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Catechins protect neurons against mitochondrial toxins and HIV proteins via activation of the BDNF pathway

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Abstract

Currently, there is no effective treatment for neurological complications of infection with the human immunodeficiency virus that persists despite the use of combination antiretroviral therapy. A medium throughput assay was developed for screening neuroprotective compounds using primary mixed neuronal cells and mitochondrial toxin 3-nitropropionic acid. Using this assay, a library of 2000 compounds was screened. Out of 256 compounds that showed variable degrees of neuroprotection, 9 were related to epicatechin, a monomeric flavonoid found in cocoa and green tea leaves that readily crosses the blood brain barrier. Hence, catechin, epicatechin and the related compound, epigallocatechin gallate (EGCG) were further screened for their neuroprotective properties against HIV proteins Tat and gp120, and compared to those of resveratrol. Epicatechin and EGCG targets the brain-derived neurotrophic factor (BDNF) and its precursor proBDNF signaling pathways, normalizing both Tat-mediated increases in proapoptotic proBDNF and concomitant Tat-mediated decreases in the mature BDNF protein in hippocampal neurons. Epicatechin and epigallocatechin gallate were more potent than catechin or resveratrol as neuroprotectants. Due to its simpler structure and more efficient blood brain barrier penetration properties, epicatechin might be the best therapeutic candidate for neurodegenerative diseases including HIV associated neurocognitive disorders where oxidative stress is an important pathophysiological mechanism.

Introduction

Infection with the human immunodeficiency virus (HIV) can result in severe complications affecting the nervous system, such as HIV sensory distal neuropathy (HIV-SN) and HIV-associated neurocognitive disorders (HAND). These complications occur and persist despite the advent and implementation of combined active antiretroviral therapy (CART)[1]. As effective CART allows HIV-infected patients to live longer, a growing proportion of patients experience some form of neuropathy and cognitive deficits. These complications are a result of oxidative stress-induced neurotoxicity that results directly from HIV infection in tissue

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reservoirs, where effective levels of antiretroviral drugs may be hard to achieve and low level HIV replication may continue. The coat protein of HIV, gp120, can cause neurotoxicity by multiple mechanisms. Exposure of neurons and glial cells to toxic viral proteins such as Tat, which can be produced by HIV-infected cells despite the use of CART, can set up positive feedback loops resulting in wide-spread neuronal dysfunction. Oxidative stress is the final common pathway for the neurotoxicity induced by both viral proteins [2]. Development of therapeutic strategies to protect against this neuronal damage and the ensuing clinical deficits remains a major unmet medical need. Neuroprotective compounds, if they can be identified and developed preclinically, may provide a treatment for HIV-SN and HAND.

One approach to identify potential neuroprotective agents would be to develop a medium throughput screening assay to assess the protective efficacy of a wide array of diverse chemical compounds. We have developed assays that utilize mixed primary cultures of neurons and glial cells to replicate the cellular conditions in situ. These cultures have been adapted to microtiter plates and exposed to oxidative stressors, such as 3-nitropropionic acid (3-NP), to mimic oxidative damage which results from HIV infection of these tissues. Confirmatory assays are then performed using HIV toxic proteins gp120 and Tat. Thus, we screened a library of 2000 compounds comprised of natural substances, FDA-approved drugs and compounds with prior human exposure (Spectrum Collection, Microsource Discovery). We identified epicatechin, epigallocatechin gallate (EGCG) and other catechin flavonoids as potential neuroprotective agents. These compounds are found in high concentrations in green tea leaves, purple grapes [3] and in seeds of the cacao tree which is used to make chocolate. While these compounds have reported antioxidant activities, these compounds haven’t been evaluated against mitochondrial toxins, or HIV toxic proteins.

Materials and methods

Cell culture

Neuronal cultures from rodent cerebral cortex and from hippocampus were prepared from embryonic day 18 Sprague–Dawley rats using methods similar to those described previously [4]. Tissues were dissociated by gentle trituration with a firepolished glass pipette in calcium-free Hank’s balanced salt solution. The single cell suspension was centrifuged at 1000 g and re-suspended in minimal essential medium containing 10% heat-inactivated fetal bovine serum and 1% (v/v) antibiotic and antimycotic solution (penicillin G 10^4 units/mL, streptomycin 10 mg/mL and amphotericin B 25 mg/mL; Sigma, St Louis, MO, USA). Cells were allowed to attach for 3 hours before the media was replaced with serum-free neurobasal medium containing 2% (v/v) B-27 supplement (Gibco, Rockville, MD, USA) and 1% (v/v) antibiotic and antimycotic mix (Sigma). Rat mixed hippocampal neurons were generated from freshly cultured rat hippocampi in neurobasal media containing 5% (v/v) fetal bovine serum and 2% (v/v) B27 supplement. Hippocampal neurons were plated in 96-well plates at a density of 4×10^5 cells per milliliter for neurotoxicity studies.

Human neuronal cultures were prepared as described previously [5]. Briefly, the cells were mechanically dissociated, suspended in Opti-MEM with 5% (v/v) heat-inactivated fetal bovine serum, 0.2% (v/v) N2 supplement (Gibco) and 1% (v/v) antibiotic and antimycotic

solution and plated in flat-bottomed 96-well plates. The cells were maintained in culture for at least 1 month before use. Mixed neuronal cultures were grown in low serum conditions with N-2 supplement to encourage neuronal growth and inhibit glial proliferation. These cultures contain 70% neurons, 30% astrocytes and <1% microglia. These cultures can be maintained for several months. Previous experiments in our laboratory and those of others have shown that prolonged cultures of neurons ensures the expression of excitatory amino acid receptors and growth of processes [5, 6]. The original screening assays were done with 3–4 replicates per plate, including an internal positive control of 10 uM resveratrol as a neuroprotectant.

Neurons were plated on polyethyleneimine coated 15-mm diameter glass coverslips at a density of 2×10^5 cells/mL for calcium imaging and in 35-mm diameter plastic plates at a density of 4×10^5 cells/mL for neurotoxicity studies.

Cell survival assay

Neuronal cell viability was assessed with 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay. This assay [7] is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals. These crystals are largely impermeable to cell membranes, and thus, accumulate within healthy cells. The resultant formazan precipitates are solubilized with dimethyl sulfoxide (DMSO) and read on a multiwell scanning spectrophotometer (SpectraMAX M5e, Molecular Devices). The number of surviving cells is directly proportional to the level of the formazan product created.

Compound screening

The Spectrum Collection of compounds by Microsource Discovery contains 2000 compounds, of which about half are FDA approved drugs and the remaining compounds are natural products or other compounds with some prior human exposure and safety testing data. The compound collection is dispensed and maintained on 96 well plates at a concentration of 10 mM in 100% DMSO and stored at −80°C. The compound mother plates were thawed one time in order to make 4 sets of daughter plates, and one set of daughter plates was used for these screening assays.

