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Didehydro-Cortistatin A inhibits HIV-1 Tat mediated neuroinflammation and prevents potentiation of cocaine reward in Tat transgenic mice

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Abstract

HIV-1 Tat protein has been shown to have a crucial role in HIV-1-associated neurocognitive disorders (HAND), which includes a group of syndromes ranging from undetectable neurocognitive impairment to dementia. The abuse of psychostimulants, such as cocaine, by HIV infected individuals, may accelerate and intensify neurological damage. On the other hand, exposure to Tat potentiates cocaine-mediated reward mechanisms, which further promotes HAND. Here, we show that didehydro-Cortistatin A (dCA), an analog of a natural steroidal alkaloid, crosses the blood-brain barrier, cross-neutralizes Tat activity from several HIV-1 clades and decreases Tat uptake by glial cell lines. In addition, dCA potently inhibits Tat mediated dysregulation of IL-1 β , TNF- α and MCP-1, key neuroinflammatory signaling proteins. Importantly, using a mouse model where doxycycline induces Tat expression, we demonstrate that dCA reverses the potentiation of cocaine-mediated reward. Our results suggest that adding a Tat inhibitor, such as dCA, to current antiretroviral therapy may reduce HIV-1-related neuropathogenesis.

Keywords

Tat; HAND; HIV-1; didehydro-Cortistatin A; neuroinflammation; cocaine; reward; conditioned place preference

Introduction

HIV-1 infection of the central nervous system (CNS) promotes neuronal damage and apoptosis that result in HIV-1 HAND, which covers a large spectrum of cognitive and locomotor impairments [1–4]. The virus, transported to the brain by infected macrophages

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Conflict of interest

The authors declare no conflict of interest.

and monocytic cells, can infect and replicate in microglial cells, but neurons are believed to be resistant to HIV infection [4]. Astrocytes are susceptible to HIV-1 but do not support replication; however, early viral transcripts were detected during persistent infection [5–7]. HIV-1 infection in the brain is accompanied by neuroinflammation, characterized by aberrant expression of cytokines and chemokines by glial cells [1–3]. Furthermore, the abuse of psychostimulants by HIV-1 infected subjects further fuels the infection and the progression of HAND. Unfortunately, 20–50% of patients under antiretroviral therapy (ART) are still affected by HAND as various antiretroviral regimens do not cross the blood-brain barrier (BBB) [1, 2, 8, 9].

The HIV-1 Tat (transactivator of transcription) protein is an early expressed gene product encoding a 101-amino acid protein (14 kDa) (Figure 1). A few laboratory-adapted virus strains (e.g. LAI, HXB2, pNL4-3) present a functional 86-amino acid form of Tat that results from an early stop codon [10]. Tat binds via its basic domain to the 5'-terminal region of HIV mRNA's stem-bulge loop structure, the trans-activation response element (TAR) and activates HIV-1 transcription by recruiting several cofactors at the viral promoter long-terminal repeat (LTR) [11]. Besides the crucial role in HIV-1 expression, Tat also displays pleiotropic effects in HAND [12]. Tat can be excreted from HIV-1 infected cells and up-taken by non-infected cells directly or through membrane receptors [13–17]. For instance, Tat uptake by astrocytes depends on Tat's basic domain interaction with the heparin sulfate proteoglycans (HSPG), and also with the Tat's Arg-Gly-Asp motif (RGD, located at its C-terminus) interaction with integrin receptors [18, 19]. The low-density lipoprotein receptor-related protein (LRP), an endocytic receptor of several ligands [20, 21], was also shown to interact with Tat via its core domain [22]. Tat binding to LRP leads to 20% apoptosis in astrocyte cells, but in a general manner apoptosis is very limited, which explains the wide spread use of this model to express Tat protein.

Upon cellular Tat uptake or its interaction with specific receptors [12, 23, 24], it stimulates excessive release of cytokines and chemokines, such as the Interleukin 1-beta (IL-1 β) [12, 25, 26], tumor necrosis factor alpha (TNF- α) [26–31] and the chemokine monocyte chemoattractant protein-1 (MCP-1) [30, 32]. IL-1 β is key to drive the neuroinflammation process, and is not only elevated in the cerebrospinal fluid of HIV-associated dementia patients [33–35], but is also up-regulated in Alzheimer's disease, Parkinson disease, multiple sclerosis and other neurodegenerative diseases [36, 37]. IL-1 β signaling through IL-1 receptor leads to the activation of the pro-inflammatory cytokines interferon γ (IFN- γ), TNF- α , IL-6, neutrophil recruiting chemokines CXCL1 and CXCL2, which increase glia activity, BBB permeability, leukocyte invasion, all of which hallmarks of brain damage [38–42]. Tat up-regulation of TNF- α triggers demyelination, infiltration of lymphocytes, astrocytosis and microgliosis [2].

The chemokine MCP-1, was shown to be up-regulated by Tat in astrocytes, and correlated to the infiltration of immune cells to the CNS in patients affected by HAND [32, 43]. However, more recent studies highlighted a beneficial role of MCP-1 in neuropathologies. For instance, neuroprotection induced by the neurotransmitter, noradrenaline, is mediated by up-regulation of expression and release of MCP-1 in astrocytes [44]. MCP-1 was also shown to protect human neurons and astrocytes from Tat or N-methyl-D-aspartate (NMDA)

induced apoptosis [45]. MCP-1 was shown to protect from viral infection; however, when infection occurs, this chemokine contributes to the increased risk of HAND through its pro-inflammatory properties [46]. Altogether, these results argue that expression of MCP-1 may have opposite contributing activities in HAND.

The exposure to HIV-1 Tat protein is also known to contribute to dysfunction of the dopaminergic system, which is involved in essential functions such as survival, movement, attentiveness, motivation, learning, memorization and drug reward [47]. Tat influences the dopamine recycling, uptake kinetics, and causes rapid dopamine transporter dysfunction [48–54]. Several studies demonstrate that the administration of cocaine and Tat together damage neurons to a greater extent than individually. For instance, cocaine enhances oxidative stress, neuronal dysfunction, and apoptosis in Tat-treated cells [54, 55]. Together, administration of cocaine and Tat results in dopamine neurotoxicity in rat fetal midbrain primary cell cultures and BBB damage [3]. Importantly, HIV-1 Tat was shown to potentiate cocaine-mediated psychostimulation and reward in the GT-tg transgenic mice model [56, 57] demonstrating the synergy between drugs of abuse and the neurotoxin Tat.

Tat is a very attractive target for therapeutic intervention because 1) it is expressed early during virus replication, 2) has no cellular homologs, 3) direct inhibition of Tat blocks exponential production of viral progeny and 4) inhibition of Tat blocks the neurotoxic activity of Tat in HAND. Even though the dependence of HIV-1 on Tat for transcription and pathogenesis has made it a highly sought after drug target, there is still no therapeutic agent in the clinic to inhibit this viral protein.

We recently reported that didehydro-Cortistatin A (dCA), an analog of a natural steroidal alkaloid, potently inhibits Tat mediated transactivation of the viral genome at subnanomolar concentrations [58]. Here, we extend our previous findings to demonstrate the interaction of dCA with Tat's basic domain and specifically with the 45–55 amino acid sequence. The basic domain of Tat is fairly well conserved throughout different HIV-1 clades, thus we confirmed the ability of dCA to inhibit HIV-1 Tat clades A–E. As our pharmacokinetic studies demonstrated dCA permeability of the BBB, we showed dCA ability to decrease Tat extracellular uptake by glial cell lines. Furthermore, we showed the ability of dCA to reverse Tat mediated regulation of cytokines and chemokines by the astrocytic cell line, U87MG, without associated cellular toxicity. Most importantly, using the GT-tg bigenic mouse model, where brain selective Tat expression is induced by activation with doxycycline (Dox) [56], we tested the effects of dCA on Tat potentiation of cocaine mediated conditioned place preference (CPP).

