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Celastrol Inhibits Tat-mediated Human Immunodeficiency Virus (HIV) Transcription and Replication

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Abstract

Current drugs used for anti-retroviral therapy against HIV have a narrow spectrum of activity, and more often have associated toxicities and severe side effects in addition to developing resistance. Thus, there is a need to develop new therapeutic strategies against HIV/AIDS to complement the already existing ones. Surprisingly, Tat, an early virus encoded protein required for the efficient transcription of the HIV genome, has not been developed as a target for small molecular therapeutics. We have previously described the ability of an endogenous Michael acceptor electrophile (MAE), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), to inhibit Tat-dependent transcription, by targeting its cysteine (Cys) rich domain. In an effort to identify other MAEs possessing inhibitory activity against HIV-1 Tat, we tested a collection of plant-derived compounds with electrophilic properties, including curcumin, rosmarinic acid, and gambogic acid, for their ability to inhibit Tat. Celastrol (Cel), a triterpenoid MAE isolated from *T. wilfordii*, exhibited the highest inhibitory activity against Tat. Using biochemical techniques, we demonstrate that Cel by covalently modifying the cysteine thiols inhibits Tat transactivation function. Using circular dichroism (CD) spectroscopy, we show that alkylation of Tat brought about a change in the secondary structure of Tat, which inhibited the transcription elongation of the HIV proviral genome by effecting mechanisms other than Tat-TAR interaction. Our results demonstrate the underlying mechanism of anti-retroviral activity of the plant-derived MAEs, and suggest that Cel could serve as a lead compound to develop novel anti-viral therapeutics.

Keywords

Michael acceptors; natural products; Cys domain; interaction; alternative therapy; circular dichroism spectroscopy; structure; RNA polymerase II

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Introduction

Human immunodeficiency virus-1 (HIV) provirus transcription is controlled by the upstream LTR (long-terminal repeat), which includes *cis*-elements that bind both cellular and viral factors. Some cellular factors shown to be required for efficient HIV transcription include Sp-1, NF- κ B, C/EBP, HATs and Swi/Snf, to the LTR^{1; 2; 3; 4}, whereas the viral transactivating factor Tat is required for transcriptional elongation and HIV replication.^{5; 6; 7; 8} Tat binds the RNA stem loop structure formed by TAR (transactivation-responsive region)^{9; 10} and by recruiting P-TEFb to the LTR, enhances processive transcription¹¹. P-TEFb, which is composed of cyclin T1 and cyclin-dependent kinase 9 (Cdk9), modifies RNA polymerase II (RNAPII) activity by hyperphosphorylating the carboxy-terminal domain (CTD) of RNAPII as well as negative elongation factors, NELF and DSIF^{12; 13; 14; 15}. In the absence of Tat, transcription elongation by RNAPII from the HIV promoter is very inefficient leading to the accumulation of short transcripts^{16; 17}. The accumulation of short initiated transcripts and the identification of a site in this region, where purified RNAPII strongly pauses elongation, has led to the speculation that transcription of the integrated provirus is repressed by premature transcription termination^{6; 18; 19}. The activity of Tat substantially increases the production of elongated transcripts^{6; 16; 20}. Furthermore, Tat recruits chromatin remodeling machinery to assure efficient transcription^{1; 2; 21}. Since Tat is necessary for HIV-1 transcription and replication, regulation of its activity using a small molecule approach would be novel and complementary to current anti-retroviral treatments targeting reverse transcriptase, integrase, and protease.

Mutagenesis-based mapping studies has shown, four domains in Tat, N-terminal domain (residues 1–19), cysteine (Cys) rich domain (residues 20–39), core domain (residues 40–47), and basic domain (residues 48–56), are recognized to be essential for its transactivation function²². Of particular interest is the Cys rich domain in Tat, which is well conserved within the various strains of HIV-1²². Previous work from our and other laboratories has shown that the redox status of the Cys rich domain is a key determinant of the transactivation function of Tat^{22; 23; 24; 25}, where four out of the seven Cys residues were shown to form disulfide bonds in the active Tat molecule²³. Disrupting this Cys rich domain presents a novel approach to blocking Tat function and controlling HIV replication.