Toxicity assays

Rat mixed hippocampal cultures were treated with test compounds for 1 hour at 37°C prior to addition of 3-NP, Tat or gp120. Cells were then exposed to these toxins for 18 hours, after which time cell viability was assessed by MTT assay [7]. For Tat toxicity assays, purified recombinant Tat 1–72 was utilized at 500 nM final concentration. After 18 hours of exposure to Tat, the MTT assay was utilized to assess cytotoxicity. Hydrogen peroxide neurotoxicity was achieved with 2 hour exposure to 100 μM H_2O_2, which is applied to the cultures following a 1 hour preincubation with the neuroprotectant. Cell viability was quantified by MTT assay. Moderate throughput functional screening assays were done in triplicate or quadruplicate wells, while confirmatory dose response assays were done with N=8 per treatment group and replicated three times.

Fluorescence Axodendritic Degeneration assays

Rat mixed hippocampal or cortical neurons were transfected with a plasmid containing the neuron-specific CaMKII promoter, with a fusion construct of tau protein and td-Tomato genes or βIII tubulin-tdTomato genes (Nucleofector 2S, Lonza, MD), according to the manufacturer's instructions. Transfection efficiency was about 60%. After 7–10 days in culture, cells were utilized in neurotoxicity assays to assess the effects of catechins on Tat-mediated axodendritic degeneration. Following catechin treatment and Tat exposure, images were captured via live imaging on a Zeiss AxioObserver Z1 inverted microscope and the relative fluorescence of Tau-tdTomato was determined with a SpectraMax M5e multimode plate reader (Molecular Devices, CA).

HIV-1 proteins and reagents

The preparation of recombinant $Tat_{1–72}$ protein has been described previously [8]. Tat protein varies in length from 86 to 101 amino acids and is made from two exons. The first exon form the first 72 amino acids and the neurotoxic epitope resides in this region [6], hence we have utilized recombinant Tat $_{1–72}$ for these studies. The toxic effects of $Tat_{1–72}$ can be blocked by anti-Tat Ig and likewise $Tat_{\Delta 31–61}$ has been used as a control [9]. Gp120 derived from HIV_{SF} was acquired from the NIH-AIDS repository (Bethesda, MD, USA).

Statistical analysis

All data are represented as mean +/− SEM and analyzed by one-way analysis of variance (ANOVA). Group-wise post hoc comparisons were assessed by Newman-Keuls multiple comparison tests, with statistical significance at $p<0.05$.

Results

Development of a neurotoxicity assay for screening neuroprotectants

To develop a reliable and reproducible neurotoxicity assay, we used primary neuronal cultures derived from rat hippocampi. We kept the species and age of the animal, the anatomical region from which neurons were obtained and the culture conditions including days in culture constant for all experiments. 3-nitropropionic acid (3-NP) was chosen as a toxin since it is stable and is a well-characterized mitochondrial toxin that causes oxidative stress. We found that the compound also has a large dynamic range and its toxic properties in neurons were highly reproducible. For these reasons, we were able to adapt the neurotoxicity assay to microtiter plates, which allowed the manual screening of a large number of compounds. Based on the dose response, we chose 3 mM 3-NP for all subsequent experiments since it provided 20–35% toxicity (Figure 1). Our intra-assay variability was 8–10%.

Neuroprotection by the catechin class of compounds

The Spectrum library of compounds contains 2000 FDA approved drugs and natural substances. We screened the entire library at a single concentration of 10 μM of each compound. We chose 10 μM as a reasonable in vitro screening dose because it may be a maximal level attained in the CNS following systemic administration. We observed that

256 compounds showed >50% protection. Of these, 146 showed >100% protection and 53 showed >200% protection (See Fig 2). The library contained 13 compounds that were derived from or related to epicatechin. Of these, one was toxic, three had no effect and the remaining nine showed variable amounts of neuroprotection (See Table 1). Of these compounds we chose epicatechin, (+) catechin and epigallocatechin gallate for further experiments since they showed the most neuroprotection and had the simplest structure. Their structure also shows similarity to that of resveratrol, in that all of them have aromatic phenolic substituents. They have the basic structure of two phenolic rings linked by three carbons that form an oxygenated heterocycle (Fig 3). The catechins may exist in *cis* ((−) epicatechin; Fig 3 A) or *trans* ((+) catechin; Fig 3B) configuration. The subfamilies of catechins (Table 1) are formed by linkage of various groups to the heterocycle, such as gallic acid (Fig 3D). Resveratrol is a known neuroprotective agent [10], hence it was used as a positive control in subsequent experiments. In addition to its use as a positive control for our experiments, resveratrol was also part of the Spectrum library of compounds and showed about 40% neuroprotection in our screening assay.

Dose response curves of neuroprotection by epicatechin and catechin

Using the 3-NP neurotoxicity assay, we found that epicatechin and (+) catechin protected in a dose-responsive manner. Each of these compounds had a linear dose-response. They showed nearly complete neuroprotection at 20 μM concentration. Resveratrol was used as a positive control which showed similar neuroprotective properties, and required about 20 μM concentrations to demonstrate greater than 50% protection. It is interesting that the EGCG compound was more active than the other three, with near complete protection at about 10 μM (Figure 4).

Epicatechin and catechin protect against HIV proteins

To determine if epicatechin can protect against the neurotoxic effect of HIV proteins, we exposed neurons to HIV proteins Tat and gp120 in the presence of epicatechin. We found that epicatechin protected in a concentration dependent manner against Tat and gp120 with significant effects at a dosage of 10 μM or above (Figure 5A and B). In comparison, resveratrol showed protection against Tat protein at a dosage of 2.5 μM or above (Figure 5C). EGCG was the most potent catechin compound, which exhibited nearly complete protection from Tat neurotoxicity at concentrations of 2 μM or greater (Figure 5C).

Neuroprotective properties against hydrogen peroxide induced toxicity

Minimal protection was seen with each of the compounds against H_2O_2 induced neurotoxicity with significant protection only at 20uM concentration for epicatechin, catechin and resveratrol. EGCG was protective at lower concentrations (Figure 6). We also discovered that the neuroprotective properties of epicatechin were light sensitive, and that exposure to ambient light at atmospheric oxygen for more than 1–2 hours resulted in loss of its neuroprotective properties (data not shown). Hence all assays were performed with freshly prepared solutions of these catechins compounds and additional precautions were taken using light barriers. Collectively it suggests that likely these compounds are easily oxidized resulting in loss of activity.

Protection from Tat-mediated neuritic damage by catechins

In order to determine whether catechins can protect against the neurotoxic effect of HIV proteins on neuronal processes as well as cell bodies, we exposed fluorescently-labeled neurons to HIV proteins Tat in the presence of epicatechin or EGCG (Figure 7). We found that epicatechin protected in a concentration dependent manner against Tat with significant effects at a dosage about 10 μM (Figure 7A). In comparison, EGCG was the more potent catechin compound, which exhibited nearly complete protection from Tat neurotoxicity at concentrations of 1 μM or greater (Figure 7B). The exposure of cortical neurons to HIV Tat resulted in fewer labeled neurons per field, as well as a simplified neuronal axodendritic profile (Figure 7C). Similarly, mixed hippocampal neurons labeled by transfection with βIII tubulin-td Tomato displayed significant losses of neuritic processes following exposure to Tat (Figure 8). The catechins protected the neurites from Tat mediated damage at 1–10 μM. The effect of epicatechin and EGCG treatment was observed to be protection of neurons, as well as the neuritic processes from the neurotoxic effects of Tat.