Materials and Methods

Bacterial expression and purification of Tat

The plasmids pTatC6H-1 (NIH AIDS Reagent Program) encoding C-terminal 6xHistidine Tat₁₋₈₆ (HXB3, subtype B) and pTatC6H-1-Mut encoding Tat with a mutation in the sequence ⁴⁹RKKRRQRRRPP⁵⁹ (arginine residues replaced with alanine) were expressed in *E. coli* BL 21 to obtain Tat and Tat Mut respectively. Recombinant proteins were induced with isopropyl β-D-thio-galactopyranoside (IPTG) and purified as described in the AIDS

Reagent Program protocol, with an added second purification step. Proteins were subjected to a 30 kDa filtration (Millipore) to retain only the active monomeric form of Tat [59]. Proteins were then solubilized in acidic phosphate buffer (pH \approx 4) to maintain cysteine residues in a reduce form important for Tat activity [60]. The mass and purity of the proteins was verified by SDS-page and western blot.

HeLa-CD4-LTR-Luc transactivation assay

The activity of Tat was verified by a transactivation assay as previously described [58]. Briefly, HeLa-CD4-LTR-Luc cells were plated at 1×10^5 cells per well of a 6-well plate. The following day, recombinant Tat protein (1.25 μ g or 0.6 μ g) was added to the cells in culture media without serum and in the presence of 100 μ M of Chloroquine. Five hours later, serum was added at 5 % to the culture media. Cells were lysed 24 h later, protein concentration determined by Bradford assay and luciferase activity was measured. Results corresponding to luciferase activity per protein concentration of each sample are shown as relative light unit (RLU). Tat is denatured by heating at 90°C, for 20 min, in acidic phosphate buffer (pH \approx 4) supplemented with 3 M urea.

ELISA - Interaction of Biotinylated dCA (Bio-dCA) with full-length Tat or Tat peptides

Full-length Tat protein or 18 peptides of 15-amino acids each spanning full-length Tat with overlaps (NIH AIDS Reagent Program) (1.25 μ M) were coated onto 96-well plates and incubated overnight at 4°C. Non-specific signal was controlled by coating bovine serum albumin (BSA) at the same concentration. Upon blocking with 2 % BSA in phosphate buffer saline (PBS), plates were incubated for 2 h with 100 μ l of 0.2 % BSA in PBS supplemented with a 2-fold excess of Bio-dCA. As previously shown [58], the activity of Bio-dCA is similar to dCA. Anti-biotin antibody (Invitrogen, cat # 03-3720) was added at 1:1000 followed by ABTS substrate (Roche Diagnostics). The absorbance was measured at 405 nm 1 h post incubation.

Dot blot assay - Interaction of Bio-dCA with recombinant Tat

Tat or BSA (2 μ g) were spotted onto a nitrocellulose membrane. The membrane was blocked with 5 % BSA in PBS followed by a 2-fold excess incubation with Bio-dCA overnight at 4°C. The anti-biotin antibody (Invitrogen, cat # 03-3720) was used at 1:1000 dilution and the interaction revealed with ECL western blotting system (Amersham).

Transfections

Transfection of Flag-Tat, Flag-Tat Mut and GFP in HeLa-LTR-Luc cells. HeLa-LTR-Luc cells were plated at 2×10^6 in a 10 cm tissue culture dish and transfected with 4 μ g of the constructs expressing Flag-Tat (PGK-Flag-Tat), Flag-Tat Mut (PGK-Flag-Tat Mut), and green fluorescent protein, GFP (PGK-GFP) driven by the murine phosphoglycerate kinase-1 (PGK) promoter, with TransIT-LT1 Transfection Reagent (Mirus Bio LLC) according to the manufacturers protocol. The GFP vector was used to determine transfection efficiency and was used as negative control for Tat expression. The cells were split 24 h post transfection and treated with dCA, Raltegravir or dimethyl sulfoxide (DMSO) at different

concentrations. Luciferase activity per protein concentration of each sample was determined 48 h later.

Transfection of Flag-Tat from clade A through E in HeLa-LTR-Luc cells. HeLa-LTR-Luc cells stably expressing luciferase driven by the LTR from clade B were plated in 10 cm dishes (1×10^6 cells per dish) and 24 h later transfected with expression vectors encoding N-terminally Flag-Tat from clades A through E or GFP. At 24 h post transfection, cells were split and treated with 100 nM dCA or DMSO. At 48 h post drug treatment, cells were lysed and luciferase activity was quantitated using a luminometer. Cell lysates were subjected to western blot analysis for Flag-Tat expression. Beta-actin was used as a loading control. Results corresponding to luciferase activity per protein concentration of each sample are shown as RLU. The data was normalized to Flag-Tat treated with DMSO set to 100 %.

Transfection of Flag-Tat (clade A–E) and matching LTRs in HeLa cells. Cells were plated at 1×10^6 cells per 10 cm dish and 24 h later transfected with expression vectors encoding N-terminally Flag-tagged Tat from clades A through E or empty vector (E.V.), plasmids encoding the matching clade LTR driving a destabilized form of the firefly luciferase gene (pGL4.22 – Promega), and Renilla luciferase driven by the HSV thymidine kinase promoter to be used as a transfection control (pGL4.74 – Promega). Each dish received three plasmids: Flag-Tat (or empty vector), the matching LTR-LucDS, and pGL4.74. At 24 h post transfection, cells were split and treated with 100 nM dCA or DMSO. At 48 h post treatment, cells were lysed and firefly/Renilla luciferase activity was quantitated using the Dual Luciferase reporter assay kit (Promega). Cell lysates were also subjected to western blot for Flag-Tat expression. Beta-actin was used as a loading control. Firefly values were divided by Renilla values for each well. The mean was calculated for the triplicate data points and the data were normalized to Flag-Tat treated with DMSO set to 100 %.

Transfection of Flag-Tat, Flag-Tat Mut and GFP in U87MG cells. U87MG cells obtained from the AIDS Reagent Program were plated at 0.25×10^6 per well of 6-well plates and transfected with 2.5 μ g of PGK-Flag-Tat, PGK-Flag-Tat Mut and PGK-GFP. dCA or Raltegravir at 250 nM or DMSO were added at the time of transfection. Cell lysates were collected at different times post-transfection and used to assess protein and mRNA expression by western blot and RT-qPCR respectively.

Western Blot

Detection of recombinant Tat and Tat Mut in microglia-like cells (differentiated U937 cells) and astrocyte U87MG cells. U937 cells (0.5×10^6) were differentiated into macrophages with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) for 48 h at 37°C. Then, differentiated U937 (0.5×10^6) and U87MG cells (1×10^5) were incubated with 500 ng of recombinant Tat or Tat Mut in 1 ml or 500 μ l of serum free Dulbecco's Modified Eagle Medium (DMEM) supplemented with DMSO, dCA or Raltegravir (500 nM) respectively. Cells were washed 4 h later with PBS, and protein extracts were subjected to western blot. Primary antibody anti-Tat serum (NIH AIDS Reagent Program, cat # 705) was used at 1:2000 dilution and horseradish peroxidase (HRP) conjugated anti-rabbit IgG polyclonal antibody (Bio-rad, cat # 170-6515) was used at 1:10000. Actin was detected with anti-actin and used as a loading control.

Detection of Flag-Tat and Flag-Tat Mut in U87MG cells and HeLa-LTR-Luc cells. After transfection and incubation with compounds, cells were washed with PBS and lysed. Protein extracts were analyzed by western blot. Flag tagged proteins were detected with anti-Flag antibody (Sigma, cat # F3165) at 1:5000 dilution and anti-mouse IgG polyclonal HRP antibody at 1:10000 (Bio-Rad, cat # 172-1011).