Several drugs have entered the market as anti-HIV agents. Most FDA approved drugs are synthetic and have associated toxicities and serious side effects such as rash, hypertension, fatigue, anemia, granulocytopenia, pancreatitis, and peripheral neuropathy^{26; 27; 28}. These adverse effects, coupled with the narrow specificity of the available drugs and drug resistance, have turned many towards natural sources to combat HIV/AIDS. Thus, the use of herbal medicine as a complementary and alternative medicine (CAM), in the form of antioxidant botanicals, has become prevalent among HIV-positive individuals worldwide^{29; 30; 31}. Furthermore, due to the fact that the plant kingdom has served as a source for many of our modern day therapeutic drugs, we selected five compounds, ferulic acid (FA), curcumin (Cur), rosmarinic acid (RA), gambogic acid (GA) and celastrol (Cel) (see Fig. 1), extracted from medicinal plants with prior history of anti-retroviral and anti-inflammatory properties^{32; 33; 34; 35; 36; 37}, to determine their anti-HIV potential. The compounds, termed “Michael acceptor electrophiles” (MAEs) by virtue of the presence of an α,β -unsaturated carbonyl functionality (enone), have the potential to form covalent Michael adducts by interacting with the cysteine sulfhydryls of certain proteins. Along these lines, our laboratory has recently demonstrated that 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), an arachidonic acid derived endogenous MAE, covalently interacted with the thiol groups in Tat to inhibit its transactivation function³⁸. Here we report the ability of some of the compounds of natural origin to inhibit the transactivation function of Tat and begin to

delineate the underlying mechanism. Our data suggest that such MAEs could be developed as anti-retroviral therapeutics for HIV/AIDS, which may serve to complement the already existing therapies.

Results

Michael acceptor electrophiles (MAEs) inhibit Tat-mediated HIV transcription

To examine the effect of the compounds on Tat-dependent HIV transcription, human promonocytic U937 cells were transfected with the HIV LTR-Luc reporter and Tat expression vector (pCI-Tat-1) or vector control (pCI), followed by treatment with the five MAE compounds shown in Fig. 1. It was observed that FA, Cur, GA, RA and Cel inhibited Tat-dependent transcription by ~ 50%, ~ 55%, ~ 40%, ~ 60%, and ~ 80% respectively (Fig. 2a). Analogs of Cur and GA, tetrahydro curcumin (tCur) and dihydro gambogic acid (DGA) (where the α,β -unsaturated ketone moiety is reduced; Fig. 1) were used as negative controls and did not inhibit Tat-dependent transcription (Fig. 2a). Viability of transfected U937 cells upon treatment with the compounds at all the concentrations tested was not affected (Fig. 2a). Because Cel had the largest impact on Tat-mediated transcription, we focused our studies on this compound. We used the highest concentration of Cel (0.15 μ M), which had no effect on cell viability in our assays, and that showed the most inhibition of Tat-mediated transcription (Fig. 2b and 2c).

Inhibition of HIV Tat-dependent transcription by Cel is NF κ B-independent

Given that Cel is known to inhibit the NF- κ B signaling pathway³⁹, and that NF- κ B activation positively affects HIV transcription^{40; 41}, we examined the contribution of NF- κ B inhibition in modulation of Tat function in our experiments. We used a LTR-Luc reporter that lacked NF- κ B sites (Δ κ BLTR-Luc) in a transient transfection in U937 cells as described earlier. As shown in Fig. 3, even in the absence of NF- κ B binding sites, there was a significant decrease (~80%), in Tat-dependent LTR activity suggesting that Cel inhibits HIV transcription via a NF- κ B-independent mechanism.