Potential neuroprotective mechanism(s) of catechin-mediated protection from HIV

In order to gain some insight into the neuroprotective mechanism of the catechins, neuronal cultures were treated with vehicle, Tat, Tat plus epicatechin (1 μM) or Tat plus EGCG (1 μM) for 24 hours (Figure 9). Cell lysates of the treated neurons were probed with antibodies to numerous proteins, such as inducible nitric oxide synthase (iNOS), IL-6 as well as potential neurotrophic proteins, such as NGF, BDNF and GDNF. HIV Tat treatment resulted in nearly a 2 fold increases in iNOS and IL-6, but only the Tat-mediated increase in iNOS was attenuated by the catechins (Figure 9). Tat exposure resulted in a decrease in the neurotrophic BDNF, but concomitantly stimulated an increase in the pro-apoptotic proBDNF. The catechins, utilized at neuroprotective 1 μM concentration, increased neurotrophic BDNF levels to nearly 50% above control media/DMSO vehicle treated levels, while decreasing the proapoptotic proBDNF to more than 50% less than control levels. The effects of catechins on the BDNF proteins were specific, since no effect of these compounds was evident on NGF or GDNF proteins (data not shown). These data suggest that the neuroprotective effects of catechins may result by modulating the BDNF proteins and their signaling pathways.

Discussion

High throughput assays have been the mainstay of pharmaceutical companies for drug discoveries. Recently, the National Institutes of Health and other academic institutions have put in major effort towards the development of such assays for drug discovery that would particularly target rare disorders or other diseases that pharmaceutical companies have traditionally ignored. The neurological disorders associated with HIV infection fall into this category. Most throughput assays are either enzyme based or binding assays. However, cellular functions are very complex and involve multiple pathways, hence we developed a moderate throughput assay using primary mixed neuronal cultures and used rigorous experimental conditions to minimize variability and maximize reproducibility. We used 3-NP for causing neurotoxicity since its acts on the mitochondria and mitochondrial dysfunction have been implicated in most neurodegenerative diseases. Further, 3-NP is

a stable compound with a wide and reproducible dose curve. Using this assay, we have identified several groups of compounds that have variable neuroprotective properties. We focused our initial efforts on catechins since nine related compounds had neuroprotective properties and have been shown to cross the blood brain barrier [11]. Further these compounds are natural substances present in green tea and other plants although the gallate group is predominant in green tea [12, 13] Dark chocolate from the plant Theobroma cocao is also a rich source of epicatechin [14]. Hence, humans have been regularly exposed to these compounds in food products for hundreds or thousands of years.

Several studies have investigated the neuroprotective properties of epigallocatechin gallate (reviewed in [15, 16]), the main catechin polyphenol constituent in green tea, and found that it protects neurons against 3-NP [17] amyloid beta peptide [18], traumatic injury [19], cerebral ischemia [20], cisplatin-induced neurotoxicity [21] and rotenone-induced neurotoxicity [22]. However, one study failed to show any neuroprotection against 6-hydroxydopamine [23]. Although the exact mechanism of action of EGCG remains unknown, prevention of generation of reactive oxygen species and nitric oxide synthesis has been implicated [24]. These compounds have also been shown to inhibit ERK and NF-κB pathways [13, 21]. It can also cause proliferation of neural progenitor cells [25]. Our data in this study implicates the BDNF pathway in the neuroprotective actions of the catechins. The proapoptotic proBDNF [26] induces neuronal apoptosis via activation of a receptor complex of $p75^{NTR}$ and sortilin. proBDNF is converted to the mature and neuroprotective BDNF by extracellular proteases [27]. Perhaps exposure of neurons to Tat caused increases in proBDNF (along with decreases in BDNF) by blocking the proteolytic processing of the proBDNF, which was reversible by catechin treatment. Interestingly, gp120 treatment also caused decreased BDNF levels in vitro and in vivo after 24 hours of exposure [28], preceding caspase-3 activation and neuronal cell death. It is possible that catechin mediated protection from gp120 (Figure 5) may also utilize this mechanism. Recent studies in the CSF of HIV-infected patients demonstrate a strong correlation with increasing severity of HIV-mediated neurologic disease and a decline in growth factors, particularly BDNF and NT-3 [29]. Thus, a potential mechanism of catechin-mediated neuroprotection which promoted BDNF availability and signaling may be an effective strategy against HIV-mediated neurotoxicity and HAND. While the potential role of BDNF as a neurotherapeutic agent has long been considered [30], its use has been limited due to the need to directly administer it to the brain as a recombinant protein or via viral vectors [31]. Catechins thus represent a novel set of compounds by their ability to act via the BDNF pathway and may thus open a new area in neurotheraputics research.

Importantly, epigallocatechin has been shown to be neuroprotective following oral administration in an animal model of acute neurotoxicity [32] and aging related oxidative injury in the brain [33]. Other compounds related to the catechins have not been well studied. We found that epigallocatechin gallate was only moderately neuroprotective against 3-NP neurotoxicity. Catechin, epicatechin and epicatechin monogallate were much more potent than epigallocatechin in our screening assay. Of these compounds epicatechin had the best potency against 3-NP induced neurotoxicity although they seemed to be similarly protective against HIV proteins with apparent EC_{50} of 1 μM compared to an EC_{50} of 10 μM for 3-NP neurotoxicity. Epicatechin gallate has been shown to have cytoprotective

effects in a glial cell line at much lower concentrations of 0.1 to 1 μM [34]. However, catechin and epicatechin have a smaller molecular mass and are efficient in crossing the blood brain barrier [11]. Catechin has also been shown to improve blood flow by causing cerebral vasodilatation [35]. The bioavailability of the compounds improves with repeated dosing. In one study, catechin and epicatechin were undetectable in the brain of rodents after administration of a single dose, however, after daily administration for 10 days, it reached 576.7 +/− 227.7 and 290.7 +/−45.9 pg/g of brain tissue respectively [36]. Defining the therapeutic window of these compounds will be critical since at very high dosages, they may have proapoptotic properties and can cause neurotoxicity [37]. Our observations also suggested a similar trend. We found that resveratrol was also neuroprotective against HIV-Tat protein induced neurotoxicity however, due to its hydrophilic properties, it does not cross the blood brain barrier as efficiently.

Oxidative and nitrosative stress play an important role in mediating the neuropathogenesis of HIV infection and protein carbonyls, nitrosylated and hydroxynonenol-modified proteins can be found in brain and CSF of these patients, where they correlate with the severity of neurocognitive impairment [38, 39]. HIV proteins Tat and gp120 have been implicated in causing excitotoxic neurotoxicity by directly acting on neurons or via indirect mechanisms leading to activation of macrophages and glial cells. Oxidative stress is the final common pathway in all these mechanisms [2]. Currently available antiretroviral medications are unable to control complete viral replication in the brain and they do not have any effect of Tat production once proviral DNA has been formed. In this study, we found that the catechins were able to block the neurotoxic effects of the HIV proteins and thus may be of therapeutic potential in HAND. However, conducting clinical trials in this patient population carries many challenges and to date all trials with potential neurotherapeutic agents have failed in phase 2 studies. The availability of good surrogate markers is critical for monitoring such clinical trials so that early decisions on the continuation of these trials can be made. Thus, measurement of oxidized or nitrosylated proteins in CSF represents an excellent marker for monitoring clinical trials [40]. It is important to note that even though catechins have been shown to be protective in a variety of models of neurodegenerative diseases, to date no clinical trials have been conducted with these compounds. This is in part because monitoring clinical end points in these diseases requires large sample sizes and monitoring of patients for long periods of time. CSF is not routinely obtained from these patients for either diagnostic purposes or for monitoring clinical progression. In patients with HAND, CSF monitoring of viral load and inflammatory markers is common practice, hence this population might represent an excellent opportunity for monitoring the therapeutic efficacy of these compounds.