Real-Time PCR of cell-associated RNA

Total RNA was extracted using the RNA extraction kit (Qiagen) following the manufacturers instruction. DNA contamination was removed using Turbo-DNAse kit (Ambion). First-strand cDNA was synthesized using SuperscriptIII™ first-strand cDNA kit (Invitrogen), following the manufacturers instructions, and random hexamers as first-strand primers. Real time qPCR was performed with an aliquot of cDNA as template, using LightCycler® 480 SYBR Green I Master (Roche) in a 20 µL reaction according to the manufacturer's instructions. The mRNAs from each sample were used as negative control. We used the same validated primer sequences of GAPDH, IL-1β and TNF-α as in Chanput *et al.* [61]. The primer sequences of MCP-1 and Tat were:

MCP-1: 5'CTCGCGAGCTATAGAAGAATCAC³/3'CATGGAATCATGAACCCACT
T⁵

Tat: 5'ATGGAGCCAGTAGATCCTAG³/3'GGGTTGCTTTGATAGAGAACTTG⁵

Immunofluorescence

Astrocyte cells were grown on fibronectin treated coverslips and transfected with the PGK-Flag-Tat or PGK-Tat Mut. Following transfection, cells were treated with DMSO or dCA (250 nM). Cells were fixed with 4 % formaldehyde, and after fixation, permeabilized with 0.1 % triton X-100 in PBS. Tat or Tat Mut were detected with the primary antibodies mouse anti-Flag M2 (Sigma) at 5 µg/mL followed with the Alexa Fluor conjugated secondary antibodies from donkey (Invitrogen, cat # A21202) at 1:2000 dilution. The coverslips were analyzed on the FluoView FV 1000 laser-scanning microscope.

Cell viability

U87MG cells were plated at 2×10^4 per well of a 96-well plate. The next day, cells were incubated with a 1 nM-10 µM range of dCA, Raltegravir or DMSO for 48 h and an MTT (3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide) assay was performed according to the manufacturers' protocol (ATCC). U87MG cells were plated at 2×10^4 per well of a 96-well plate. The next day, cells were incubated with Tat and Tat Mut (10 ng/ml up to 30 µg/ml) in presence or absence of dCA, Raltegravir (250 nM or 1 µM) or DMSO for 24 h and an MTT assay was performed according to the manufacturers' protocol (ATCC).

Animals and housing

Adult male GT-tg bigenic mice (n = 77, 8–11 weeks of age) were generated from colonies in the Torrey Pines Institute for Molecular Studies vivarium (Port Saint Lucie, FL). GT-tg bigenic mice were created by the laboratory of J.J. He as described previously [56]. Colony progenitors came from breeders that were previously back-crossed seven generations onto

the C57BL/6J strain. The confirmation of the inducible and brain-specific expression of HIV-Tat protein in these animals was described previously [56, 57, 62].

Pharmacokinetics of dCA assessed in C57BI-6 mice

Pharmacokinetics experiments were performed at the Scripps Florida pharmacokinetics core facility. A solution of 1 mg/mL of dCA was prepared and injected intraperitoneally (i.p.) in three mice at 2 mg/Kg and other three mice at 5 mg/kg via the tail vein twice daily for 10 days. dCA levels were measured in plasma and brain by LC-MS/MS, 7 h after the last dosing on day 10.

Induction of Brain-specific Tat with Dox Treatment

Tat₁₋₈₆ protein was expressed in GT-tg bigenic mice via daily i.p. injections of Dox (Sigma-Aldrich, St. Louis, MO) (100 mg/kg/day, in 0.9 % saline) as described earlier [57, 62, 63]. Induction experiments administered Dox once daily for 7 days, as previously determined to be the best dosing for the highest Tat expression [62]. GT-tg mice administered saline ('uninduced') served as negative controls.

Intracerebroventricular administration technique

Intracerebroventricular (i.c.v.) injections (of vehicle 50 % DMSO or 20 nM dCA) were made directly into the lateral ventricle according to the modified method of Haley and McCormick [64]. The volume of all i.c.v. injections was 5 μ L. The mouse was anesthetized with isoflurane, an incision made in the scalp, and the injection made 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm using a 10 μ L Hamilton syringe.

Conditioned place preference

Mice were conditioned via a counterbalanced cocaine-CPP paradigm as described previously [65], using the apparatus and automated measurement system (San Diego Instruments, San Diego, CA) described by Carey *et al.* [66]. Animals were treated with vehicle (0.9 % saline) or Dox (100 mg/kg/day) for 7 days as described above. This treatment was administered before starting the place conditioning. An initial preconditioning preference was determined by measuring the amount of time the individual mice spent in each chamber during a 30-min testing period. Prior to place conditioning, mice did not demonstrate significant differences in their time exploring the left (615.7 ± 23.4 s) versus right (669.0 ± 20.6 s) compartments ($n = 0.14$; Student's paired *t*-test), resulting in an average preconditioning response of -31.0 ± 23.4 s and demonstrating the apparatus was balanced. Each day for the next 2 days, mice were administered vehicle (0.9 % saline, s.c.) and confined in a randomly assigned outer compartment, half of each group in each chamber [57]. Four hours later, mice were administered cocaine (10 mg/kg, s.c.) and confined to the opposite compartment for 30 min. This saline/cocaine conditioning 'cycle' was repeated for two cycles, one cycle per day. Mice were assessed for place preference 24 h after the completion of conditioning by allowing the mice free access to the apparatus and measuring the time they spent in each chamber over a 30-min testing period [57]. Conditioned place aversion [67] was not detected under any conditions.

Statistical Analysis

Two-tailed *t*-tests were assessed on the effect of dCA on cytokines and chemokine expression in U87MG cells. Data are presented as mean \pm SEM.

Conditioned place preferences were statistically assessed as previously described [57].

Results

dCA specifically interacts with active recombinant Tat

We produced and purified recombinant HIV-1 Tat protein (clade B, HXB3 isolate) in *E. coli* using nickel column chromatography followed by a 30 kDa cut-off filtration. The activity of Tat was assessed via transactivation of HeLa-CD4 cells stably expressing the HIV-1 5'LTR promoter driving luciferase expression (HeLa-CD4-LTR-Luc) (Figure 2A). In this system, Tat activity directly correlates with luciferase production. Tat activated the LTR promoter activity, while denatured Tat, used as negative control, was unable to transactivate the reporter, confirming previous results demonstrating that the proper folding of Tat is required for the transactivation activity [60]. Of note, we observed the same activity with 58 nM of our purified recombinant Tat as with 100 nM of recombinant Tat obtained from the NIH AIDS Reagent Program [58].

Next, we confirmed that dCA potently inhibits the activity of recombinant and transfected Tat in this transactivation assay. HeLa-CD4-LTR-Luc cells were incubated with recombinant Tat or Tat mutated at the basic domain (Tat Mut) (28 nM) in the presence of 10 nM or 1 μ M of dCA or the integrase inhibitor, Raltegravir (used as negative control) (Figure 2B). Tat transactivated the LTR promoter but not Tat Mut, and while dCA inhibited the activity of wild-type Tat, Raltegravir had no effect. In parallel, expression vectors encoding Tat or Tat Mut were transfected into HeLa-CD4-LTR-Luc cells and treated with dCA or Raltegravir (Figure 2C). Again, transfected wild type Tat activated the LTR promoter and Tat's activity was inhibited by dCA but not by Raltegravir. As expected, Tat Mut was unable to activate the viral promoter. Altogether, these results confirm that our produced Tat is active and dCA specifically inhibits Tat transactivation activity of the HIV LTR promoter.

dCA recognizes the basic domain of Tat

We have previously shown that a biotinylated version of dCA (Bio-dCA) presents a similar EC₅₀ than dCA [58]. We have now confirmed the interaction of Tat with Bio-dCA using a dot blot assay (Figure 3A). Recombinant Tat or BSA was spotted onto a nitrocellulose membrane, incubated with Bio-dCA and the interaction revealed using an anti-biotin antibody. Bio-dCA specifically recognized Tat but not BSA, confirming the interaction between transfected Tat and Bio-dCA in pull-down assays shown by Mousseau *et al.* [58].

To identify the sequence of Tat recognized by dCA, we took advantage of a set of 18 peptides composed of 15 residues that span the full-length Tat protein (Figure 3B). These peptides along with full-length Tat were coated onto a 96-well plate and we assessed their interaction with Bio-dCA upon incubation with an anti-biotin antibody. Bio-dCA associated with two peptides spanning the basic region of Tat (45–55 a.a. sequence) equally as well as

the full-length Tat (Figure 3C). This result corroborates the absence of interaction between Bio-dCA and Tat mutated in the basic domain demonstrated by Mousseau *et al.* [58] in pull-down assays.