Interaction of Cel with rTat *in vitro*

To address the possibility that a direct interaction of Cel with the Cys residues in Tat affects its biological activity, we performed binding analyses with celastrol biotinamide (bCel; synthesized in the lab; Fig. 4a) and rTat. We bacterially expressed rTat. rTat was incubated with bCel at a molar ratio of 1:10 (rTat:bCel) in 50 mM Tris-Cl buffer, pH 7.5 for 3 h at 37°C. Samples were separated by PAGE followed by Western blot analysis with streptavidin-HRP. As shown in Fig. 4b, bCel covalently bound rTat. As a control, we incubated bCel with GSH prior to incubating it with rTat. GSH will form an adduct with bCel across the α,β -unsaturated moiety. The binding of such a GSH modified bCel to rTat was found to be much reduced. We used Cel to compete with bCel for rTat binding, which resulted in reduced biotinamide signal as observed by probing with streptavidin-HRP indicating specific binding. Using AMS (an iodoacetamide derivative) to alkylate the Cys-thiols in rTat prior to treating it with bCel, we showed that the binding of Cel to Tat occurs specifically with the Cys. Furthermore, using UV-Vis spectroscopy, we demonstrated that Cel binds to Tat. As in Fig. 4b, rTat (0.9 μ M) and Cel (9.0 μ M) were incubated with each other at a molar ratio of 1:10 at 37 °C. The reaction mixture was subjected to extraction with ethyl acetate five times to remove any unreacted Cel, and the resulting aqueous phase was used to perform UV/Vis spectroscopy. As a control, 9.0 μ M of Cel alone was incubated in buffer under the same conditions, and then extracted with ethyl acetate five times. This extracted all of the free (unbound) Cel from the solution, resulting in the sample showing no absorption in the visible range, where Cel displays a λ_{max} at 419 nm. As seen in Fig. 4c, rTat showed no absorption in the visible range; however, Cel-modified rTat exhibited a λ_{max} at

404 nm. The increase in absorbance of rTat-Cel complex (modified rTat) in the visible region, as compared to rTat alone, accompanied by a hypsochromic shift in the λ_{max} of the compound is suggestive of an interaction of Cel with rTat.

As a proof of principle, we incubated rTat and bCel in the presence of U937 cell lysates to determine if Cel can interact with Tat in the presence of cellular proteins. rTat (250 ng, 0.023 nmol) and bCel (173 ng, 0.23 nmol) were incubated with each other in a molar ratio of 1:10 in the presence of 100 μg of U937 cellular protein in a total volume of 100 μl . The mixture was subjected to pull-down by neutravidin-agarose beads. Immunoblotting clearly demonstrated that bCel could interact with Tat even in a cellular milieu (Fig. 4d).

Cel inhibits HIV transcription and replication

To determine if Cel inhibits HIV replication in infected U937 cells, 0.15 μM Cel was added exogenously to U937 cells infected with HXB.2 as described. The extent of HIV replication was measured by p24 ELISA every 24 h for 5 d. We observed that, treating infected U937 cells with Cel significantly decreased HIV replication when compared to vehicle treated cells even at day 5 (Fig. 5a).

We also analyzed Tat-mediated transcriptional elongation using SYBR Green quantitative PCR (qPCR) from virus infected U937 cells treated with Cel. Infected U937 cells were treated with 0.15 μM Cel for 5 d as before. After 5 d, the total RNA from cells was harvested with Trizol (Invitrogen) and elongated transcripts were examined by qPCR with prevalidated primers. We observed that Cel significantly inhibited Tat-mediated transcriptional elongation in infected cells (Fig. 5b).

Inhibition of Tat-dependent transcription may be due to structural changes in Tat

Cel is a MAE that would be expected to modify the cys rich domain of Tat promoting a conformational change inactivating Tat. To formally test this possibility we performed circular dichroism (CD) spectroscopic studies on free and modified rTat, to detect any changes in the structure of modified Tat when compared to the native form. rTat was incubated in buffer alone, or with Cel at different molar ratios (1:1, 1:5, 1:10, the concentration of rTat was kept constant at 50 $\mu\text{g/ml}$), for 3 h at 37°C, and subsequently the samples were subjected to CD spectroscopy. The CD activity of proteins in the far-UV region (220 – 260 nm) are due to the amide linkages in the protein backbone, and a change in this region signifies a shift in the conformation of the protein backbone. The bands in the near-UV region (260 – 350 nm) in the CD spectrum of proteins are due to aromatic amino acids and prosthetic groups, like the thiol-group in cysteines. As seen in Fig. 6a, a shift in the spectrum of Cel-modified Tat as compared to native Tat in the near-UV and far-UV regions was clearly evident. While the spectrum of native rTat was constant around zero in the near-UV region, it became negative in the far-UV region. When the molar concentrations of both rTat and Cel were equal, the spectra of native rTat and modified rTat were similar. As the concentration of Cel in the reaction was increased, the spectra of modified rTat became more positive in both the far-UV and near-UV regions (between 260 – 280 nm). At 10 times the molar concentration of Cel as compared to rTat, the spectrum of modified rTat became slightly more negative in the 320 – 350 nm range of the near-UV region. The spectra of the various concentrations of Cel, used as controls, were increasingly negative in the far-UV region, while they became increasingly positive in the 320 – 350 nm region of the near-UV region (data not shown). CD spectra in the visible range of native and Cel-modified rTat were not significantly different (Fig. 6b). The change in the far-UV region could be attributed to a change in the secondary structure of the Tat protein, while the modification of Cys thiols in Tat could explain the shift in the near-UV spectrum of modified Tat as compared to native Tat. On examining the ellipticity of rTat at 260 nm, we

found that it increased steadily as the concentration of Cel increased in the reaction mixture (Fig. 6c).