In conclusion, we have developed a novel screening assay for identification of neuroprotective compounds and identified the family of catechins as a class of compounds with potent neuroprotective properties. Of these compounds, epicatechin is the most promising therapeutic candidate because of its high potency, simple structure, excellent bioavailability following oral administration and efficient ability to cross the blood brain barrier.

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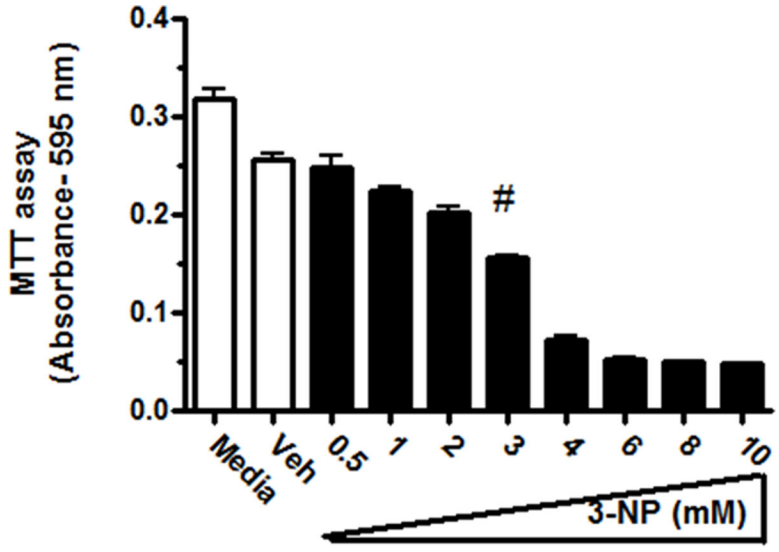

Figure 1. Titration of 3-NP toxicity in rat mixed hippocampal neurons and assay validation

Rat hippocampal cultures were exposed to various concentrations of 3-nitropropionic acid (3-NP) for 18 hours and cell viability was determined with an MTT endpoint (See Methods for assay details). An average toxicity of between 20–35% was observed with 3 mM 3-NP exposure; hence this concentration was used for subsequent assays.

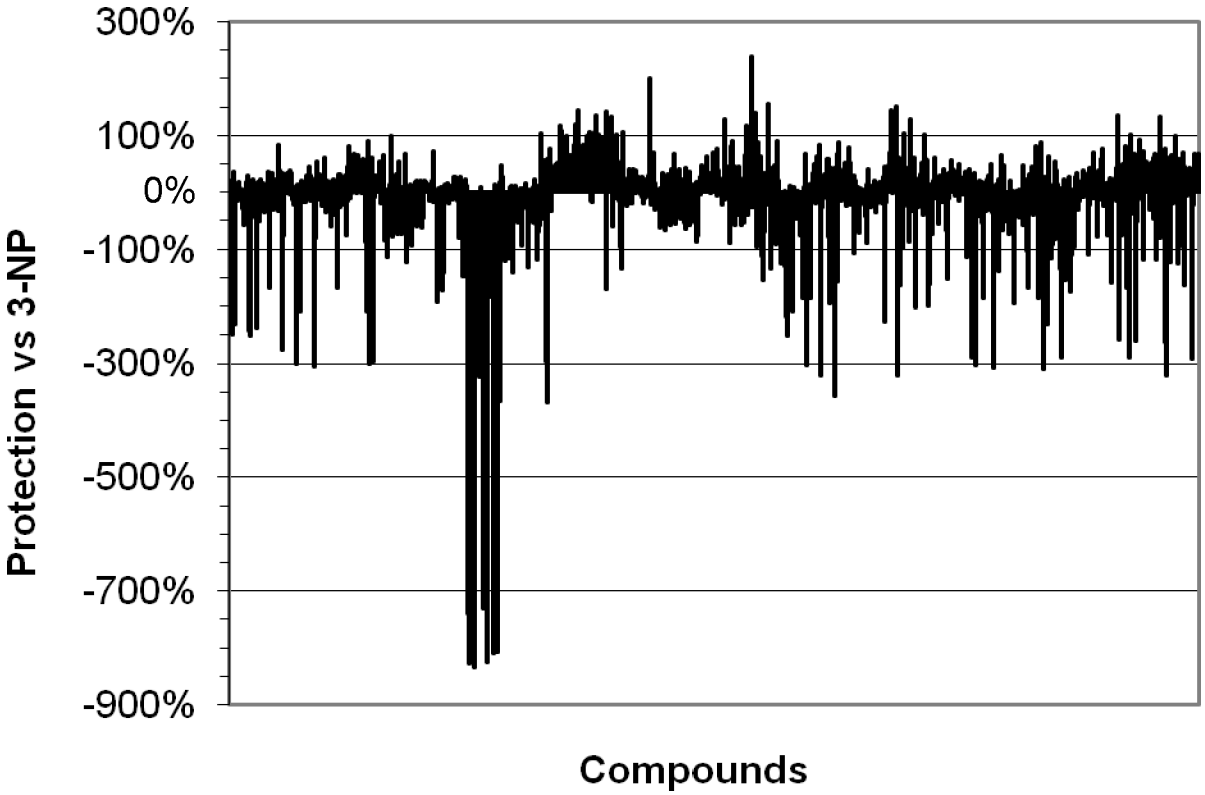

Figure 2. Screening of the Spectrum collection with the 3-NP neurotoxicity assay

A total of 2000 compounds were screened using the 3-NP Neurotoxicity assay in rat mixed hippocampal cultures, with screening at 10uM drug concentration against the toxic effects of 3mM 3-NP. From these screening assays, 256 compounds showed >50% protection. Additionally, of these “hits”, 146 compounds displayed complete (100% protection) and 53 demonstrated >200% protection.

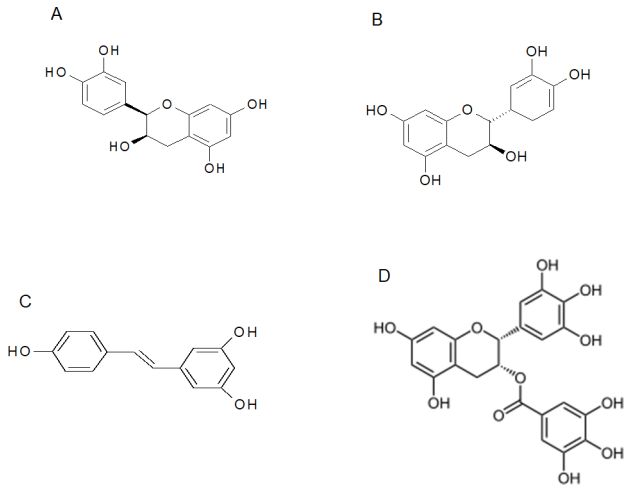

Figure 3. Chemical structures of catechins compounds used in these studies

(A) (−) Epicatechin (molecular mass 290) which is the cis form of (B) (+)-catechin. (C) Resveratrol (molecular mass 228) and (D) Epigallocatechin gallate (molecular mass 458)