Activity of dCA on Tat from different HIV-1 subtypes

HIV-1 is known for its large sequence mutation and its main group M can be subdivided into several clades (A–D, F–H, J and K) [68]. Sequence mutation can be directly correlated to their overall fitness and pathogenesis. For example, variations within the HIV promoter region of main subtypes B, C and E include differences in the TATA box, the NF- κ B enhancer, the TAR element as well as Sp1 binding sites. Their Tat protein also exhibits amino acid divergences, which may influence binding and transactivation activity. Despite this divergence, the basic domain is a rather well conserved sequence [10, 15]. As dCA binds to this highly conserved region, we tested whether dCA may exhibit a pan activity against Tat from the various subtypes of group M.

We built expression vectors encoding the 86 amino acid version of Tat from a reference strain of subtypes A–E. HeLa-CD4-LTR-Luc cells (LTR subtype B) were transfected with the various constructs expressing the different Tat subtypes. At 24 h post transfection, cells were split and treated with DMSO vehicle control or with 100 nM dCA and Luciferase was measured an additional 24 h later (Figure 3D). dCA potently inhibited the transactivation of a stably integrated LTR-driven reporter by Tat from the various subtypes tested (Figure 3D, upper) without affecting the expression of the proteins (Figure 3D, lower). Next, we explored whether dCA could inhibit LTR and Tat from each individual subtype HIV-1 clade A–E. The LTR that matches the reference strain selected for each Tat was cloned into a reporter vector expressing a destabilized form of firefly luciferase. HeLa-CD4 cells were transiently transfected with the LTR-driven destabilized luciferase reporters along with a vector encoding Renilla luciferase under the control of the herpes simplex virus thymidine kinase promoter (low to moderate expression) as a transfection control. Along with these constructs, plasmids encoding Tat matching the transfected LTR reporter (or empty vector) were delivered. Twenty-four hours post-transfection, the cells were split evenly and treated with either DMSO or 100 nM dCA. dCA was able to inhibit Tat-mediated stimulation of the matching LTR (Figure 3E), which suggests that dCA is a potent and broad-spectrum inhibitor of Tat, a conclusion further supported by our previous report of dCA's ability to inhibit Tat from HIV-2 [58].

dCA crosses the blood brain barrier

As Tat crosses the BBB and induces neurotoxicity [69], we wondered whether dCA was able to cross the BBB to neutralize Tat activity. Mouse experiments were conducted to test this hypothesis. dCA was formulated at 1 mg/mL in water and dosed by intraperitoneal (IP) injection into three C57Bl-6 mice at 2 or 5 mg/kg twice daily for a period of 10 days. dCA concentration was determined in plasma and the brain by LC-MS/MS, 7 h after the last dosing on day 10. The three mice that received dCA at 2 mg/kg presented an averaged concentration of dCA in the brain 50 fold higher than the EC₅₀ value found in cell based-assays (~2 nM), while the mice that received 5 mg/kg of dCA presented 350 fold higher levels without obvious signs of toxicity (Table 1). These results confirm the ability of dCA

to cross the BBB and prompted us to study the effect of dCA in neurotoxic events mediated by Tat.

dCA inhibits extracellular Tat uptake by different cell lines

It is well known that Tat's basic domain binds HSPG on different cellular membranes resulting in Tat uptake into the cytoplasmic compartment [13–17, 19]. Thus, we hypothesized whether dCA was able to inhibit Tat uptake in microglial-like cell lines such as the differentiated U937 and the astrocytic cell line U87MG. These cells were incubated with 500 ng of recombinant Tat or Tat Mut in the presence or absence of dCA for a period of 4 h, and after thorough washing of unincorporated Tat, cells were disrupted and total lysates analyzed by western blot with an anti-Tat serum (Figure 4). dCA reduced by about $40.6 \pm 0.9\%$ and $45.6 \pm 6.8\%$ the uptake of Tat in U937 and U87MG respectively, while Raltegravir used as negative control had no significant impact on Tat uptake. The Tat Mut protein was incorporated by U87MG cells (19.1 ± 6.9), and as expected dCA did not significantly alter its uptake (17.1 ± 7.3). Tat Mut was not internalized at all by differentiated U937 cells.

dCA and Tat do not affect the viability of the astrocyte cell line U87MG

The induction of apoptosis by Tat in astrocytic cells is very limited [21]. Eugenin *et al.* [21] showed by TUNEL that Tat (used at 10 ng/ml) via the LRP receptor could induce a 20 % apoptosis in astrocyte cells in co-culture with neurons. Moreover, Maletinska *et al.* [20] showed that U87MG cells are rich in this receptor. We wondered whether our recombinant Tat could induce apoptosis and whether dCA was able to reverse this effect. First, we assessed whether dCA alone could reduce U87MG cell viability (Figure 5A). For that, a range of concentrations of dCA and Raltegravir (as negative control) were incubated with U87MG cells for 48 h and cell viability determined with a mitochondrial metabolic activity (MTT) assay. As expected, dCA did not alter U87MG cell viability. Next, Tat and Tat Mut were incubated with U87MG for 24 h in the presence of an array of DMSO, dCA and Raltegravir concentrations, and cell viability was revealed with an MTT assay (Figure 5B, C). Only upon incubation with very high concentrations of Tat ($>10 \mu\text{g/ml}$) a decrease in cell viability could be observed. It is worth noting that this Tat concentration is 1000 times higher than reported by Eugenin *et al.*, and a much higher concentration than observed in patient's serum [70, 71]. The core domain of Tat has been postulated to be involved with the apoptosis observed in astrocyte cells, and thus as expected, dCA (as well as Raltegravir) did not rescue cellular viability at high concentrations of Tat. In addition, these results assured us that transfection of astrocyte cells by Tat should not result in loss of viability, as also previously reported [28].

Transient transfection of Tat and Tat Mut in the astrocytic cell line U87MG

Astrocytosis is the most frequent pathology occurring in the brain of HIV-1 infected patients [72, 73]. It is especially characterized by an up regulation and release of different cytokines and chemokines. Consistently, transgenic mice expressing Tat in astrocytes (GT-tg) presented a reactive astrocytosis [56]. To determine whether dCA was able to reverse Tat up-regulation of cytokines in astrocytes; we transiently transfected Tat or Tat Mut DNA into

U87MG cells and assessed IL-1 β , TNF- α and MCP-1 production. First, the transfection efficiency was assessed by co-transfecting Tat or Tat Mut with the enhanced GFP plasmid in U87MG cells and by detecting fluorescence by microscopy at 24 h, 36 h and 48 h post-transfection (Figure 6A). The transfection efficiency determined by FACS was approximately 70% with the first signs of eGFP expression observed at 24 h post-transfection and peaking at 48 h. To confirm Tat and Tat Mut expression, we extracted total mRNA from these cells and upon cDNA conversion, real time quantitative PCR (RT-qPCR) was performed using common primers directed to the C-terminus of Tat (Figure 6B). The expression of Tat and Tat Mut increased with time with maximum expression observed at 48 h. This result was also confirmed by the highest expression of Tat proteins by western blot assay at 48 h post transfection (Figure 6C). It is interesting to note that for similar Tat and Tat Mut mRNA expression, higher protein expression of Tat Mut is observed, possibly due to increased protein stability. Subsequently, mRNA expression of IL-1 β , TNF- α and MCP-1 were analyzed by RT-qPCR (Figure 6D). At all time points post transfection, the mRNA level of IL-1 β was increased with Tat expression as compared to Tat Mut or GFP negative control. The level of TNF- α mRNA expression was similar between Tat, Tat Mut and the GFP negative control at all tested time points. However, upon normalization of the mRNA TNF- α per Tat mRNA expression, a difference could be observed between Tat and Tat Mut, but even so, the mRNA TNF- α expression was low compared to mRNA IL-1 β expression. MCP-1 levels did not seem to change between Tat proteins and control GFP until 48 h post Tat transfection. At this time point, both Tat proteins mediated a reduction of MCP-1 levels by approximately 50%. The expression of MCP-1 in the literature has been contradictorily associated with either neuroinflammation or neuroprotection. Our results suggest that in the specific case of U87MG cells, Tat expression leads to an MCP-1 down-modulation.

