM1-43 uptake (Figure 8F), in keeping with its role as a mediator of pre-synaptic glutamate release [Clark, G.D., et al., Neuron, 1992. 9(6): p. 1211-6].

Since reactive oxygen species (ROS) can be a final common mediator of neuronal dysfunction in HAD and other neurodegenerative diseases, and because neuronal synapses and synaptic function can be particularly vulnerable to oxidative stress [Mattson, M.P. and...
D. Liu, Neuromolecular Med, 2002. 2(2): p. 215-31], ROS production was measured in response to Tat and PAF. Twenty-four hour treatment with either PAF or Tat increased ROS production in primary rodent cortical neurons by approximately 50% and 100% respectively (Figure 9).

Interestingly, the Tat-induced increase in FM1-43 uptake was completely blocked by the antioxidant bile liver acid antioxidant tauroursodeoxycholic acid (TUDCA) (Figure 10). These data are in concordance with recent evidence that ROS can directly enhance synaptic transmission [Kamsler, A. and M. Segal, Mol Neurobiol, 2004. 29(2): p. 167-78] [Giniatullin, A.R. and R.A. J Physiol, 2003. 552(Pt 1): p. 283-93] [Chen, B.T., M.V. J Neurophysiol, 2001. 85(6): p. 2468-76]. It is important to point out that enhanced synaptic transmission can come with a cost to the ultimate fate of the neuron.

Changes in mitochondrial function that could be associated with the increased vesicular activity were evaluated. Mitochondria generate the energy for synaptic transmission in the form of ATP, and as a byproduct of this oxidative phosphorylation, they are also one of the primary producers of intracellular ROS. Two key mitochondrial parameters were examined in response to Tat and PAF: 1) mitochondrial membrane potential (Δψm) [as assessed by mitochondrial uptake of the lipophilic cationic dyes tetramethylrhodamine ethyl or methyl ester (TMRE or TMRM respectively)], and 2) ATP production (as measured by luciferase assay). The electronegative mitochondrial membrane potential provides the driving force for calcium buffering and ATP production by the mitochondria, and as such, Δψm is frequently used as an indicator of mitochondrial health and energetic capacity. Moreover, fluctuations in Δψm can trigger mitochondrial release of pro-apoptotic factors, an event most frequently associated with a loss of Δψm.

To the contrary, however, Tat treatment of rodent cortical neurons resulted in a dose-dependent, biphasic increase in Δψm over 48 hours (Figure 11A). A low dose of Tat (100 ng/ml) caused a gradual increase in Δψm, peaking with a 17% increase in mitochondrial TMRE uptake at 4 hours (p<.004), then declining to baseline by 14 hours, before rising again (6% increase vs. control vehicle) 26 hours after application, followed by a plateau at 36 hours that persists until the end of the analysis (48 hours) (19% increase vs. control vehicle) (Figure 11A, squares). In contrast, a higher dose of Tat (2.5 μg/ml), caused a sharp increase in Δψm versus control, peaking at a 39% increase over control by 1 hour (p<.0003) before declining to control level by 10 hours, then increasing again to a second peak of 27% vs. control at 36 hours, followed by a plateau of 23% vs. control at 44
hours that also persisted until the end of the analysis (48 hours) (Figure 11A, circles). Incubation of cortical cultures with the biologically inactive Tat mutant (Δ31-61) at either 1 or 26 hours resulted in TMRE uptake values that were indistinguishable from control vehicle (Figure 11A, diamonds), demonstrating that Tat’s effect on mitochondrial hyperpolarization was biologically specific.

In addition, both PAF and TNF-α treatments also caused similar dose dependent increases in Δψm, suggesting that this effect can be broadly generalizable to HIV neurotoxins in the context of HAD. Notably, the mitochondria-depolarizing protonophore trifluoromethoxy carbonyl cyanide phenylhydrazone FCCP (5 μM), substantially diminished TMRE/M signal, thus serving as an additional control that Tat, PAF, and TNF-α were indeed increasing Δψm.

There was also a concomitant, dose-dependent rise in ATP production and ATP/ADP ratios with the Tat-induced mitochondrial hyperpolarization, as would be expected from the increased driving force on the mitochondrial F,−F,+ -ATPase (Figure 12). After 1 hour treatment with 0.1 and 2.5 μg/ml Tat, ATP but not ADP levels were slightly elevated versus control, and the ATP/ADP ratio was significantly elevated for the 0.1 μg/ml dose of Tat. After 24 hour treatment, 0.1 and 2.5 μg/ml Tat elevated both ATP and ADP levels, but the ATP/ADP ratio was only significantly increased for the 2.5 μg/ml dose of Tat. By 48 hours, ATP and ADP levels were still elevated for both doses of Tat, but less so, and the ATP/ADP ratio had begun to decline, perhaps indicating that the neurons were entering a stage of ATP deprivation from constant over-activity.

The dose-dependent, Tat-induced rise in mitochondrial membrane potential could, however, be blocked by the potassium ATP (KATP) channel antagonist Tolbutamide (Figure 13A), which had the further effect of attenuating apoptotic cell death (Figure 13B).


These data demonstrate that blocking mitochondrial hyperpolarization can have a protective effect on neurons. Thus, this approach represents a therapeutic avenue of tremendous importance. A related therapeutic avenue is the use of protease inhibitors to
restore mitochondrial bioenergetics, or more specifically, to block a detrimental rise in mitochondrial polarization.

**Example 4: Approaches to normalize synaptic transmission in models of post-synaptic injury**

An additional pathologic consequence of exposure to HIV-1 neurotoxins is an increase in oxidized cellular phospholipids that can bind to and activate the PAF-R [Marathe, G.K., et al., Vascul Pharmacol, 2002. 38(4): p. 193-200]. Both energetic stress and PAF-R signaling have the potential to impair post-synaptic neurotransmission. Thus, a morphologic correlate of dendrite damage in HAD was identified, which is activity-induced dendritic swelling or “beading” in the presence of the HIV neurotoxin cPAF. This beading is also accompanied by impairment of synaptic activity. Figure 14 shows that exposing hippocampal neurons *in vitro* to sublethal (130 nM) concentrations of cPAF for 60 hours results in loss of dendritic spines (but not neurites) and dendrite beading, without cell death.