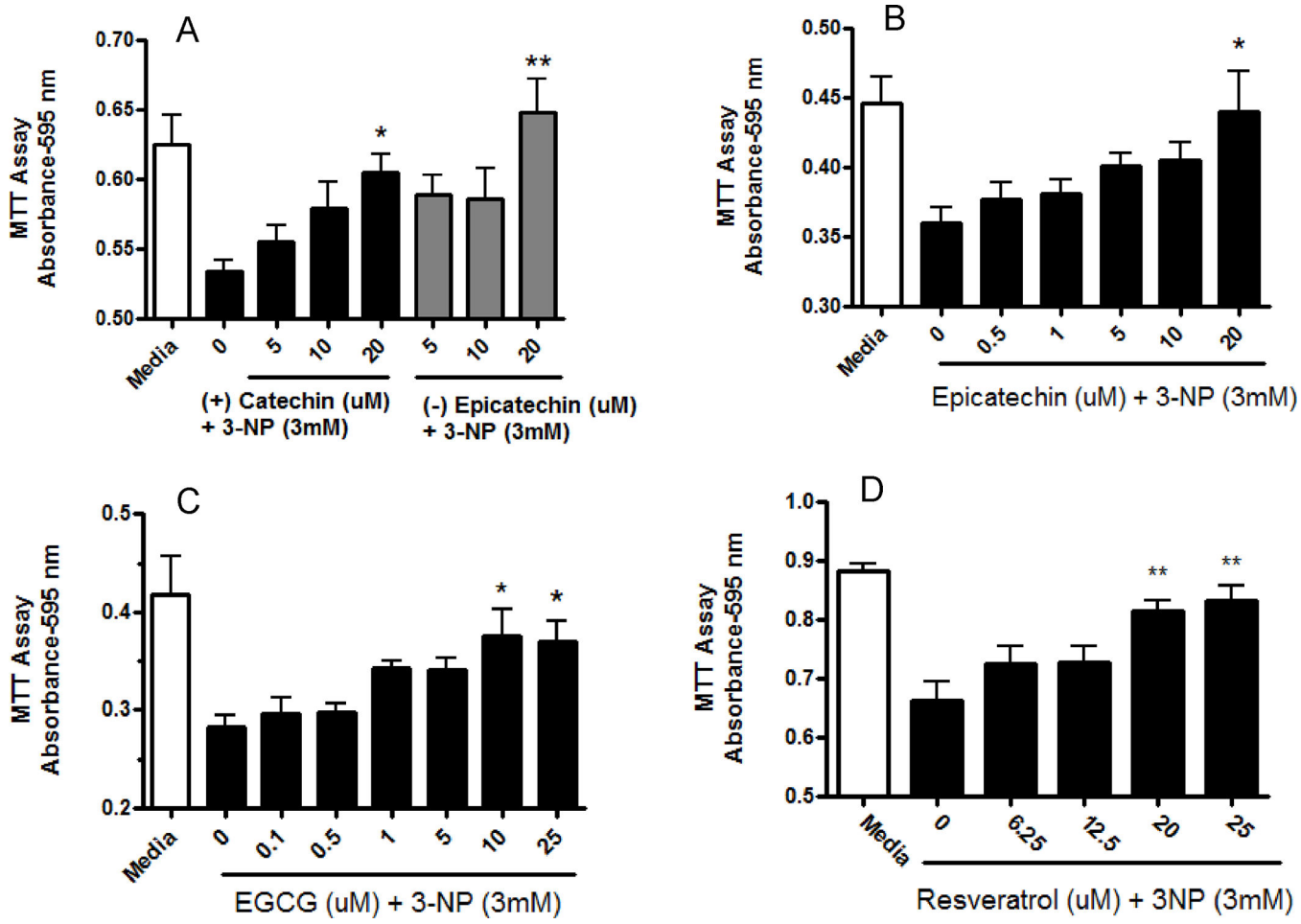

Figure 4. Catechins protect against 3-NP mediated oxidative stress in hippocampal cultures

(A) Epicatechin and (+) catechin (5–20 μM) were preincubated with rat mixed hippocampal cultures for 1 hour, followed by 18 hour exposure to 3-NP. Neuronal cell viability was assessed by MTT endpoint. The data from 8 replicates were utilized for each treatment group, and were evaluated by ANOVA for significance. Group-wise post hoc comparisons were assessed by Newman-Keuls multiple comparison test, with statistical significance at $p<0.05$ versus 3-NP. Epicatechin was more effective than Catechin at all concentrations. Full dose response with (B) Epicatechin, (C) EGCG and (D) Resveratrol demonstrate concentration-dependent neuroprotective effects of these compounds.