Modification of Tat by Cel does not affect its interaction with the HIV TAR element

To determine how Cel inhibits Tat function, we adapted a previously described binding assay to test the interaction of Cel-modified Tat with a 29 bp RNA ³²P-labeled oligonucleotide representing a mini HIV TAR element⁴². Incubation of mini TAR with immobilized Tat showed a ~ 3 fold increase in binding. Surprisingly, Cel-modified Tat also bound to mini TAR equally well suggesting that Cel does not affect the binding of Tat with the TAR RNA oligo in an *in vitro* system (Fig. 7).

Discussion

We have demonstrated that MAEs have the ability to inhibit Tat-mediated transactivation of HIV LTR activity. Our studies, for the first time, demonstrate that the inhibitory property of Cel towards Tat involves an intriguing mechanism of modifying Cys thiols in Tat towards Cel in a covalent reaction.

The binding of Tat to the TAR element is a key event that recruits the positive transcription elongation factor P-TEFb to the HIV promoter⁷. pTEFb causes the hyperphosphorylation of the CTD of RNAPII, which enhances the processivity of RNAPII and increases HIV proviral transcription. We used a reporter construct consisting of the LTR region of HIV coupled to luciferase gene to specifically address the role of MAEs on transcription and not other potential steps in the virus replication cycle. We cotransfected this construct with pCI-Tat-1 into U937 cells, then treated these cells with MAEs to determine if the compounds had any inhibitory activity towards Tat. Our studies demonstrated that Cel brought about the highest inhibitory activity against Tat among the MAEs tested. While the analogs of MAEs (lacking the α,β -unsaturated carbonyl functionality) had no inhibitory effect, all the MAEs inhibited the transactivation activity of Tat by varying degrees. This suggests that there is some level of specificity to the interaction of the compounds with Tat, despite containing the reactive electrophilic carbon.

As NF- κ B is a pivotal transcription factor in the formation of RNAPII initiation complex to effect HIV transcriptional initiation at the LTR and is known to also be a target of Cel³⁹, we decided to test if the observed inhibition of Tat-dependent transcription was indeed due to a reduction of Tat activity, or due to an inhibition of NF- κ B. Our data with LTR-Luc lacking the NF- κ B binding sites (Δ κBLTR-Luc; Fig. 3), suggests that the observed inhibition by Cel of HIV transcription is independent of NF- κ B inhibition.

Based on the data presented above and our previous studies with endogenous MAEs³⁸, albeit structurally unrelated to Cel, we hypothesized that Cel could interact covalently with the Cys thiols in Tat. To test this hypothesis, we incubated rTat and bCel together and analyzed the mixture via pull-down with neutravidin-agarose beads, and immunoblotting. We observed that Cel indeed covalently interacts with the Cys thiols in Tat and pretreatment of Tat with AMS (to alkylate Cys) completely abrogated the interaction of Tat with Cel. Our findings are consistent with the general notion that electrophiles alkylate key Cys residues in proteins^{43; 44; 45; 46; 47; 48; 49}. This may be due to the structural permissivity of different electrophiles and the accessibility of Cys thiols for such an interaction to complement a sterically feasible and thermodynamically stable complexation. Also, previous studies suggest that the binding of such electrophiles to Cys thiols in proteins depends on the context of the Cys residue to effectively ionize Cys-SH to a thiolate anion (Cys-S⁻) nucleophilic form⁴⁵. As a proof of principle, we tested the interaction of Cel with Tat in the presence of other cellular proteins. We used U937 cell lysates with rTat to show that bCel