Together, these results confirm that Tat triggers the expression of IL-1 β and TNF- α in the astrocytic line U87MG and that the Tat basic domain is involved in the process. Moreover, MCP-1 expression was down-regulated by Tat irrespective of the basic domain.

dCA reverses Tat mediated cytokines/chemokine dysregulation in U87MG cells

We tested the activity of dCA (250 nM) on the mRNA expression of IL-1 β , TNF- α and MCP-1 induced by Tat at 48 h post Tat transfection (Figure 6E). We chose this time point to reflect the highest level of Tat mRNA (or Tat Mut) expression versus the peak of expression of the different cytokines or chemokine (Figure 6B–D).

dCA significantly reduced the Tat mediated up-regulation of IL-1 β and TNF- α mRNA (Figure 6E). Raltegravir, used as negative control, had no significant impact on IL-1 β ; however, even if not significantly, it seemed to mediate a decreased in the expression of TNF- α . This effect of Raltegravir on TNF- α expression had been previously reported [74, 75]. The down-modulation of MCP-1 expression by Tat was potently counteracted by dCA but not affected by Raltegravir (Figure 6E). The increase in MCP-1 expression in the presence of dCA was similar to control levels obtained with control GFP (Figure 6D). These results demonstrate the ability of dCA to inhibit to various degrees the inflammation mediated by Tat, by inhibiting production of IL-1 β and TNF- α and promoting an increase in MCP-1.

dCA alters Tat nucleolar localization in U87MG cells

Tat was shown to traffic through the nucleolus via its nucleolar localization signal located in the basic domain [28, 76, 77]. As dCA interacts with Tat through its basic domain, we assessed whether dCA altered the nucleolar accumulation of Tat in U87MG (Figure 6F). U87MG cells transfected with Flag-Tat or Flag-Tat Mut were treated with dCA or DMSO for 24 h and the subcellular localization of Tat assessed by fluorescence microscopy. As expected Tat was found to be mostly nuclear with a clear accumulation at the nucleolus. Tat Mut was detected in the cytoplasm and nucleus but was excluded from the nucleolus, as previously observed by Chauhan *et al.* [28]. dCA caused a redistribution of Tat to the periphery of the nucleolus, forming a distinctive ring-like structure. This effect seemed to mimic the phenotype caused by the basic domain mutation, confirming previous results described for HeLa cells [58]. These results further support that dCA binds to the basic domain of Tat, inhibiting Tat trafficking through the nucleolus, while not affecting expression of Tat or Tat Mut proteins.

dCA blocks Tat potentiation of cocaine conditioned place preference

A recent study showed that Tat potentiates the CPP response to cocaine [57]; here we tested whether dCA can reverse this process. Transgenic mice expressing a Tat 'knock in' gene encoding the first 86-amino acids of HIV-1 HXB2 Tat protein was used. In this model, Tat expression is driven by the astrocyte-specific glial fibrillary acidic protein promoter and is controlled by a Tet-on inducible system, which becomes transcriptionally active when Dox is introduced [56]. Male mice were treated daily with saline (0.9%, i.p.) or Dox (100 mg/kg, i.p.) for 7 days followed by 2 days of cocaine place-conditioning (Figure 7A). Although there were no significant differences in initial preference of all mice ($F(3,74)=0.22$, $p=0.88$; one-way ANOVA, data not shown), all mice demonstrated significant cocaine CPP as compared to preconditioning responses ($F(1,143)=31.2$, $p<0.0001$; two-way ANOVA) (Figure 7B). Consistent with previous results [57], exposure to Tat protein significantly potentiated the cocaine conditioned place preference response of GT-tg bigenic mice [56], ($F(3,69)=4.79$, $p=0.004$; one-way ANOVA; left pair of bars) (Figure 7B).

To counter the behavioral deficits induced by Tat protein, GT-tg bigenic mice were pretreated daily with dCA 30 min prior to the administration of saline (i.p.) as a control (yellow bar), or Dox (100 mg/kg, i.p.; right-most brown bar) to induce Tat protein expression. After 7 days treatment, mice were place conditioned with cocaine (Figure 7A). All mice treated with dCA demonstrated cocaine CPP responses that did not significantly differ from vehicle-treated, uninduced mice ($p>0.05$, Tukey's post hoc test). However, dCA-pretreatment ($n=10$) prevented Tat-induced potentiation of cocaine-CPP ($p<0.05$; Tukey's post hoc test) (Figure 7B).

Discussion

Here, we show that dCA, an analog of a natural steroidal alkaloid that inhibits HIV-1 infection at subnanomolar concentrations [58], also potently cross-neutralizes the transactivation activity of HIV-1 Tat from different clades on heterologous or homologous LTRs by binding to the highly conserved basic domain. Several studies involved the basic

domain of Tat in reactive microglia, astrocytosis and neuron death [28, 42, 78]; however, the mechanism is not well known. We also demonstrated that dCA, when intraperitoneally injected in mice, crosses the BBB and is detected in the brain at high levels. These two characteristics should be highlighted, since the low efficiency of ART in the brain is mainly due to its low BBB penetration [1, 8, 9], and in addition, current ART targets HIV subtype B more specifically than other subtypes (cf. A and C) that are highly prevalent around the world [68, 79].

The basic domain of Tat is not only involved in the interaction between Tat and the HIV-1 TAR [11], but also in translocation through cell and nuclear membranes. It has been widely established that proteins with high molecular weight linked to the basic domain of Tat are easily transported into the cytoplasmic compartment [80, 81]. Our results demonstrate the ability of dCA to partly reduce the cytoplasmic uptake of Tat by glial cells, which are at the epicenter of the neuroinflammatory process [2, 3]. For this study we used differentiated U937 as microglia like cell model and the astrocytic U87MG cell line. U87MG cells, unlike differentiated U937, also uptake Tat Mut, which confirms studies implicating the RGD motif located at the C-terminus of Tat [18, 19] and LRP in Tat up-take by astrocyte cells [21]. The RGD domain is present only in HIV subtypes B and D [15], suggesting that dCA might more potently neutralize uptake of Tat from other HIV clades. To our knowledge, there is no study investigating the mechanism of entry of Tat in microglia cells or macrophages. A study done on monocyte cells indicated that Tat is up-taken through its basic domain [82], confirming our own results in differentiated U937 cells.

The nucleolus is involved in numerous cell functions including transcriptional regulation, cell cycle control, maturation of rRNA and apoptosis [83–86]. We showed that dCA potentially blocked Tat localization at the nucleolus in U87MG cells, similar to the pattern observed with Tat Mut, which is in agreement with results obtained in HeLa cells [58, 77] and in primary human fetal astrocyte cells [28]. This result confirms that dCA binds to the basic domain of Tat; however, exactly why Tat traffics through the nucleolus remains unclear [87].

Astrocytes are the most abundant cell type in the brain and are involved in homeostasis and regulation of neurons and maintenance of the BBB, thus any dysregulation of these cells affects neighboring cells. The apoptosis of astrocyte cells induced by HIV infection and Tat is very limited, which may be explained by the expression of the restrictive factor SAMDH1 [88], the constitutive expression of calcium/calmodulin-dependent protein kinase II [89], NF-E2-related Factor-2-dependent Genes [90] or the effect of the P2Y₆ Nucleotide Receptor [91]. Here we showed that Tat did not cause apoptosis of U87MG cells except at very high concentrations (>10 µg/ml), substantially higher than what is found in patients serum (0.1 ng to 40 ng/ml) [70, 71] or the concentration of Tat used by Eugenin *et al.* [21] that resulted in 20% apoptosis measured by TUNEL. The different sensitivity of the methods used, or the different buffer conditions, may explain the divergent results. Eugenin *et al.* [21] also showed that Tat triggered apoptosis by interaction of its core domain with the LRP receptor, which may explain why dCA had no apparent rescue effect at higher Tat concentrations when apoptosis was observed.