Additionally, in an acute hippocampal slice model, shorter cPAF exposures (1μM for 30-60 minutes) increased neuronal susceptibility to beading in response to synaptic activity, and results in failure of long-term potentiation in the beaded dendrites (Figure 15). Increased neuronal susceptibility to beading in response to synaptic activity was also observed in hippocampal cultures following brief (1 hour) cPAF exposures.

Thus, PAF can lower the threshold for synaptic injury, and that by impairing synaptic function, beading can serve as an important functional marker of dendritic injury, and can underlie the reversible impairments of neuronal function seen in HAD. Equally important, the *in vitro* and *in vivo* models of dendritic injury disclosed herein are sensitive and reproducible, and have great utility to determine the ability of adjunctive therapies to restore function (i.e., synaptic transmission) during exposure to HIV-1 neurotoxins.

**Example 5: Bioenergetic defects in neurons exposed to HIV-1 neurotoxins**

HIV neurotoxins can increase vesicle recycling, mitochondrial membrane potential, ATP/ADP ratios, and reactive oxygen species. Furthermore, blocking the Tat-induced rise in Δψm protects the neurons against apoptotic cell death. Thus, the observed increases in Δψm are not simply a compensatory response to increased metabolic demand, since in that case blocking the rise in Δψm would likely lead to energetic failure and exacerbate cell death.
Thus, increased $\Delta \psi_m$ in response to HIV neurotoxins could be a pathogenic mechanism leading to excessive production of ROS and ATP, resulting in synaptic stress, excitotoxicity, and eventual demise of synaptic contacts and dendritic arbor, i.e., "synaptic apoptosis" [Mattson, M.P., J.N. Keller, and J.G. Begley, Exp Neurol, 1998. 153(1): p. 35-48]. These pathogenic mechanisms could contribute to the reversible component of HAD, but if left unchecked, could ultimately result in permanent neurologic deficit with or without cell loss.

In order to determine if HIV-neurotoxin-induced mitochondrial hyperpolarization is associated with a rise in NADH/NAD$^+$ ratio and increased oxygen consumption by the electron transport chain (ETC) and to further clarify Tat and PAF's effects on oxidative phosphorylation primary rodent cortical or hippocampal neurons are treated with a range of doses of Tat (10 ng/ml, 100 ng/ml, and 2.5 $\mu$g/ml) and cPAF (2 nM, 20 nM, and 500 nM) over 1, 4, 8, 12, 24, 36, 48, and 72 hours, and NADH/NAD$^+$ ratio and rates of $O_2$ consumption at these time points assessed. ATP/ADP ratios and $\Delta \psi_m$ are also assessed at these time points to verify previous findings. If all components of oxidative phosphorylation are functioning properly, then by increasing the proton driving force through the mitochondrial $F_1F_0$-ATPase, a mitochondrial hyperpolarization is associated not only with increased ATP production as disclosed herein, but also with increased $O_2$ consumption and increased NADH/NAD$^+$ ratio.

The doses of Tat and PAF described herein have been determined by previous studies to represent sub-threshold (i.e., no measurable effects), sub-lethal (measurable effects on intracellular parameters but induces little cell death at 18-24 hours), and toxic (strong effects on intracellular parameters and induces apparent cell death at 18-24 hours or greater) in neuronal culture systems. These doses have been selected to cover the range of responses expected for the cellular functions being assessed. However, for any given assay, a full dose-response calibration is performed, and Tat or cPAF doses altered, if warranted. Additionally, specificity of effects is confirmed by substituting Tat with an equimolar amount of a biologically inactive mutant Tat protein ($\Delta$Tat31-61) [Gurwell, J.A., et al., Neuroscience, 2001. 102(3): p. 555-63] or applying cPAF with the PAF receptor antagonists WEB 2086 (10 $\mu$M) or BN52021 (10 $\mu$M).

In order to determine if the ETC or the mitochondrial $F_1F_0$-ATPase are responsible for the rise in $\Delta \psi_m$, if blocking the hyperpolarization results in return of ATP/ADP ratios to baseline levels, and to determine if the $F_1F_0$-ATPase or the ETC is responsible for the
Tat or PAF-induced rise in $\Delta\psi_m$, primary cortical neuron cultures are treated with Tat or PAF at the doses and time points above, in the presence of the F$_i$F$_o$-ATPase inhibitor oligomycin (5 $\mu$g/ml, strong depolarizing dose) [Ward, M.W., et al., J Neurosci, 2000. 20(19): p. 7208-19], and the effects on mitochondrial membrane potential and ATP/ADP ratios are monitored.

If oligomycin partially or fully blocks the Tat or PAF-induced rise in $\Delta\psi_m$, then reversal of the F$_i$F$_o$-ATPase is at least in part responsible for the mitochondrial hyperpolarization. In this case, Tat or PAF + oligomycin induces a rise in ATP/ADP ratios versus Tat or PAF alone, due to reduced consumption of ATP by the reversed F$_i$F$_o$-ATPase.

If the F$_i$F$_o$-ATPase is functioning normally – i.e. passing protons into the mitochondria and generating ATP – then co-incubation with oligomycin augments the hyperpolarizing effects of Tat and PAF, and reduce ATP/ADP ratios, thus suggesting that the ETC, and not the F$_i$F$_o$-ATPase, is responsible for a hyperpolarized $\Delta\psi_m$ measured after Tat or PAF exposure.