could interact with Tat even in the presence of cellular proteins. This lends further credence to our hypothesis that Cel binds to Tat with some selectivity to inhibit its transactivation activity in cells. Pull-down studies with 0.15 μ M Cel added to cells transfected with pCI-Tat were unsuccessful perhaps owing to lack of sensitive methods to assess the interaction between the two in intact cells. Most importantly, we have demonstrated that Cel inhibits HIV replication in HXB.2 infected U937 cells. As mentioned earlier, Tat plays an important role in increasing the processivity of RNAPII to enable the production of elongated viral transcripts. In the absence of Tat, the formation of these elongated transcripts is reduced^{6; 17}. Treatment of infected cells with Cel inhibited the formation of elongated viral transcripts, as tested by SYBR Green qPCR. Based on the inability of rTat-Cel complex to drive the LTR-Luc expression, and the fact that Tat is a critical player in recruiting host transcription machinery to LTR, we believe that Tat, once covalently modified, is unable to transactivate even though the binding to the TAR element is not affected. Furthermore, CD analyses of the interaction of Cel and rTat confirmed changes in structure of rTat. The spectrum of native rTat is indicative of a random coil structure. A steady positive shift in the spectra of Cel-modified rTat might indicate that the protein structure is altered to a more “ordered” form, presumably a α -helix. We speculate that such a change in the structure was likely responsible for its impaired transactivational property.

We also investigated the effect that such a modification of Tat by Cel would have on its interaction with the HIV TAR RNA, to try and identify the step that is inhibited in viral transcription. Using an oligonucleotide binding assay, we observed that Cel modification did not affect the interaction of Tat with the TAR element. Thus, modification of Tat probably affects its interaction with host proteins such as pTEFb and RNAPII affecting its ability to recruit host transcription factors to the HIV promoter. Such studies are currently being performed in our laboratory and will be reported in the near future.

In summary, we have demonstrated the anti-retroviral property of Cel to mainly inhibit the transactivation property of Tat by covalently binding to the free Cys thiols in Tat. We have shown that Cel causes a change in the conformation of Tat thereby impairing its ability to transactivate viral transcription. We believe that our work will open a new avenue in developing anti-retroviral agents targeted towards the highly conserved Cys region of Tat, which will complement the existing therapies for HIV/AIDS.

Materials and Methods

Michael Acceptor Electrophiles

RA and Cel were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Cur and tCur were a kind gift from Dr. Bharat B. Aggarwal. GA and dihydro gambogic acid DGA was purchased from Gaia Chemical Corporation (CT, USA). FA was a kind gift from Dr. Devin Peterson, University of Minnesota. Celastrol-biotinamide (bCel) was synthesized in the laboratory by reacting celastrol with 5-(biotinamido)pentylamine (Pierce) in a molar ratio of 1:2.5, in the presence of 1-ethyl-3-(3-diaminomethylaminopropyl)carbodiimide hydrochloride (EDC) in MES buffer (100 mM, pH 5.5). The reaction was allowed to proceed for 4 hours, and extracted using ethyl acetate. This protocol was modified from a previously published one³⁸. bCel was purified by column chromatography on a silica gel column. bCel was subjected to LC-MS in our laboratory to confirm the modification. All compounds were tested for the presence of endotoxin and were found to be below the limits of detection using the QCL-1000 chromogenic LAL endpoint assay from Lonza (Basel, Switzerland).

Cell Culture

The human promonocytic U937 cell line (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 (Cellgro, Manassas, VA, USA) supplemented with 10 % defined fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (Cellgro, Manassas, VA, USA), 100 U/ml Penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA, USA). HEK 293T cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 4 mM L-glutamine, and 100 U/ml Penicillin and 100 µg/ml streptomycin.

Plasmids, transient transfections and luciferase assays

Mammalian HIV-1 Tat-1 expression vector, pCI-Tat-1, pHXBnPLAP-IRES-N+, VSV-G and RSV-Rev plasmids were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health. pCI vector (Promega, Madison, WI, USA) was used as an empty vector control. LTR reporter constructs, LTR-Luc and ΔκBLTR-Luc, representing the wild type –205LTR and ΔκB mutated –205LTR HIV-1 promoters, respectively, were prepared as described earlier⁵⁰. DNA for transfections was prepared using the endotoxin-free plasmid purification systems from Qiagen using the manufacturer's protocol. U937 cells were transfected using the *TransIT*-Jurkat transfection reagent from Mirus (Madison, WI, USA). Such cells were treated with DMSO or indicated concentrations of FA, Cur, tCur, GA, DGA, RA, and Cel. The final concentration of DMSO was kept constant at 0.1 %. Cell viability of treated cells was assessed using the CCK-8 cell counting kit from Dojindo (Gaithersburg, MD, USA). Luciferase assays were performed using a luciferase assay kit from Promega and a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA). The results were normalized to total protein in the lysates using the 660 nm protein assay reagent (Pierce, Rockford, IL, USA).