Astrocytosis is generally accepted to contribute significantly to HAND [72] either by releasing cytokines and chemokines, but also viral proteins such as Tat, leading to stimulation of the infected microglia cells and neuron death. Recombinant Tat, transfected Tat or pseudo-virus incubated with astrocyte cells cause an increased expression of different cytokines such as IL-1 β [12, 26] and TNF- α [26–31], but also chemokines such as MCP-1 [29, 32]. In our studies, upon transient transfection of Tat or Tat Mut in the human astrocytic cell line, U87MG, we confirmed Tat's ability to induce IL-1 β at different time points post-transfection. Tat's mediated up-regulation of TNF- α was low, which was already observed by Chen *et al.* [27].

Interestingly, at the highest concentration of Tat expression (48 h post transfection), we observed down-regulation of MCP-1, which is in opposition to previously published results. However, in a recent study adult astrocytes stimulated by IL-1 β presented a decrease of MCP-1 expression, but not in fetal astrocytic cells [92]. The decrease of MCP-1 expression in our experiment might be an indirect consequence of the IL-1 β up-regulation mediated by Tat [92]. Surprisingly, MCP-1 was also down-regulated by Tat mut. This suggests that the reverse effect of MCP-1 expression mediated by dCA may be the result of dCA binding to Tat, possibly with low affinity, to regions that cover more than just the basic domain.

The expression of IL-1 β and TNF- α was not up-regulated by Tat Mut, sustaining the importance of the basic domain in inducing the expression of these cytokines. Most importantly, treatment of cells with dCA tended to reverse IL-1 β , TNF- α and MCP-1 expressions back to control levels, suggesting the potential benefit of a Tat inhibitor, such as dCA, in therapeutic strategies for treating HAND.

Finally, using Tat transgenic mice we clearly demonstrate the ability of dCA to reverse Tat potentiation of cocaine place preference and reward. This result is extremely relevant since drug abusers constitute one of the fastest growing groups of HIV infected individuals and the co-administration cocaine and Tat additively damage neurons to a greater extent [3, 54, 55]. Numerous studies demonstrated that IL-1 β , and to a lesser extent TNF α , were greatly responsible for the sickness behavior which is defined by “the expression of adaptive reorganization of the properties of the host during an infectious episode triggered by pro-inflammatory cytokines” [93, 94]. Future studies will correlate the cytokines and chemokines expression profile in mice with the behavioral data.

In conclusion, an ideal drug candidate to target Tat, should be directed to either a conformational structure of Tat shared by many variants [10] or a conserved sequence/motif. It should neutralize the pleiotropic activities of Tat, be soluble, inexpensive, with low posology and able to penetrate in viral sanctuary sites, such as the brain. There are several candidate molecules that were reported during the last decade but none reached phase III clinical trials [11, 30, 95]. In this study, we demonstrated that dCA permeates the BBB, inhibits the five main HIV variants in the world, potently suppresses Tat induced astrocytosis and Tat induced cocaine potentiation, placing dCA as an ideal drug candidate. As such, dCA should be considered as promising therapeutic agent for the treatment of HAND.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ART	Antiretroviral therapy
RGD	Arginine-glycine-glutamine
Bio	Biotinylated
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
CPP	Conditional place preference
dCA	Didehydro-Cortistatin A
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
DMEM	Dulbecco's Modified Eagle Medium
E.V	Empty vector
e	Enhanced
GFP	Green fluorescent protein
HSPG	Heparin sulfate proteoglycan
HAND	HIV-associated neurological diseases
HRP	Horseradish peroxidase
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IL- 1β	Interleukin- 1 β
IL-6	Interleukin- 6
icv	Intracerebroventricular
ip	Intraperitoneal
LTR	Long terminal repeat
LRP	Low-density lipoprotein receptor-related protein

MCP1	Monocyte chemotactic protein 1
MTT	3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide
NMDA	N-methyl-D-aspartate
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate buffer saline
PGK	Phosphoglycerate kinase
PCR	Polymerase chain reaction
RLU	Relative light unit
sc	Subcutaneous
TAR	Trans-activation response
Tat	Transactivator of transcription
TNF-α	Tumor necrosis factor- α

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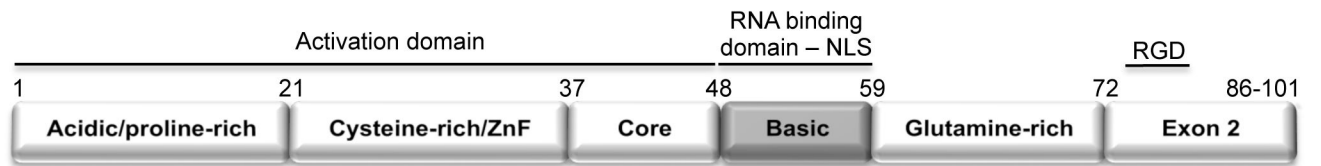


Figure 1.
Schematic representation of Tat protein's domains. NLS: nuclear localization signal; RGD: Arg-gly-Asp.

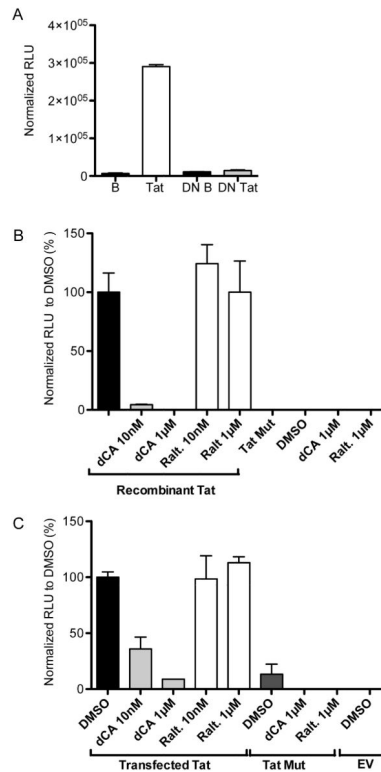
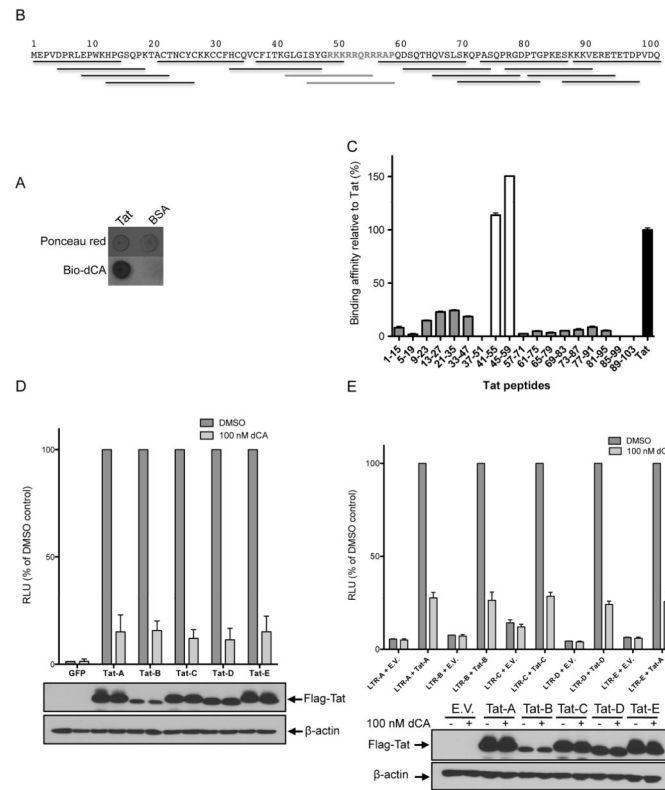


Figure 2.

dCA inhibits Tat induced HIV-1 LTR activation. (A) Tat activates the HIV-LTR. HeLa-CD4-LTR-Luc cells were treated with recombinant Tat protein and luciferase activity was measured 24 h later. Results are represented as relative light units (RLU, luciferase activity per protein concentration). DN: denatured; DN B: denatured buffer. (B, C) dCA inhibits Tat transactivation of the HIV-1 LTR. dCA prevents transactivation of the HIV-1 promoter from HeLa-CD4-LTR-Luc cells by recombinant Tat (B) or Transfected Tat (C). Raltegravir (Ralt.) was used as negative control. Tat mutated in the basic domain (Tat Mut), does not transactivate the promoter, and is used as negative control. Results obtained with Tat in the presence of DMSO were set as 100 %. EV: Empty vector control. Results are representative of three independent experiments.