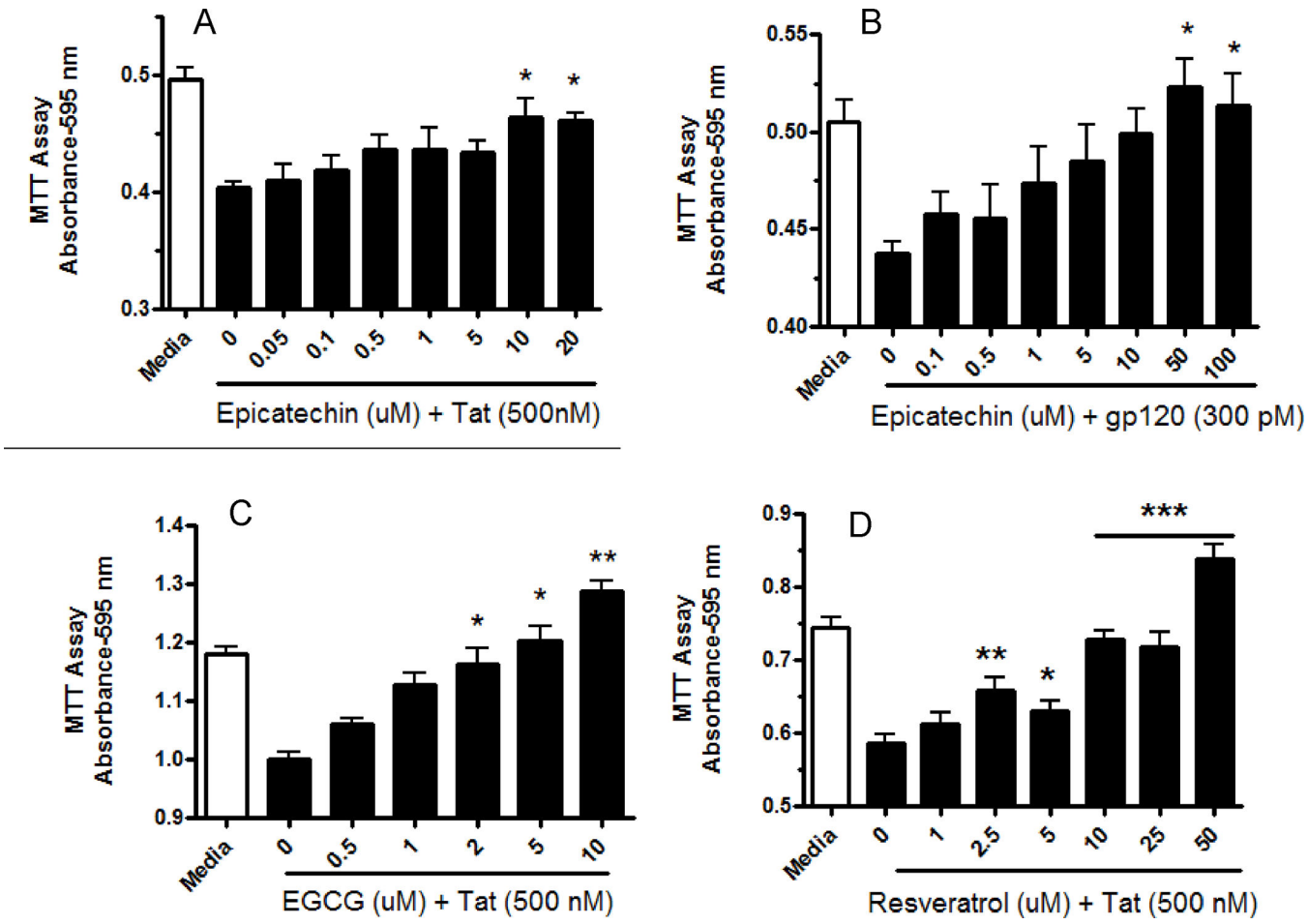

Figure 5. Protection by catechins and resveratrol against HIV protein induced neurotoxicity

Using rat hippocampal cultures, we assessed the neuroprotective efficacy of Epicatechin against HIV proteins, (A) Tat and (B) gp120; and the efficacy of (C) EGCG as well as D) Resveratrol against the neurotoxicity elicited by HIV Tat. Various concentrations of these compounds were pre-incubated with hippocampal cultures for one hour, followed by 18 hour exposure to HIV Tat1–72 or gp120. Neuronal cell viability was quantified by MTT endpoint. The data from 8 replicates were utilized for each treatment group, and were evaluated by ANOVA for significance. Group-wise post hoc comparisons were assessed by Newman-Keuls multiple comparison test. *p<0.05, **p<0.01, ***p<0.005 versus Tat or gp120. With each of these compounds, nearly complete protection is seen with concentrations of ≥10 μM. However of all the catechin compounds, EGCG showed the most potent neuroprotection.

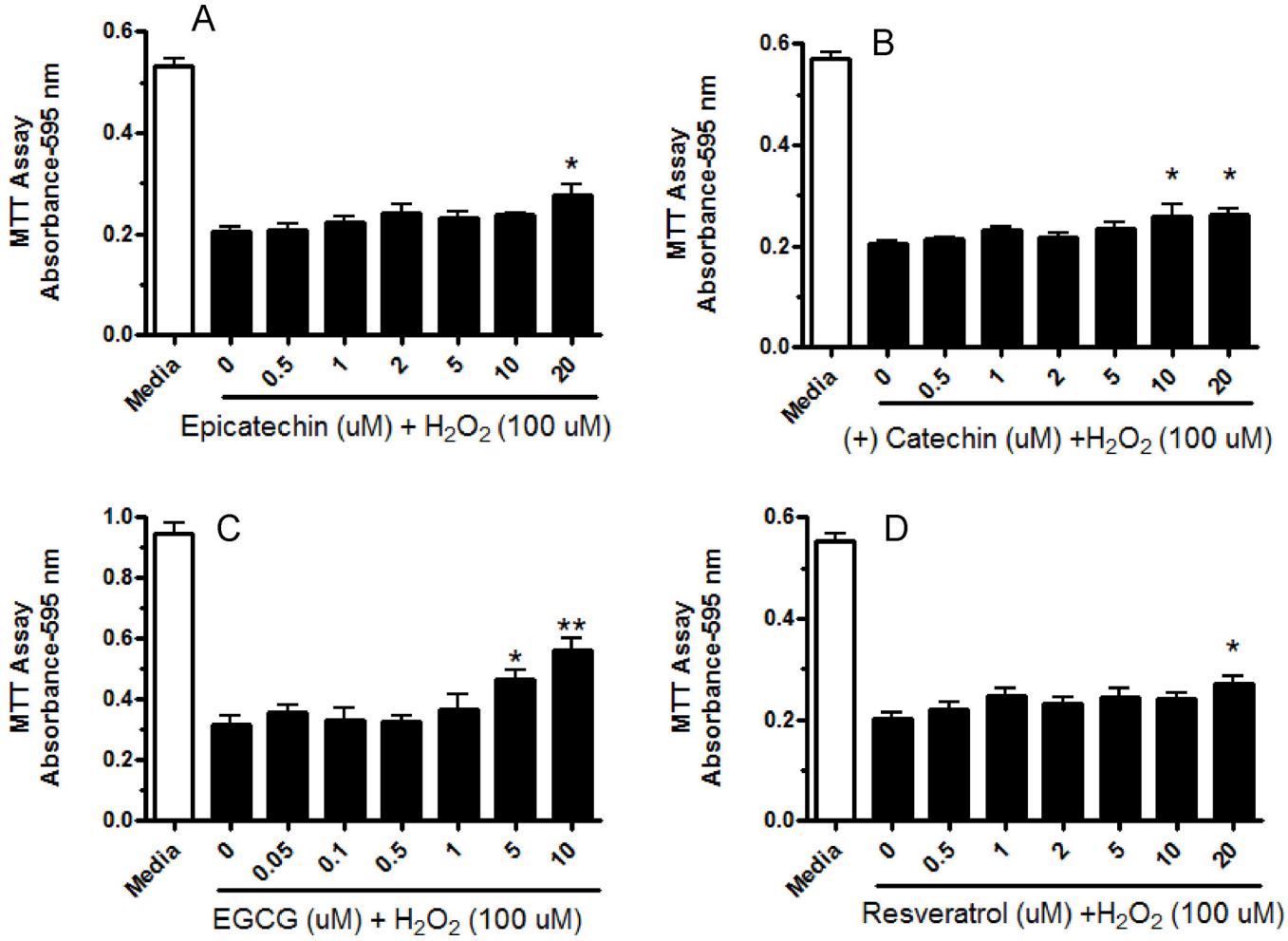

Figure 6. Catechins protect against hydrogen peroxide-mediated neurotoxicity

(A) Epicatechin, (B) (+) Catechin, (C) EGCG and (D) Resveratrol were preincubated with hippocampal neurons for 1 hour prior to 2 hour exposure to 100 μM hydrogen peroxide (H_2O_2). Cell viability was quantitated by MTT analysis. The data from 8 replicates were utilized for each treatment group, and were evaluated by ANOVA for significance. Group-wise post hoc comparisons were assessed by Newman-Keuls multiple comparison test. *$p<0.05$; **$p<0.01$ versus hydrogen peroxide. Minimal protection is seen with each of these compounds.