In order to determine if the rise in $\Delta\psi_m$ is accompanied by increased ROS production, Cytochrome-C (CytC) release by the ETC, and caspase activation, and to determine if increased ROS production from the ETC is a consequence of the Tat or PAF-induced rise in $\Delta\psi_m$, primary cortical neuron cultures are treated with Tat (10 ng/ml, 100 ng/ml, and 2.5 $\mu$g/ml) and PAF (2 nm, 20 nm, and 500 nm) over 1, 4, 8, 12, 24, 36, 48, and 72 hours, with or without the complex I inhibitors diphenyleneiodonium and rotenone or the complex III inhibitors antimycin and myxothiazole.

Mitochondrial complexes I and III are the chief ROS producers of the ETC [Nicholls, D.G., Int J Biochem Cell Biol, 2002. 34(11): p. 1372-81]. ROS levels are assessed by with the oxidizable dye indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CMH$_2$DCFDA, abbreviated as "DCF") (Molecular Probes, Eugene, OR) as described herein. If ROS levels are higher in Tat/PAF treated cultures, and this effect is blocked by rotenone and/or myxothiazole, then the hyperpolarization is contributing to enhanced ROS production from the ETC in Tat/PAF treated neurons.

To determine if Tat/PAF-induced mitochondrial hyperpolarization results in increased CytC release from mitochondria, rodent cortical neurons are treated with Tat or PAF as above, then assayed for release of cytochrome c using a commercially available kit
(http://www.emdbiosciences.com/product/QIA87). If increased release of CytC from mitochondria is observed, it can be confirmed that mitochondrial hyperpolarization is responsible for this effect by blocking the hyperpolarization with low-dose FCCP (100 nM) and the ETC complex inhibitors listed above, as well as tolbutamide and any other hyperpolarization-inhibiting compounds identified herein.

Complex II and IV inhibitors, succinate-q reductase (TTFA) and potassium cyanide (KCN) respectively can also be used herein. Complexes II and IV contribute to ROS production, but generally not as much as complexes I and III [Nicholls, D.G., Int J Biochem Cell Biol, 2002. 34(11): p. 1372-81]. Further, in addition to Caspase 9, activation of Apaf1, another key component of the CytC/Apaf1/Caspase9 apoptosome, and Caspase 3 are also examined.

Further, Tat or PAF ± glucose or NADH are co-incubated, and Δψ_m, O2 consumption, NADH levels, ATP/ADP ratios, and apoptotic cell death assessed at the 24, 48, and 72 hour time points. NADH and glucose are titrated to select doses that are not toxic to the neuronal cultures.

To assess the possibility that the decreased ATP production that results from blocking the Tat-induced rise in Δψ_m simply shifts the cell death continuum from apoptosis to necrosis, dual labels for apoptosis and necrosis are used as previously described [Perry, S.W., L.G. Epstein, and H.A. Gelbard, Biotechniques, 1997. 22(6): p. 1102-6].

**KATP channel antagonists:** As disclosed herein, the KATP channel antagonist and hypoglycemic agent Tolbutamide block the rise in Δψ_m from the HIV-1 virotoxin Tat and protect against cell death. Tolbutamide’s principal action is thought to occur via inhibitions of KATP channels [Liss, B. and J. Roepner, Mol Membr Biol, 2001. 18(2): p. 117-27], although it activates glycolysis as well [Kaku, K., Y. Inoue, and T. Kaneko, Diabetes Res Clin Pract, 1995. 28 Suppl: p. S105-8], and has mitochondrial uncoupling properties [Smith, P.A., P. Proks, and A. Moorhouse, Pflugers Arch, 1999. 437(4): p. 577-88]. The first and third mechanisms are the most likely candidates for tolbutamide’s protective and stabilizing effects based on the data disclosed herein, as enhancing glycolysis would only further increase Δψ_m, ATP, and ROS production. Controlled uncoupling, or regulation of mitochondrial permeability to H+ or other ions could underlie protective actions of several drugs disclosed herein, notably those that link pro-apoptotic mitochondrial proteins with mitochondrial membrane permeability (see rationale for
"protease and enzyme inhibitors" below). In addition, aberrant control of mitochondrial polarity represents a link between mitochondrial status and dendrite beading, via volume-regulated anion channels (VRACs).

However, because there is concern with reports of cardiac toxicity with
Tolbutamide [Meier, J.J., et al., Heart, 2004. 90(1): p. 9-12] other KATP antagonists with known neuronal action, but exhibiting lower (e.g. glibenclamide aka glyburide) [Riveline, J.P., et al., Diabetes Metab, 2003. 29(3): p. 207-22] or no (e.g. meglitinide analogs) [Schwanstecher, C. and D. Bassen, Br J Pharmacol, 1997. 121(2): p. 193-8] cardiac toxicity are expected. The mitochondrial KATP channel specific antagonist 5-hydroxydecanoic acid (5-HD), and the KATP channel agonist diazoxide, are used as controls to further dissect KATP antagonist protective mechanisms.

**Antioxidant compounds:** As disclosed herein, Tat and PAF induce ROS production in neurons, which could stem from increased oxidative phosphorylation and ATP production. Mitochondrial production of ROS leads to enhanced excitotoxic synaptic activity, leading to synaptic damage, or activates cell death pathways directly. Synapses are particularly vulnerable to oxidative stress. As disclosed herein, the antioxidant compound Tauroursodeoxycholic acid (TUDCA) protects against Tat-induced increases in synaptic activity. Together these data indicate that antioxidant compounds protect against synaptic damage and dysfunction in HAD.


Candidate compounds are tested for their efficacy in preventing pathologic outcomes against Tat and PAF treatment in primary assay systems that provide physiological and morphologic correlates of neuronal dysfunction HAD, i.e. whether they block mitochondrial hyperpolarization and cell death, prevent aberrant synaptic transmission and dendrite loss, and/or prevent dendrite swelling or "beading".