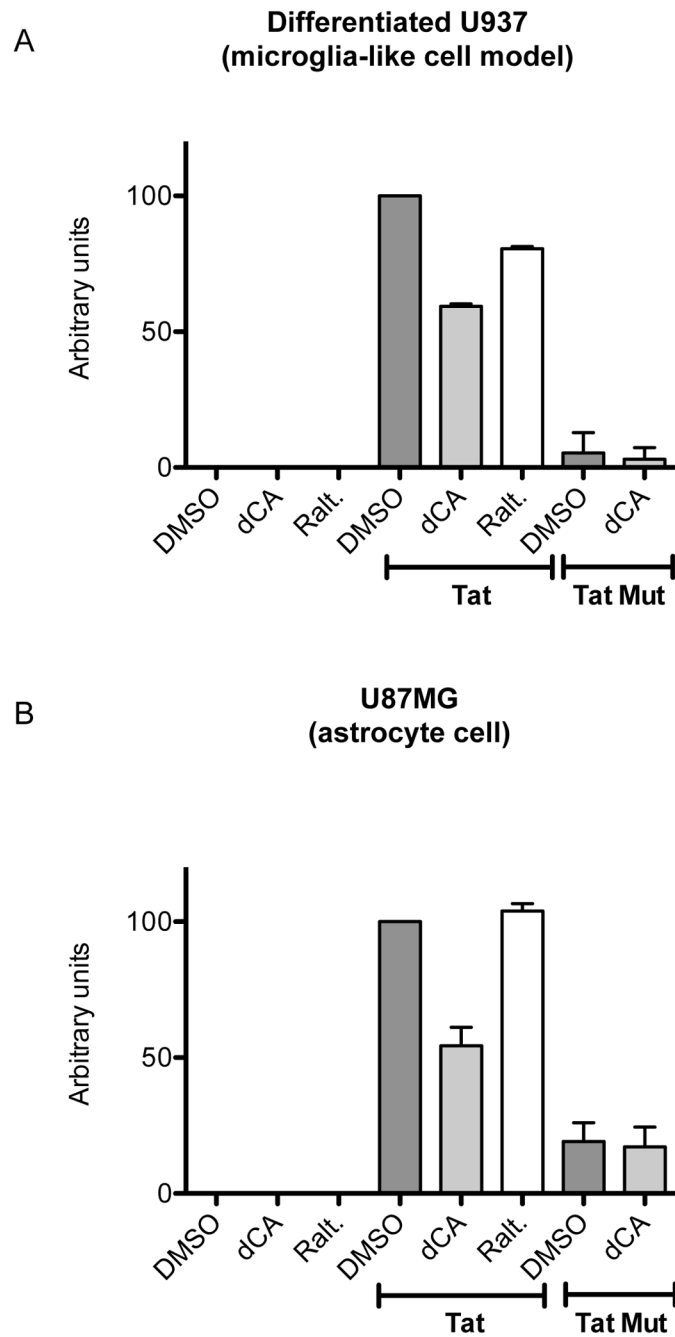


Figure 4.

dCA reduces the extracellular uptake of Tat. Recombinant Tat was added to the PMA differentiated monocyte cell line U937, used as microglia-like cells, and to the astrocyte cell line U87MG, in the presence of DMSO, dCA or Raltegravir (Ralt.). After 4 h incubation, cells were thoroughly washed and intracellular Tat detected by Western blot, with actin serving as loading control. Results are represented as the ratio of cellular up-taken Tat per total Tat input. Shown is the mean \pm SD between two independent experiments.

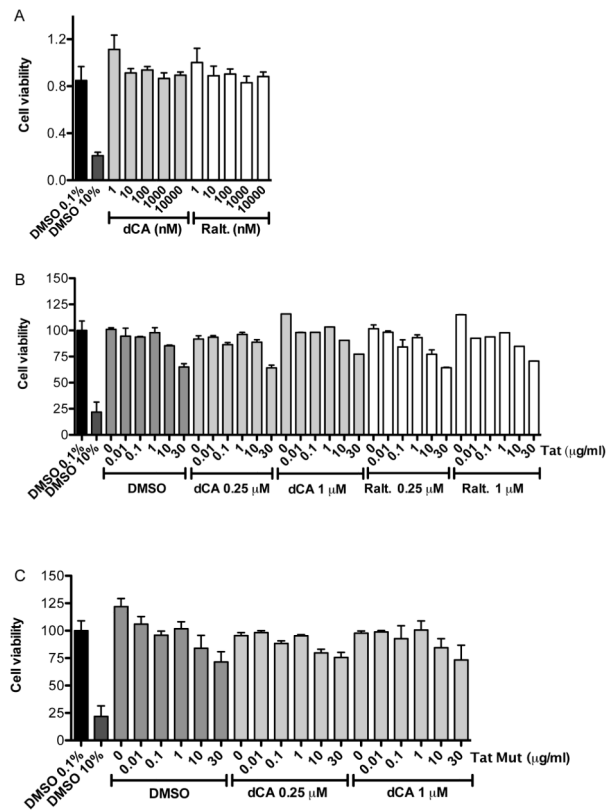


Figure 5. Effect of dCA and Tat on the cellular viability of the astrocytic cell line U87MG. A) MTT assay on U87MG cells incubated with increasing concentrations of dCA, Raltegravir (Ralt.) and DMSO for 48 h. B, C) MTT assay on U87MG cells incubated with dCA and Tat. Cells were incubated with Tat (B) or Tat mut (C) in presence of dCA or Ralt. at 250 nM or 1 μ M for 24 h. Raltegravir and DMSO (0.1%) were used as controls. DMSO 10% was used as positive control. Results are representative of three independent experiments.