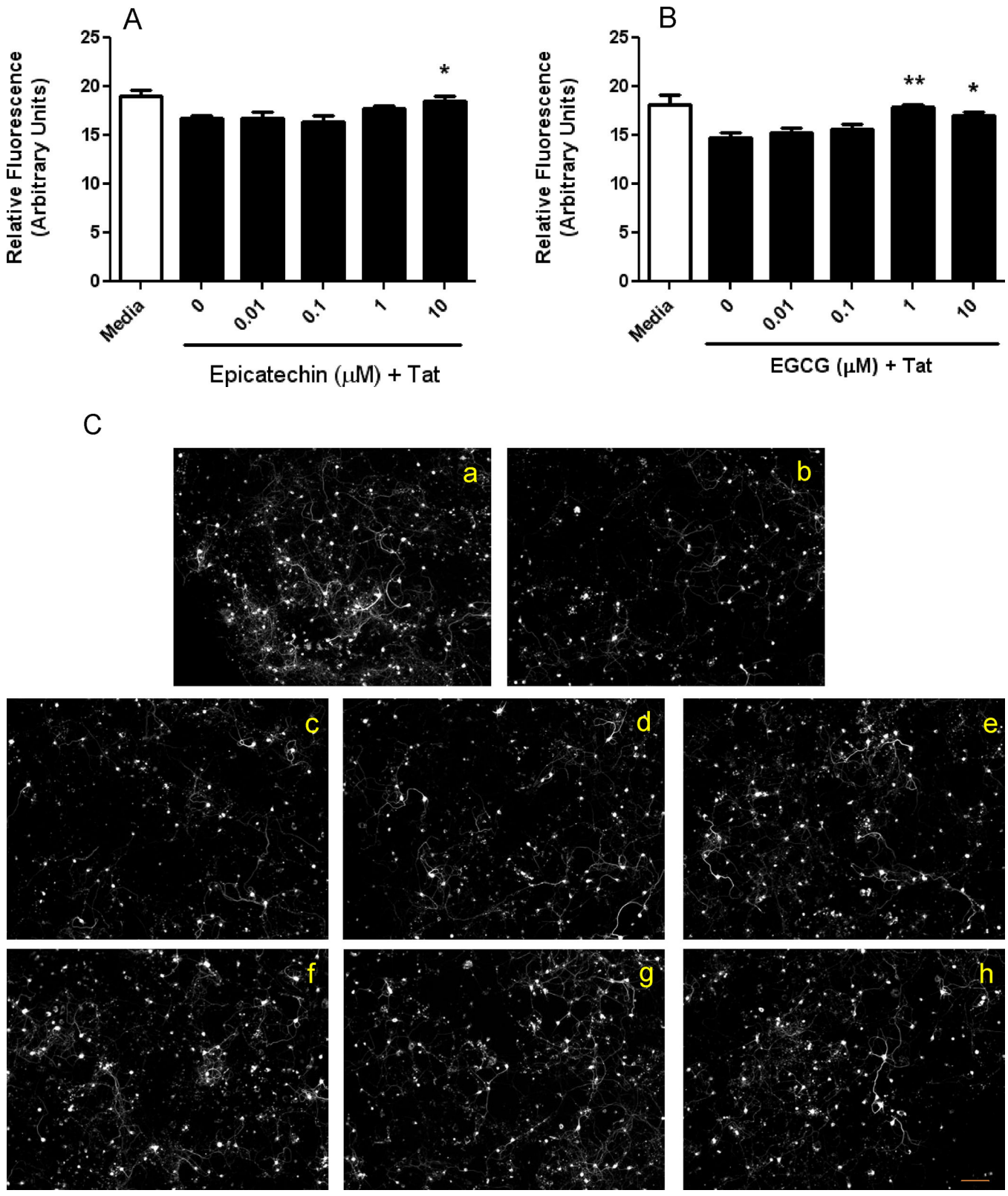

Figure 7. Protection by catechins against HIV Tat induced neurotoxicity

Using rat cortical cultures which were expressing the Tau-tdTomato gene under control of the CamKII promoter, we assessed the neuroprotective efficacy of epicatechin (Left panel, A) and EGCG (Right panel, B) against the neurotoxicity elicited by HIV Tat. Various concentrations of these compounds were pre-incubated with cortical cultures for one hour, followed by 24 hour exposure to 500 nM HIV Tat1 −72. Images were acquired by live imaging in an unbiased manner from a Zeiss AxioObserver inverted microscope. Fluorescence intensity was measured with a SpectraMax M5e plate reader. The data from 6 replicates were utilized for each treatment group, and were evaluated by ANOVA for significance. Group-wise post hoc comparisons were assessed by Newman-Keuls multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.005$ versus 500 nM Tat. Nearly complete protection is seen with each compound, with epicatechin requiring concentrations of 10 μM, while EGCG at 1 μM was maximally protective. Representative fluorescence images of these treatments are depicted in Panel C, where Media (Top row, a), 500 nM Tat (top row, b), Epicatechin + Tat (Middle row, 0.1 μM (c), 1 μM (d) and 10 μM (e)), and EGCG + Tat (Bottom row, 0.1 μM (f), 1 μM (g) and 10μM (h)).