Using the Tat and PAF doses and a time course disclosed herein, the compounds identified herein are tested for their ability to block Tat and PAF-induced mitochondrial hyperpolarization, as assessed by the TMRM assay (below), and cell death, as assessed by
a combined assay for both necrosis and apoptosis [Perry, S.W., L.G. Epstein, and H.A. Gelbard, Biotechniques, 1997. 22(6): p. 1102-6]. All potential therapeutic compounds are first be titrated in full dose response curves to determine non-toxic doses for neurons. Dose ranges of Tat and PAF are extended if necessary to clearly determine whether protective effects exist.

Understanding potential interactions between HAART (particularly protease inhibitors [PIs] and nucleoside reverse transcriptase inhibitors [NRTIs]) and adjunctive neuroprotective agents disclosed herein is of great value in terms of defining future regimens of HAART that are tailored specifically to the amelioration of CNS disease and/or use in combination with specific adjunctive regimens that target mitochondrial bioenergetics, synaptic dysfunction, and neuronal apoptosis. For example, pro- and anti-apoptotic proteins could participate not only in pathologic mechanisms at the synapse, but also physiologic processes of synaptic regulation, such as growth cone rearrangement or synaptic strengthening [Jonas, E., J Bioenerg Biomembr, 2004. 36(4): p. 357-61]. Thus PIs that alter the processing of mitochondrial apoptotic regulatory proteins such as BCL-xL could both impact mitochondrial and cellular/synaptic function - as well as cell fate.

Thus, the effect of PIs (e.g. Ritonavir) and NRTIs (e.g. Tenofovir) (alone and in combination), as well as standard PI-using (kaletra [lopinavir/ritonavir] + efavirenz) and PI-sparing (efavirenz + AZT + 3TC) HAART regimens, are evaluated on the protective and functional outcomes of neuronal exposure to promising adjunctive neuroprotective agents described herein. Outcome measures include normalization of mitochondrial bioenergetics and synaptic vesicle recycling after Tat or PAF exposure. These assays reveal whether potential mitochondrial toxicity by NRTIs, or mitochondrial protection by PIs [Matarrese, P., et al., J Immunol, 2003. 170(12): p. 6006-15], modulates the effect of candidate therapies. These assays also determine whether a CNS-penetrating PI such as Indinavir exerts effects on mitochondrial hyperpolarization in neurons in the same way that some PIs have been shown to exert such effects on T cells [Matarrese, P., et al., J Immunol, 2003. 170(12): p. 6006-15].
Table 2. Antiretrovirals

<table>
<thead>
<tr>
<th>Drug Category</th>
<th>Specific compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>Indinavir (<em>penetrates the CNS</em>), Lopinavir, Ritonavir, Atazanavir</td>
</tr>
<tr>
<td>NRTI</td>
<td>AZT, 3TC, Tenofovir</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Efavirenz</td>
</tr>
<tr>
<td>HAART</td>
<td>1) Atazanavir + Tenofovir + Ritonavir</td>
</tr>
<tr>
<td></td>
<td>2) AZT + 3TC + Efavirenz</td>
</tr>
<tr>
<td></td>
<td>3) Lopinavir + Ritonavir + Efavirenz (<em>HAART regimens</em>)</td>
</tr>
</tbody>
</table>

Adjuvont neuroprotective agents provided herein (at doses known to be effective in the in vitro assay systems) are combined with different antiretrovirals, starting with individual drugs alone (PIs, NRTIs) and then progressing to the three defined HAART regimes (see Table 2, above). Selection of dose ranges is based on physiologically achievable drug levels and/or the EC50 and EC99 for each drug. Thus, as an example, Atazanavir is used at doses between 1-100 nM [Colombo, R.J., et al., Antimicrob Agents Chemother, 2003. 47(4): p. 1324-33] based on its ED50 dose (2-8 nM) for inhibiting viral replication in HIV-1 isolates resistant to other protease inhibitors.

The effects of other adjuvont neuroprotective agents that do not directly affect mitochondrial bioenergetics (e.g., compounds disclosed herein) are evaluated for their effects on mitochondrial membrane potential and synaptic activity in neurons exposed to Tat or PAF.

**Reagents:** Carbamyl-PAF (c-PAF; abbreviated “PAF” herein), a non-hydrolyzable form of PAF, is obtained from Biomol (Plymouth Landing, PA).

**Cell Culture:** Cells that are used in the compositions and methods disclosed herein include:

(1) *Primary rat cerebellar granule neurons (CGNs).* CGNs represent a highly homogenous population of primary neurons which are susceptible to Tat and PAF-mediated cell killing and have been used in many of previous studies. CGN cells are isolated according to published procedures [Maggirwar, S. B., et al. 1999. J Neurochem 73:578-86] [Tong, N., et al. 2001. Eur J Neurosci 13:1913-22].

(2) *Primary rat cortical neurons (CN).* CN are readily established, and available in significant cell numbers; they have the particular advantage that they fully reprise all the neuronal circuitry necessary to drive striatal projection neurons and are therefore used to identify electrophysiologic parameters of synaptic transmission to develop a functional bioassay for measuring the neuroprotective efficacy of potential therapeutic agents. Like CGN, CN are susceptible to Tat and PAF-mediated cell killing as well as Tat and PAF-
mediated mitochondrial dysfunction and synaptic apoptosis. These cells are isolated and maintained using methods described herein and in [Perry, S. W., et al. 2004. J Neurosci Res], incorporated by reference herein for its teaching of these methods.