Expression, purification and refolding of rTat

Recombinant Tat (rTat) was expressed in *Escherichia coli* as a His-tagged protein using the methods previously described by Patki and Lederman⁵¹ and Kirsch *et al.*²⁴ with some modifications. Briefly, pTatC6H-1 construct from the NIH AIDS Research and Reference Reagent Program was used to transform BL21 (DE3) bacterial cells. The bacterial clone was grown in terrific broth (TB) and induced with IPTG (1 mM) overnight at 37 °C. The bacterial cell pellet was subjected to lysis in 6 M guanidine hydrochloride for 12 h. All steps were performed at 4 °C unless otherwise mentioned. The clarified cell lysates were subjected to metal affinity chromatography using Ni-NTA matrix (Novagen). The eluate was dialyzed against 0.1 N HCl for 2 d followed by an overnight lyophilization. The lyophilized powder was reconstituted in 6 M urea followed by reduction by tris(2-carboxyethyl)phosphine (TCEP, 25 mol/mole of rTat) in the dark for 8 h at room temperature. The mixture was subjected to sequential dialysis in phosphate buffer (0.1 M, pH 6.3) containing 4 M urea, 2 M urea, 1 M urea, and finally in buffer containing 200 mM NaCl. The dialysate was filter sterilized, purged with nitrogen, and stored in small aliquots at –80°C for further use. The rTat solution tested negative for the presence of endotoxin LPS using the QCL-1000 chromogenic LAL endpoint assay from Lonza (Basel, Switzerland). Using 5,5'-dithiobis-(2-nitrobenzoic acid; Ellman's reagent), the number of free Cys residues per mole of protein was deduced to be three in the rTat protein²³.

Binding studies

Recombinant Tat (0.9 µM) was incubated with bCel (9.0 µM) for 3 h at 37 °C in a reaction volume of 100 µl. The buffer used for the reaction was 50 mM Tris-Cl, pH 7.5. rTat was incubated with bCel in the presence of 9.0 µM of Cel, as a “cold” competitor. As negative controls, bCel treated with 90 µM reduced Glutathione (GSH, Calbiochem), at 37 °C and rTat treated with 9.0 µM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, disodium

salt (AMS, Invitrogen) were used. AMS is a thiol-reactive agent which forms charged adducts with proteins causing them to migrate slower when subjected to SDS PAGE. The samples were analyzed by SDS PAGE followed by Western blotting, and were probed with a polyclonal antibody to Tat (ab43014; abcam, Cambridge, MA, USA) and streptavidin-HRP (Pierce).

Recombinant Tat (0.9 μ M) was incubated with Cel (9.0 μ M) for 3 h at 37 °C. The reaction mixture was then extracted with ethyl acetate five times to remove unreacted celastrol. The aqueous phase containing the protein and bound Cel was then subjected to UV/Vis spectroscopy using a Beckman DU 7500 spectrophotometer. The spectrum was observed between 320 nm and 470 nm. Reaction mixtures containing buffer only and Cel only were subjected to the same extraction conditions as described above, and were used as controls.

Human U937 cell lysates was prepared using the mammalian protein extraction reagent (MPER, Pierce) supplemented with protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin. 100 μ g of U937 cell protein was incubated with or without 250 ng (0.023 nmol) of rTat in the presence or absence of 173 ng (0.23 nmol) bCel (1:10 molar ratio) in a reaction volume of 100 μ l. The mixtures were incubated on ice for 3 hours. These were then incubated with neutravidin-agarose beads overnight at 4 °C. Biotinylated proteins were separated by SDS-PAGE. Following Western transfer, the membrane was probed with anti-Tat antibodies.