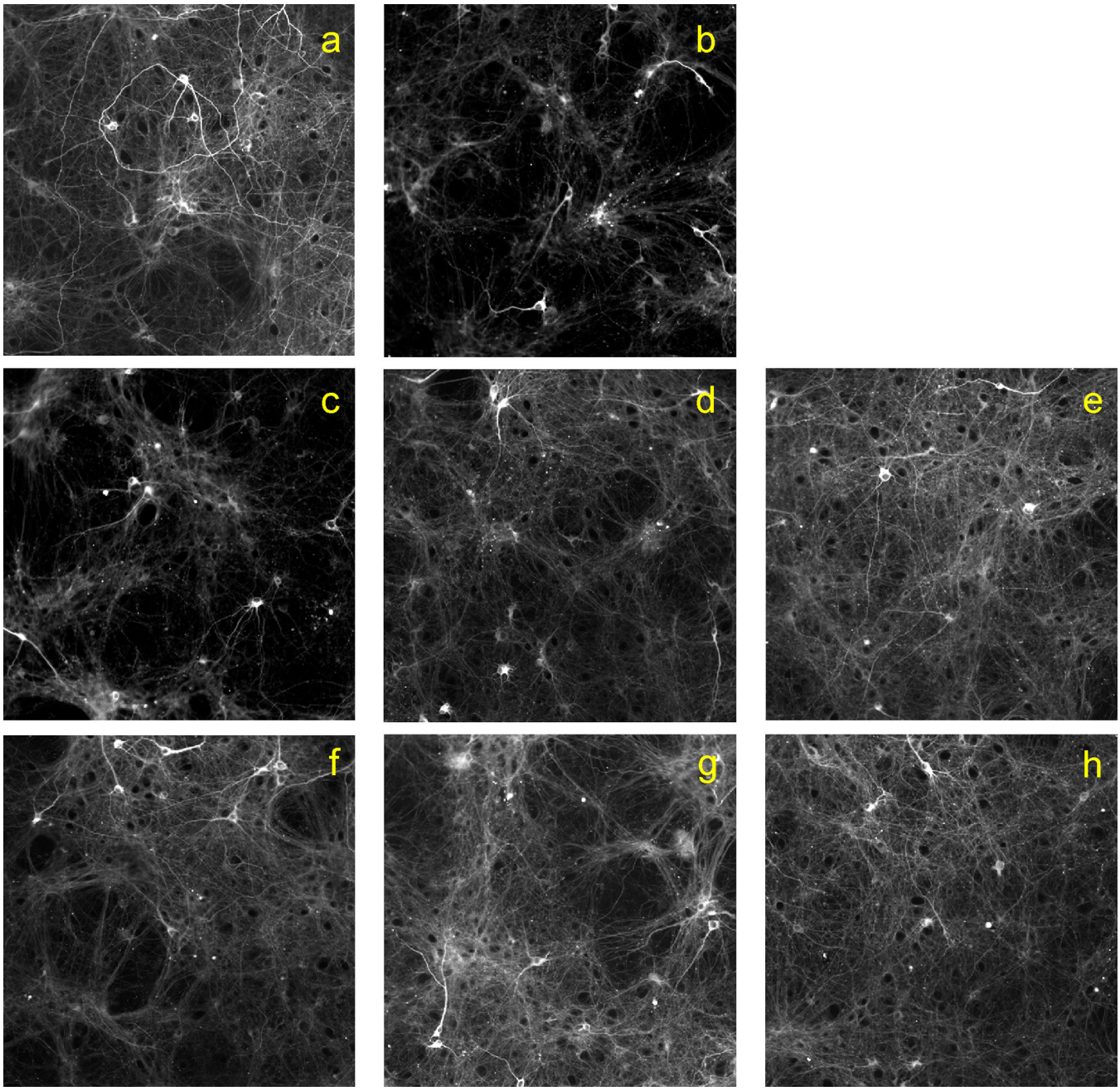

Figure 8. Protection by catechins against HIV Tat induced neurotoxicity

Using rat hippocampal cultures which were expressing the βIII Tubulin-tdTomato gene under control of the CamKII promoter, we assessed the neuroprotective efficacy of epicatechin and EGCG against the neurotoxicity elicited by HIV Tat. Various concentrations (0.1, 1 or 10 μM) of these compounds were pre-incubated with rat mixed hippocampal cultures for one hour, followed by 24 hour exposure to 500 nM HIV Tat1 −72. Images were acquired by live imaging in an unbiased manner from a Zeiss AxioObserver inverted microscope. The data from 6 replicates were utilized for each treatment group. Nearly complete protection is seen with each compound at 1–10 μM, with epicatechin requiring

concentrations of 10 μM, while EGCG at 1 μM was maximally protective. Representative fluorescence images of these treatments are depicted, where Media (Top row, a), 500 nM Tat (top row, b), Epicatechin + Tat (Middle row, 0.1 μM (c), 1 μM (d) and 10 μM (e)), and EGCG + Tat (Bottom row, 0.1 μM (f), 1 μM (g) and 10 μM (h)).

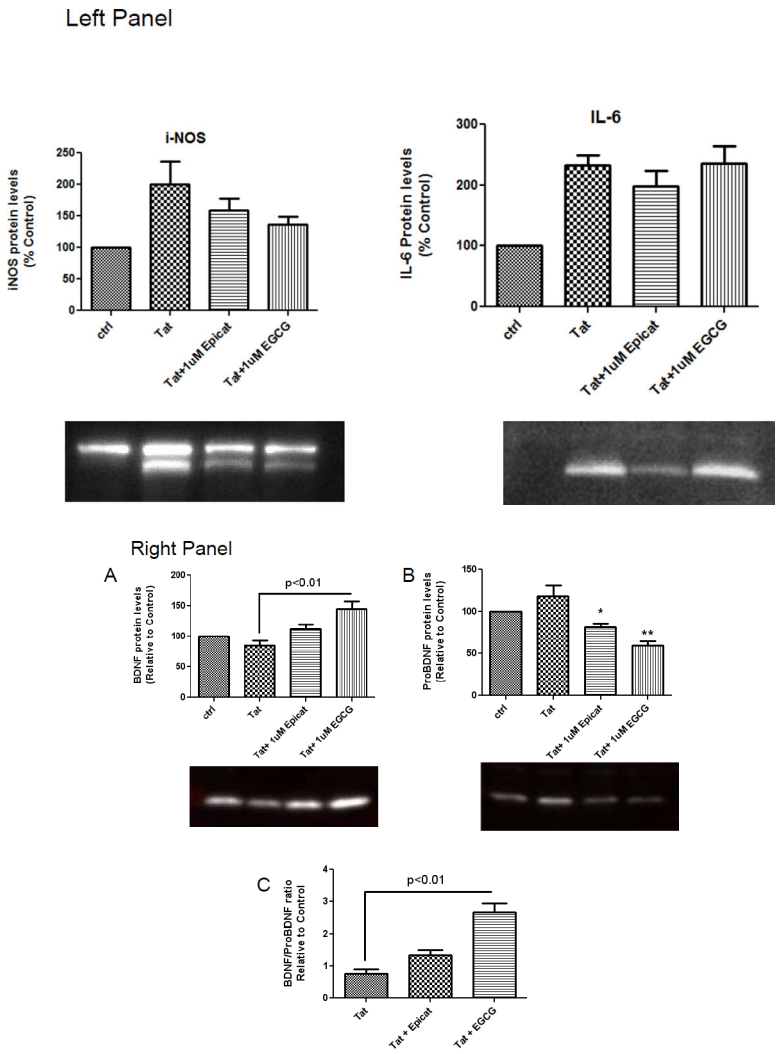

Figure 9. Tat-mediated expression of signaling proteins in mixed rat neuronal cultures

Rat mixed cortical cultures (10^6 cells per well on 6-well plates) were treated with culture media plus 0.1% DMSO vehicle, 500 nM Tat or Tat plus 1 μM Epicatechin or 1 μM EGCG for 24 hours. After 24 hours, cell lysates were generated, proteins separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes and probed with antibodies to iNOS and IL-6 (Left panel), and BDNF (Right panel). The BDNF antibody recognized the precursor of BDNF (proBDNF) at 32 kDa as well as the mature BDNF protein at 13kDa. Gel loading was normalized by BCA protein determinations, and blots were normalized by GAPDH immunoreactivity. The iNOS antibody recognized both neuronal NOS, whose expression remained constant with Tat treatment, along with the slightly smaller iNOS protein. Protein expression levels relative to control media (+ 0.1% DMSO vehicle) treatment were made by densitometry with NIH ImageJ software. The data from 3 independent replicates were utilized for each treatment group, and were evaluated by ANOVA for significance. Group-wise post hoc comparisons were assessed by Newman-Keuls multiple comparison test. *p<0.05, **p<0.01, ***p<0.005 versus 500 nM Tat.

Table 1

Neuroprotective properties of the Catechin family of compounds

Spectrum Collection Compound	% Protection vs 3-NP
EPICATECHIN	50.50%
EPICATECHIN 3,5-DIGALLATE	32.69%
EPICATECHIN MONOGALLATE	47.34%
EPICATECHIN PENTAACETATE	42.38%
EPICATECHIN, TETRAMETHYL ETHER	−28.72%
EPIGALLOCATECHIN	3.22%
EPIGALLOCATECHIN 3,5-DIGALLATE	29.78%
EPIGALLOCATECHIN OCTAMETHYL ETHER	39.88%
EPIGALLOCATECHIN-3-MONOGALLATE	2.84%
CATECHIN	42.51%
CATECHIN PENTAACETATE	29.36%
CATECHIN PENTABENZOATE	−3.62%
CATECHIN TETRAMETHYLETHER	3.40%