(3) Primary rat hippocampal neurons (HN). The hippocampus plays a central role in learning and memory – processes which are adversely affected in HIV-associated neurologic disease. Furthermore, HN are susceptible to Tat and PAF-mediated cell killing [Kruman, II, et al. 1998. Exp Neurol 154:276-88] [Nath, A., et al. 1996. J Virol 70:1475-80] as well as Tat-mediated excitotoxicity [Song, L., et al. 2003. J Neurovirol 9:399-403]. These cells also undergo reductions in dendritic arborization in response to Tat [Maragos, W. F., et al. 2002. J Neurochem 83:955-63] or secretory products from HIV-1 infected monocyte-derived macrophages [Zheng, J., et al. 2001. Neurotox Res 3:443-59]. HNs are be prepared from embryonic day 18 rats by modification of the protocol by Brewer [Brewer, G. J., et al. 1993. J Neurosci Res 35:567-76]. In brief, hippocampi are dissected from a litter of E18 embryonic rats, dissected free of meninges and other tissue, and incubated in 2.0 ml of Ca⁺/Mg⁺-free Hanks balanced salt solution (HBSS) (with 10mM HEPES, pH7.3) with PSN antibiotics (penicillin 50 mg/L; streptomycin 50 mg/L; neomycin 100 mg/L) plus 0.5 ml for 2.5% trypsin (for 0.25% final) for 15 min at 37°C per brain. After the 15 minute incubation, trypsin is removed, cells are washed twice with HBSS (with Ca⁺/Mg⁺), then dissociated in growth media (below) by Pasteur pipette trituration by 8-10 passages through a 0.9 mm bore 1000 µl blue pipette tip. Dissociated cells are counted by trypan blue viability assay and plated in cell culture plates at 0.5-0.6 X 10⁵ cells/cm², on poly-D-lysine-coated cell culture plastic or sterile glass coverslips. The plating and maintenance media consists of Neurobasal with B27 supplement (trademark Life Technologies, Gaithersburg, MD) as described by Brewer [Brewer, G. J., et al. 1993. J Neurosci Res 35:567-76]. This media formulation inhibits the outgrowth of glia resulting in a neuronal population that is 98% pure; thus glial inhibitors are unnecessary. Cells are cultured for 10 - 21 days at 37°C in a humidified atmosphere of 5% CO₂/95% air, changing media every 4 days; cells are used for experiments at days in vitro (DIV) 14-21.

human fetal neurons lack the regional homogeneity that characterizes primary rat neurons, and cannot be obtained in pure culture without contaminating astrocytes (20-25% of the cell population, typically) and microglia (~5% of the cell population, typically). These properties make primary human fetal neurons undesirable when one wishes to examine highly homogenous neuronal populations. However, the presence of glia within the cultures, combined with their human origin, offers advantages in terms of providing a confirmatory model system that more closely approximates the human condition in vivo.

(5) Primary microglia, of both rat and human origin. Primary rat microglia are isolated using previously described methods [Patrizio, M., et al. 2001. J Neurochem 77:399-407]. These cells are used for all initial screening of candidate therapeutic molecules for the potential to influence microglia activation. It should be noted that rat microglia isolated by this method are efficiently activated by HIV-1 Tat [Patrizio, M., et al. 2001. J Neurochem 77:399-407]. Human fetal microglia are used in confirmatory experiments, to verify key findings. These cells are obtained from ScienCell, which provides highly purified (>90% CD11b+) cultures. Mixed primary rat glial cultures are established from 1-day postnatal Wistar rat cerebral cortex and grown in Basal Eagle’s Medium supplemented with 10% FBS plus glutamine and antibiotics (penicillin/streptomycin). The cells are plated on poly-L-lysine-coated plastic at a density of 2.5 x 10^4 cells/cm² in 24-well culture plates. Microglial cells are removed from 12 to 15-day primary glial cultures by mild shaking and plated on uncoated plastic at a density of 1.5 x 10^5 cells/cm² in 48-well culture plates. After 20 min, the cultures are washed with fresh medium to remove non-adherent cells and grown for 2 to 3 days. These cultures are typically 99% pure microglia/macrophages, as assessed by staining for the macrophage marker ED1. Added note:

(6) Primary monocytes. Immunomagnetic isolation of CD14+ human monocytes [Wang, X., et al. 2003. J Virol 77:7182-92] is used to isolate monocytes from peripheral blood mononuclear cells immediately following phlebotomy. Purity of the isolated cell population is verified by flow cytometric analysis using a CD14-specific monoclonal antibody; cells are used if >95% CD14+. Positive immunomagnetic selection is used for CD14+ human monocytes (Miltenyi-Biotec). However, since positive selection results in cellular activation, monocyte populations are isolated from a panel of normal donors by both positive selection (using CD14-conjugated magnetic beads) and negative selection.
(i.e., “untouched” cells). Results are compared for both populations, as well as purity (as assessed by immunophenotyping using CD14, CD68 and MHC class II).

Monitoring alterations in $\Delta \psi_m$ with TMRM: In the relevant methods described herein, changes in mitochondrial membrane potential ($\Delta \psi_m$) in response to Tat or PAF treatment is assessed by TMRM, which must equilibrate across the plasma membrane before entering the mitochondria; and in whole cell applications dye concentration in the mitochondria is dependent on both plasma membrane potential ($\Delta \psi_p$) and $\Delta \psi_m$. At sufficiently low dye concentrations (TMRM = 1 nM, i.e. high cell/dye ratio) and normal membrane potentials, $\geq95\%$ of the dye is in the mitochondria, and $\leq1\%$ in the media, which results in an approximately 100x sensitivity of the dye to $\Delta \psi_m$ over $\Delta \psi_p$, given equivalent changes in plasma or mitochondrial membrane potential [Rottenberg, H. and S. Wu, Biochim Biophys Acta, 1998. 1404(3): p. 393-40].

For these methods, primary rodent neurons are treated with reagent under normal culture conditions for the indicated time period, followed by removal of the media, 1x2 minute wash in pre-warmed 37°C HBSS plus 10 mM glucose and 10 mM HEPES (HBSS+), then incubation in HBSS+ with 1 nM TMRE/M. Following equilibration of the dye for 20 minutes to ensure distribution across the mitochondrial membrane, the cells are imaged while remaining in the 1 nM TMRE/M solution, as is necessary for a continued dye equilibrium state. Random field images are taken using an Olympus IX-70 microscope and 40x objective (fluorescent excitation: 545; emission: 610) and Apogee KX32ME CCD camera, and the mean relative fluorescent unit (RFU) value of the neuronal soma and processes (excluding mitochondria deficient regions) is quantified using Scanalytics IPLab software.