Interaction studies of Tat with HIV-TAR

A 29 bp ssRNA oligonucleotide representing the HIV-TAR element was obtained from Dharmacon to determine its interaction with Cel-modified rTat. The sequence of the oligo was 5' GGCAGAU CUGAGCCUGGGAGCUCUCUGCC. 4 μ M of the oligo was labeled with 30 μ Ci of γ -³²P-ATP using 10 units of T4-polynucleotide kinase enzyme in a 30 μ l reaction. 100 μ g of rTat was incubated with 41 μ g of Cel (1:10 molar ratio) in 100 μ l of 50 mM Tris-Cl, pH 7.5, prepared in nuclease-free water. They were incubated together at 37 °C for 3 h. Buffer only, Cel only and native Tat were used as controls. The reaction mixtures were incubated with a HisPur Cobalt (Co²⁺) Resin (Thermo Scientific) for an additional 3 h on ice on an orbital shaker. The resin was washed 4 times with 50 mM Tris-Cl, pH 7.5 to remove unreacted Cel. The control and sample resins were incubated with labeled TAR (20000 cpm) for 1 h in 50 mM Tris-Cl, 20 mM potassium chloride, 0.1% triton X-100, pH 7.5 buffer (prepared in nuclease-free water) at room temperature. The resins were again washed with 50 mM Tris-Cl, pH 7.5, five times to remove unbound labeled TAR. The resins were boiled with 1X SDS gel loading dye and subject to scintillation counting on a Beckman LS6000IC.

Circular dichroism (CD) spectroscopy

Samples were prepared by incubating rTat (100 μ g, 60.60 μ M) alone or rTat and different concentrations of Cel (60.60 μ M, 303.00 μ M and 606.00 μ M) in 150 μ l of buffer (50 mM Tris-Cl, pH 7.5) at 37 °C for 3 hours. Buffer alone and Cel only at the above concentrations were used as controls. The volume was made up to 2 ml with buffer after incubation (final concentrations: rTat 4.55 μ M, and Cel 4.55 μ M, 22.75 μ M, or 45.50 μ M). These samples were subjected to CD spectroscopy using a Jasco Corp. spectrophotometer, J-810 with a cuvette (Hellma, USA) path length of 10 mm. The temperature was 25 °C. The spectrum was recorded between 220 nm and 450 nm at the rate of 2 sec per nm.

Generation of infectious HIV titers, infection of U937 cells with HIV-1, and p24 ELISA

Infectious viral stocks of the HXB.2 clone of HIV-1 were prepared by cotransfecting 293T cells with 3.6 μ g of pHBnPLAP-IRES-N+ plasmid (NIH AIDS Research and Reference

Reagent Program), 0.7 µg of vesicular stomatitis virus-glycoprotein (VSV-G) and 0.7 µg of RSV-Rev using the *TransIT*-293 transfection reagent (MirusBio, Madison, WI, USA). Supernatants were collected for 5 d post-transfection, filtered through 0.45 µm filters and combined. Transfection efficiency was measured by p24 ELISA (Bio Academia, Japan). The infectious titer was found to be 1.0×10^6 infectious units/ml. 10.0×10^6 U937 cells were incubated with 10 ml of undiluted viral stock for 24 h, after which the cells were cultured for 4 d in fresh media. As the HIV-1 clone being used is replication-competent, a progressive infection was established in the U937 cells. These cells were then treated with vehicle (DMSO, final concentration 0.1%) or Cel at 0.15 µM for 5 d. A fresh treatment of Cel was provided along with fresh media every day for the 3 d. Supernatants were collected by centrifugation at the end of the 3rd day and assayed for p24 protein by ELISA according to the manufacturer's instructions.

Quantitative PCR (qPCR)

U937 cells were infected with HIV and treated with Cel as above. At the end of the treatment, the cells were harvested in Trizol (Invitrogen), and total RNA was isolated. cDNA was prepared from 2 µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 5 ng of the cDNA were used in SYBR Green qPCR using the PerfeCTa SYBR Green SuperMix, ROX kit (Quanta Biosciences, Gaithersburg, MD, USA) with primers designed to detect elongated transcripts of HIV (~ 5 kb downstream of the LTR)¹⁹. Forward and reverse primers were: 5' ACTCGACAGAGGAGAGCAAG and 5' GAGTCTGACTGTTCTGATGA, respectively. PCR conditions used were: Initial denaturation at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 15 sec and annealing an extension at 60 °C for 1 min. Conditions for studying dissociation curves were: 95 °C for 15 sec, 60 °C for 30 sec, and 95 °C for 15 sec.

Statistical analysis

All data are represented as mean ± S.E.M. of at least three independent triplicate experiments. Statistical significance was analyzed by Student's *t*-test.