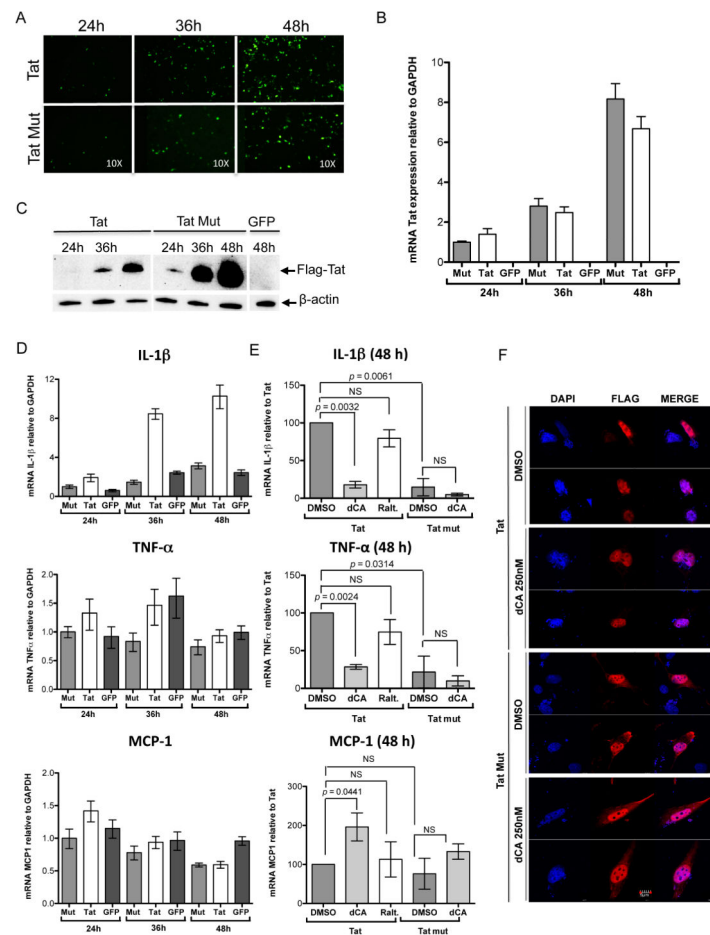


Figure 6. dCA reverses Tat-induced cytokines and chemokine dysregulation and alters Tat's nucleolar localization. (A) Flag-Tat or Flag-Tat Mut DNA were co-transfected with eGFP DNA into U87MG cells. Transfection efficiency was assessed by measuring GFP fluorescence at 24 h, 36 h and 48 h post-transfection. (B) Analysis of mRNA Tat and Tat Mut expression after transfection at different time points. Total RNA was extracted at each time point and converted to cDNA. Quantification was performed by RT-qPCR using primers recognizing Tat and Tat Mut. Results were normalized as copies of Tat per copy of GAPDH mRNAs. The arbitrary value of 1 was assigned to the amount of Tat Mut generated after 24 h. RNA samples not reverse transcribed were used as negative control. (C) Analysis of Tat and Tat Mut protein expression at different time points. Lysates from cells were subjected to western blot analysis. Anti-Flag antibody was used to reveal Tat and Tat Mut. GFP transfected in astrocyte cells and expressed for 48 h was used as negative control. Actin was used as loading control. (D) Analysis of IL1- β , TNF- α , and MCP-1 mRNA expression induced by Tat and Tat Mut transfection at different time points. Results were normalized to copies of GAPDH mRNA, with a value of 1 assigned to the Tat Mut 24 h. (E) Analysis of IL-1 β , TNF- α and MCP-1 mRNA induced by Tat and Tat Mut at 48 h post transfection upon dCA treatment. Results were normalized to copies of GAPDH and Tat (or Tat Mut) mRNA, with value of 1 assigned to the Tat alone. Raltegravir (Ralt.) was used as negative control. A two-

tailed paired *t*-test was used for statistical comparisons of the different conditions. NS: not significant. Shown is the mean \pm SD between three independent experiments. (F) dCA excludes Tat from the nucleolus U87MG cells. Confocal microscopy analysis of the sub-cellular localization of transfected Flag-Tat or Flag-Tat Mut after 24 h in presence of DMSO or dCA in astrocyte cells. Flag-Tat was recognized with anti-flag and Alexa Fluor 568 anti-IgG. Magnification 60X.

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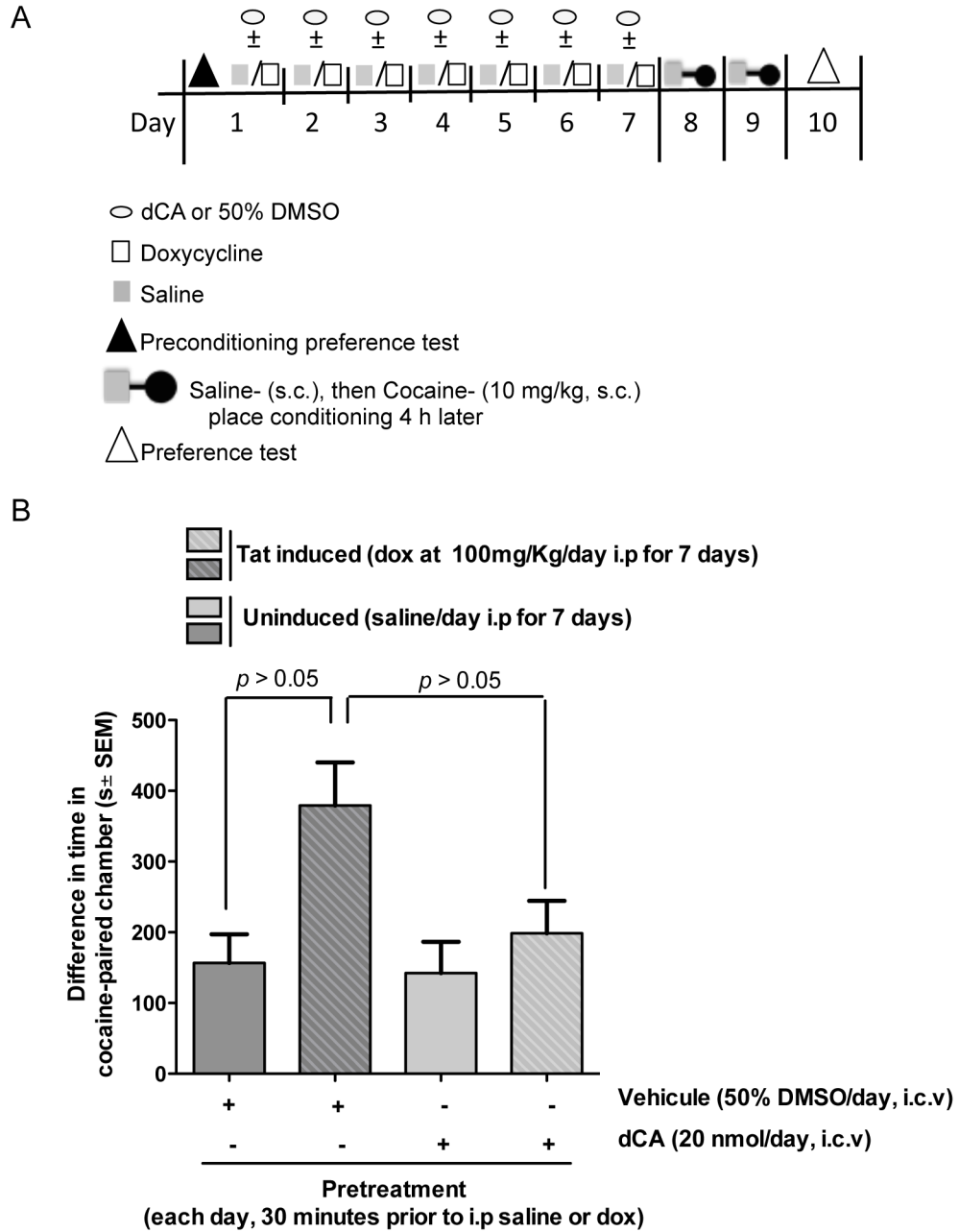


Figure 7. Tat potentiation of cocaine-conditioned place preference is reverted by dCA. (A) Experimental design for cocaine-conditioned place preference testing. GT-tg bigenic mice were treated daily for 7 days with the vehicle (50 % DMSO, i.c.v.) or dCA (20 nmol, i.c.v.) for 30 min before i.p injecting saline or Dox (100 mg/kg/d) to induce Tat expression. This was followed by 2 days of cocaine place-conditioning before the final conditioning place preference test. (B) dCA reverses the cocaine-conditioned place preference induced by Tat. All mice treated with dCA demonstrated cocaine CPP responses that did not significantly differ from vehicle-treated, uninduced mice ($p > 0.05$, Tukey’s post hoc test). However, dCA-

pretreatment (n=10) prevented Tat-induced potentiation of cocaine-CPP ($p<0.05$; Tukey's post hoc test). Bars = 16–20 mice

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Table 1

Pharmacokinetics of dCA assessed in C57Bl-6 mice. Drug levels were measured by LC-MS/MS. A sample formulation of 1 mg/mL dCA in water was prepared and dosed intraperitoneally (i.p.) via the tail vein twice daily for 10 days at either 2 mg/kg or 5 mg/kg. Values represent dCA levels in the plasma and brain of each mice 7 h after the last dosing on day 10.

	Plasma Concentration		Brain concentration	
	ng/mL	μM	ng/mL	μM
IP 2 mg/Kg of dCA, measurement 7 h after last dosing				
Mouse 1	40.3	0.09	95.3	0.2
Mouse 2	26.1	0.06	35.3	0.1
Mouse 3	38.9	0.08	67.5	0.1
Average	35.1	0.07	66.0	0.1
IP 5 mg/Kg of dCA, measurement 7 h after last dosing				
Mouse 4	96	0.20	434	0.9
Mouse 5	91.6	0.19	315	0.7
Mouse 6	107	0.23	235	0.5
Average	98.2	0.21	328	0.7