Measuring production of intracellular ROS: Generation of ROS in response to Tat and PAF is assessed with the oxidizable dye indicator 5-(and- 6)-chloromethyl-2', 7'- dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA, abbreviated as “DCF”) (Molecular Probes, Eugene, OR). Upon entering the cell by passive diffusion, the acetate groups are cleaved by intracellular esterases, and the thiol-reactive chloromethyl group binds to intracellular thiols, rendering the CM-H$_2$DCF product trapped within the cell.

Oxidation by ROS including hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO’), peroxy radical (HOO’), or peroxynitrite anion (ONOO’) [Carter, W.O., P.K. Narayanan, and J.P. Robinson, J Leukoc Biol, 1994. 55(2): p. 253-8] [manufacturer’s data] leaves the final fluorescent product CM-DCF, which is then imaged (excitation: 485, emission: 538) and
quantitated in the same fashion as TMRM (above) as an indicator of relative levels of ROS in the culture.

**ATP and ADP Measurement:** Adenosine di- and tri-phosphate (ADP and ATP respectively) levels in cortical cultures were measured after treatment using a kit from Cambrex (Rockland, ME). Briefly, cortical neurons were plated in white poly-d-lysine coated 96 well plates at a density of 32,000 cells/well. Each treatment group contained five replicate wells. After treatment, ATP and ADP levels were then measured using an ATP/ADP assay kit (Cambrex, Rockland, ME). This kit assays ATP levels via the luciferase reaction, then assays ADP levels by a proprietary method of ADP to ATP conversion; the difference in luminescence signal pre-and post-ADP conversion represents the ADP levels in the culture. Luminescent signal was integrated over 1 second and read on a Packard LumiCount microplate luminometer (Meriden, CT). For each condition, data represents the mean ± SEM ATP or ADP signal of five replicates per condition, expressed as percent increase over corresponding untreated, time-matched control. Background luminescence was negligible, but nonetheless was subtracted from all readings before data analysis. Experimental conditions were run two or more times with similar results.

**FM1-43 uptake assay:** Total spontaneous activity dependent vesicular uptake was assessed using the lipophilic styryl dye N- (3- triethylammoniumpropyl)-4-(4- (dibutylamino)styryl) pyridinium dibromide (FM1-43). FM1-43 is an amphipathic molecule with a +2 charge that prevents it from passively crossing membranes [Ryan, L.A., et al., Cell Mol Biol (Noisy-le-grand), 2002. 48(2): p. 137-50]. It is non-fluorescent in aqueous solution, but becomes fluorescent upon reversibly binding to exposed membrane by partitioning into the lipid bilayer. Therefore extracellular membranes bind FM1-43, which then becomes encapsulated into a vesicle during endocytosis, generating a fluorescent signal from the endocytosed vesicle. Upon subsequent exocytosis, the vesicle fuses with the membrane, releasing its contents, and the FM1-43 dissociates back into the extracellular solution and loses its fluorescence. These features of this dye have made it a useful tool for studying endocytosis and exocytosis, notably synaptic vesicle recycling and size estimation of various synaptic vesicle pools. FM1-43 was loaded in the absence of a depolarizing stimulus to neurons. This method loads FM1-43 into vesicles undergoing both spontaneous miniature synaptic release and spontaneous action-potential (AP) dependent release.
FM1-43 was loaded for 15 minutes, a length of time that has been determined to label spontaneous activity without saturating the vesicle pool [Prange, O. and T.H. Murphy, J Neurosci, 1999. 19(15): p. 6427-38]. Following washout of excess dye, release of the spontaneously endocytosed FM1-43 in a 100 mM KCl depolarizing bath confirmed the activity dependent nature of the staining [Pyle, J.L., et al., Neuron, 1999. 24(4): p. 803-8], and ensured that all FM1-43 vesicular uptake was released.

Immediately after addition of the depolarizing solution, cells were transferred to a Bio-rad fluoromark plate reader, and release of FM1-43 was monitored at 20 sec intervals using 438 excitation and 605 emission filters. Since background non-vesicular staining by FM1-43 is not released by KCl [Pyle, J.L., et al., Neuron, 1999. 24(4): p. 803-8], then for each condition, the total loss of FM1-43 signal during the release period equals the total spontaneous activity dependent vesicular uptake of FM1-43 in each well over the loading period. This absolute FM1-43 uptake value reflects the total activity of the culture, and is equivalent to the synaptic or vesicular release probability of the culture [Prange, O. and T.H. Murphy, J Neurosci, 1999. 19(15): p. 6427-38].

Quantifying Cell Death: Cell death is assessed by visualizing fragmented DNA per the TUNEL method as described previously as well [Perry, S.W., et al., J Neurosci Res, 2004. 78(4): p. 485-92], and where necessary, a dual stain with Trypan blue (to identify necrotic cells)/ApopTag reagent [Perry, S.W., L.G. Epstein, and H.A. Gelbard, Biotechniques, 1997. 22(6): p. 1102-6]. Briefly, after treatment in 24 well plates, cells are fixed with Histochoice MB Tissue Fixative (Amresco) then TUNEL-labeled with the ApopTag kit (Chemicon) according to kit instructions. Cells are visualized under Hoffman modulation contrast optics using a 40x objective, and images taken of ten random fields per well from 3 replicate wells per condition. Data is expressed as percent Apoptag-positive neurons [(# Apoptag positive neurons/(Total neurons)) X 100] (or Trypan bluepositive neurons where applicable) per field, then field values averaged for a mean cell death value per well. Mean values from 3 or more (see “power” calculations below) wells are averaged for a final mean cell death value for each condition ± SEM; the analyses is made without knowledge of the treatment group.