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Abbreviations

FA	ferulic acid
Cur	curcumin
tCur	tetrahydro curcumin
GA	gambogic acid
DGA	dihydro gambogic acid
RA	rosmarinic acid
Cel	celastrol
bCel	celastrol biotinamide
MAE	Michael acceptor electrophile

CD	circular dichroism
Tat	transactivator of transcription
P-TEFb	positive transcription elongation factor b
RNAPII	RNA polymerase II

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Fig. 1. Structures of different Michael Acceptor Electrophiles

Grey highlighting denotes the α,β -unsaturated enone. DGA and tCur are inactive analogs of GA and Cur, where the α,β -unsaturation has been reduced.

Fig. 2. MAEs inhibit Tat-dependent transcription

(a) U937 cells were transfected with -205LTR-Luc only or -205LTR-Luc and pCI-Tat or pCI vector. These cells were treated with the 3 μM of FA, Cur, tCur and RA, 0.25 μM of GA and DGA, 0.15 μM of Cel, or 0.1% DMSO as the vehicle control. Luciferase activities from the cell lysates were normalized to protein and compared to the activity of the vehicle treated cells, which were set at 100%. Viability of transfected cells when treated with compounds at the indicated concentrations was analyzed using the CCK-8 kit. All values are mean ± S.E.M. @; $p < 0.005$ *, $p < 0.00005$, #; $p < 0.000005$. (b) Dose dependent inhibition of Tat activity by Cel. *: $p < 0.00005$. (c) Viability of transfected U937 cells at the indicated concentrations of Cel.

Fig. 3. Cel inhibits Tat-dependent transcription independent of NF- κ B status

U937 cells were transfected with $\Delta\kappa$ BLTR-Luc only or $\Delta\kappa$ BLTR-Luc and pCI-Tat-1 or pCI vector. Cells were treated with 0.15 μ M Cel or DMSO (final concentration 0.1%) as the vehicle. Luciferase activities from the lysates of the cells were normalized to protein and compared to the activity of the vehicle treated cells, which was set at 100%. *; $p < 0.005$.

Fig. 4. Celestrol interacts with rTat

(a) Structure of celestrol-biotinamide (bCel). **(b)** 0.9 μ M of rTat was incubated with 9.0 μ M bCel (lane 2), both Cel (9.0 μ M) and bCel (lane 3), and bCel conjugated with 90.0 μ M GSH (lane 4). The Cys thiols in rTat were modified with 9.0 μ M AMS, and this rTat was incubated with bCel (lane 5). The mixtures were incubated at 37°C for 3 hours. Interaction was analyzed by SDS-PAGE followed by Western transfer and probing the membrane with streptavidin-HRP conjugate and anti-Tat antibodies. **(c)** UV/Vis analysis of the interaction of Cel and rTat. **(d)** Interaction of bCel and rTat in the presence of U937 cellular proteins (rTat:bCel = 1:10).

Fig. 5. Celastrol decreases viral replication

(a) U937 cells were infected with HXB.2 for 5 d followed by treatment with 0.15 μ M of celastrol for 72 h with a fresh dose every 24 h. Supernatants were collected every 24 h post treatment and were assayed for p24 levels by ELISA. @; $p < 0.05$, *; $p < 0.005$ **(b)** RNA isolated from the cells (after 5 d) were used for qPCR analysis of elongated viral transcripts. *; $p < 0.0005$.

Fig. 6. CD analysis of rTat and modified rTat

(a) Near-UV and far-UV CD spectra of rTat and rTat modified with various concentrations of Cel. (b) CD spectra in the visible range of the same samples. (c) Increase in the ellipticity of rTat at 260 nm when treated with increasing concentrations of Cel.

Fig. 7. Binding of HIV TAR RNA fragment to Cel-modified Tat

100 μ g of His-tagged Tat protein was incubated with 41 μ g of Cel (1:10 molar ratio) at 37 $^{\circ}$ C for 3 h, and bound to a cobalt resin. 32 P-labeled HIV TAR RNA oligonucleotide was then incubated with the resin bound modified Tat for 1 h at room temperature. The resin was then subjected to scintillation counting. Buffer incubated resin, later incubated with the labeled mini TAR oligonucleotide, was used as a control, and fold increases in cpm were determined. Native Tat was used as a positive control. N= 3, mean $\pm$ S.E.M. shown. * $p < 0.005$