Example 6: Approaches to normalize synaptic transmission in in vitro and ex vivo morphologic correlates of post-synaptic injury.

Therefore, while assessing cell death remains an important method by which to better understand and dissect the bioenergetic consequences of Tat and PAF-treated neurons and assess adjunctive neuroprotective agents’ therapeutic potential, other biologic outcome markers are needed to better model the reversible metabolic component of HAD.

Therefore, the herein disclosed adjunctive neuroprotective agents are evaluated for the ability to reverse or ameliorate synaptic dysfunction in the herein described models of HIV-1 associated neurologic disease.

HIV neurotoxins including Tat and PAF induce a reversible synaptic dysfunction that is morphologically characterized by dendrite beading, and eventually lead to permanent synaptic deficit (i.e. synaptic apoptosis) if the local concentration of HIV-1 neurotoxins increases to irreversibly toxic levels. Tat and PAF increase pre-synaptic terminal activity and induce mitochondrial hyperpolarization, contemporaneously with increased ROS and ATP production. ROS and ATP both induce synaptic activity directly [Cheng, J., et al., Neuroscience, 1998. 82(1): p. 97-106] [Kamsler, A. and M. Segal, Mol Neurobiol, 2004. 29(2): p. 167-78] [Giniatullin, A.R. and R.A. J Physiol, 2003. 552(Pt 1): p. 283-93], and excessive ROS levels lead to synaptic damage. Furthermore, excessive synaptic activity leads to disruption of mitochondrial structure and function via Δψm-dependent calcium uptake [Rintoul, G.L., et al., J Neurosci, 2003. 23(21): p. 7881-8], which could be augmented by mitochondrial hyperpolarization. Together, these mechanisms could result in a vicious cycle of synaptic disruption and damage in the context of HAD.

Explant hippocampal slices are exposed to HIV-1 neurotoxins ± agents that reverse mitochondrial hyperpolarization. Beading and LTP are then be measured:

<table>
<thead>
<tr>
<th>HIV-1 neurotoxin</th>
<th>Treatment</th>
<th>Δψm</th>
<th>Beading Prediction</th>
<th>LTP Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>FCCP (100 nM)</td>
<td>Normalized</td>
<td>Prevented</td>
<td>Achieved</td>
</tr>
<tr>
<td>Tat</td>
<td>Vehicle</td>
<td>Hyperpolarized</td>
<td>Occurs</td>
<td>Failure</td>
</tr>
<tr>
<td>cPAF</td>
<td>FCCP (100 nM)</td>
<td>Normalized</td>
<td>Prevented</td>
<td>Achieved</td>
</tr>
<tr>
<td>cPAF</td>
<td>Vehicle</td>
<td>Hyperpolarized</td>
<td>Occurs</td>
<td>Failure</td>
</tr>
<tr>
<td>Vehicle</td>
<td>FCCP</td>
<td>Slightly Depolarized</td>
<td>N/A</td>
<td>?</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>No change</td>
<td>None</td>
<td>Achieved</td>
</tr>
</tbody>
</table>

As described above, 100 nm FCCP normalizes Δψm back to baseline levels but not below; here it is used simply to determine the effects of normalizing Δψm on beading and...
LTP in this model; it is not being considered as an adjunctive therapeutic agent. Other candidate therapeutics that normalize ΔV<sub>m</sub> (e.g. glibenclamide) are tested in place of FCCP, with a view to use in future animal or human studies.

**Hippocampal slice preparation:** Brains from anesthetized P14-28 rodents are rapidly removed and cooled in sucrose based artificial cerebrospinal fluid (ACSF) containing in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 28 NaHCO<sub>3</sub>, 10 D-glucose, 0.5 CaCl<sub>2</sub>, 7 MgSO<sub>4</sub>, 0.6 ascorbate. The brains are blocked and fixed to a specimen stage with cyanoacrylate. Coronal slices (250-400 µm in thickness) are cut with a vibroslicer (World Precision Instruments, Fl, USA) equipped with a Peltier cooling system. The middle four to six slices of each hippocampus (with the entorhinal cortex removed) are placed into a holding chamber prior to use.

**Measuring dendritic beading and postsynaptic potentials in single CA1 cells in hippocampal slices:** Dendritic arbors of individual CA1 pyramidal cells are imaged while simultaneously recording excitatory postsynaptic potentials in the same cell by patch clamp recordings in whole-cell configuration. Recording pipettes (3-6 MΩ resistance) are pulled from 1.5mm borosilicate glass using a horizontal Flaming/Brown micropipette puller (Sutter Instrument Co, CA), fire-polished, and filled with intracellular recording solution (in mM: KCl 20, potassium gluconate 130, EGTA 0.5, HEPES 10, MgSO<sub>4</sub> 2, ATP 2.5, GTP 0.5, pH 7.3). In order to visualize dendrites of the recorded cell, Alexa 568 hydrazide (30 µM) is included in the recording pipette and is injected by small negative current pulses for 15-20 min prior to recording in order to fill the dendritic arbor.

Hippocampal slices are bathed in artificial CSF (containing, in mM: NaCl 125, KCl 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.25, NaHCO<sub>3</sub> 25, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, D-glucose 25, oxygenated and buffered with 5% CO<sub>2</sub>). Membrane potentials of CA1 pyramidal cells are recorded in current clamp mode, and postsynaptic potentials are evoked by test pulses of constant-current stimulation applied via a bipolar stimulating electrode placed 50-200 µm away in the stratum radiatum. Bicuculline (10 µM) is included in the bath to isolate excitatory postsynaptic potentials. After at least 10 min of consistent baseline recording, a high-frequency LTP induction stimulus (HFS, consisting of four 1 s, 100Hz trains delivered every 15 s) is applied. EPSPs are recorded for 60 min following HFS. At the same time, dendrites are imaged at 40x magnification by fluorescence microscopy, using a shutter to limit fluorescent light exposure and prevent phototoxic injury to the slice. Serial images of dendrites are compared to detect development of focal swellings along dendritic shafts.
following HFS. Cells are scored as beaded if swellings develop along *any* of their dendrites.

**Field potential recording and induction of LTP in hippocampal slices**: Single-cell recordings are complemented by extracellular field potential recordings: while single cell recordings allow correlation of electrophysiologic data with dendritic beading in the same cell, extracellular recordings sample responses from populations of dendrites and are less invasive. Extracellular recording electrodes, filled with 2 M NaCl and 2% pontamine blue (electrode impedance 2-4 MΩ), are placed within the stratum radiatum layer of area CA1. Field excitatory post-synaptic potentials (EPSPs) are evoked by stimulation of the Schaffer collateral-commissural afferents once every 30 sec and the initial (1-2 ms) slope is measured. Baseline responses are recorded for at least 20 min prior to induction of LTP by tetanic stimulation (four individual 100 Hz trains delivered for 1 sec each at the test intensity with inter-train intervals of 15 sec). Field responses are measured for 1 hr after applying tetanic stimulation; % baseline values are determined from the final 10 min interval recorded. Field potentials are recorded using an Axoclamp-2B amplifier (Axon Instruments, CA) and amplified further by an EX1 differential amplifier (Dagan Corporation, MN, USA). Data acquisition and analyses are performed using pClamp 8 software (Axon Instruments, CA, USA) on a Pentium IV PC computer (Dell, IN, USA).

**References**


CLAIMS

What is claimed is:

1. A method of protecting a neuron from dysfunction induced by an HIV-1 neurotoxin comprising contacting the cell with a modulator of adenosine receptor signaling.

2. The method of claim 1, wherein the HIV-1 neurotoxin is HIV-1 Tat.

3. The method of claim 1, wherein the modulator is an adenosine 2A receptor antagonist.

4. The method of claim 3, wherein the adenosine 2A receptor antagonist is the small molecule ATL455 or ZM241685.

5. The method of claim 1, wherein the modulator is an adenosine 2A receptor agonist.

6. The method of claim 5, wherein the adenosine 2A receptor agonist inhibits the recruitment of monocytes to the CNS.

7. The method of claim 5, wherein the adenosine 2A receptor agonist is the small molecule ATL313 or CGS21680.

8. A method of treating HIV associated dementia (HAD) in a subject comprising administering to the subject a modulator of adenosine receptor signaling.

9. The method of claim 8, wherein the modulator is an adenosine 2A receptor antagonist.

10. The method of claim 9, wherein the adenosine 2A receptor antagonist is the small molecule ATL455 or ZM241685.

11. The method of claim 8, wherein the modulator is an adenosine 2A receptor agonist.

12. The method of claim 11, wherein the adenosine 2A receptor agonist inhibits the recruitment of monocytes to the central nervous system (CNS).

13. The method of claim 11, wherein the adenosine 2A receptor agonist is the small molecule ATL313 or CGS21680.

14. The method of claim 8, further comprising administering to the subject an antiretroviral compound.
15. The method of claim 14, wherein the antiretroviral compound comprises one or more molecules selected from the group consisting of protease inhibitors [PI], nucleoside reverse transcriptase inhibitors [NRTI], and non-nucleoside reverse transcriptase inhibitors [NNRTI].

16. The method of claim 15, wherein the PI is selected from the group consisting of Indinavir, Lopinavir, Ritonavir, and Atazanavir.

17. The method of claim 15, wherein the NRTI is selected from the group consisting of AZT, 3TC, and Tenofovir.

18. The method of claim 15, wherein the NNRTI is Efavirenz.

19. The method of claim 8, further comprising administering to the subject a neurotoxin inhibitor.

20. The method of claim 8, further comprising administering to the subject an inhibitor of GSK-3β.

21. The method of claim 20, wherein the inhibitor is sodium valproate or lithium.

22. The method of claim 8, further comprising administering to the subject a compound that enhances CNS uptake.

23. The method of claim 8, further comprising administering to the subject a microglial deactivator.

24. The method of claim 23, wherein the microglial deactivator is minocycline.

25. The method of claim 8, further comprising administering to the subject an inhibitor of glutamate damage.

26. The method of claim 25, wherein the inhibitor is ceftriaxone.

27. A composition, comprising a modulator of adenosine receptor signaling and a molecule that inhibits mitochondrial hyperpolarization in a neural cell.
Fig. 3

Fig. 4
**Fig. 5**

**Fig. 6**

Diagram A: TNPα (pg/ml) vs. Treatment conditions: NT, LPS, Tat, Tat+CGS, CGS

Diagram B: Fold Induction vs. Treatment conditions: LPS, Tat, Tat+CGS, CGS

Diagram showing TNFα and IL1β levels for different treatments: No Tat, Tat alone, ATL313 (1 nM), ATL313 (2 nM), ATL455 (1 nM), ATL455 (2 nM), ATL455 (4 nM)
**Fig. 7**

Graph showing the concentration of TNFα (in $10^3$ pg/ml) against ALT313 (nM).

**Fig. 8**

Images and graphs illustrating different experimental conditions:

- **A** - Brightfield
- **B** - FM1-43 loading
- **C** - KCl
- **D** - Graph showing FM1-43 uptake (% control) for different treatments.
- **E** - Graph showing FM1-43 uptake (RFU) for control and treatment conditions.
- **F** - Graph showing FM1-43 uptake (RFU) for control and treatment conditions with a specific treatment.
**Fig. 9**

**Fig. 10**
**Fig. 11**

A diagram showing the effect of different Tat concentrations on a specific biological parameter over time.

**Fig. 12**

A bar chart illustrating the percentage control production of ATP and ADP after 1, 24, and 48 hours of treatment with various concentrations of Tat in micrograms per milliliter (µg/ml). The chart includes statistical significance labels (p-values) for each treatment group.
Fig. 13

Fig. 14
Fig. 15