17th Conference on Retroviruses
and Opportunistic Infections
February 16-19, 2010
San Francisco, California

CNS Toxicity of Antiretroviral Drugs

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Introduction

HIV-1 enters the CNS early during the course of infection and establishes a persistent viral reservoir. CNS HIV infection frequently results in neurological disease marked by a set of cognitive, motor, and behavioral symptoms. Potent combination antiretroviral (ARV) therapies have been shown to improve cognition and reduce the prevalence of severe HIV-associated neurocognitive disorders, although recent studies have shown that mild-moderate neurological and cognitive manifestations of HIV infection persist in some patients on treatment. To control viral replication in the brain, strategies are under development to increase the penetration of antiretroviral compounds across the blood-brain barrier. Although these compounds have well described toxic actions systemically and in the peripheral nervous system, little is known about the toxicity of the compounds to neurons in the CNS. One recent study (ACTG 5170) reported that cognition actually improved for up to 96 weeks in a group of immunologically and virologically stable patients who elected to come off of treatment. These results raised the possibility that even low concentrations of ARVs that penetrate the brain may have some detrimental effects. If this is true, future efforts at delivering higher concentrations of ARVs to the CNS could have significant adverse effects. To provide a comparative analysis of the neurotoxicity of antiretroviral compounds, we evaluated the effects of fifteen different antiretroviral compounds and six combinations on primary cultures of rat neurons.

Methods

Primary cultures of rat forebrain.
For primary neuronal cultures, fetuses (E17) were harvested from pregnant female Long-Evans rats, washed with ice cold HEPES-buffered Hank's balanced salt solution (HBSS) and the brain removed. The cortex/hippocampus was dissected from the brain and cleaned of dura-arachnoid membrane and visible vessels. The tissue was transferred to a 15 ml tube containing 5 ml calcium-magnesium free-HBSS + 1.2 U/ml dispase + 2 U/ml DNase I and incubated for 20-30 min at 36° C. Tissue was triturated and allowed to settle for 2 min, and the suspended cells were transferred to a 50 ml culture tube containing 25 mls of complete medium (Minimum Essential Medium (MEM) + 10% fetal bovine serum + 20 μg/ml gentamicin). The remaining tissue was resuspended in 3-5 ml calcium-magnesium free HBSS (CMF-HBSS; calcium and magnesium salts replaced by an equimolar concentration of NaCl) and run through several cycles of trituration until most of the tissue was dispersed. Dissociated cells were seeded at a density 40,000 cells/cm^2 (approximately 70-80% neurons on the first day after seeding) on poly-D-lysine-treated coverslips and 96-well plates. Cultures were fed by 50% medium exchange three times/week.

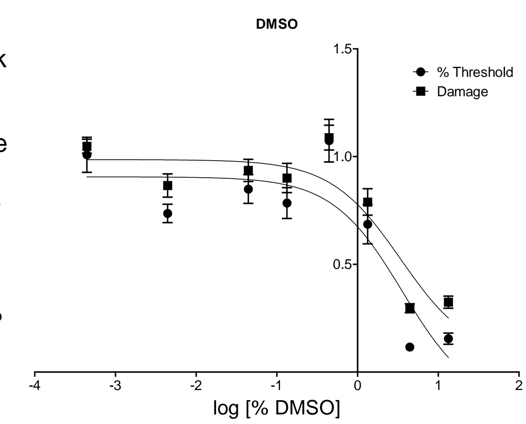
ARV Challenge
In cultured rat cortical neurons we challenged cells for 1 week with doses of 15 ARVs at concentrations ranging from 0.01 to 300 μg/ml to determine single drug toxicities. In addition, six combinations of ARVs currently in the DHHS guidelines were tested at therapeutic concentrations. We assessed neuronal pathology and cell death (MAP-2 staining), calcium signaling in response to glutamate and mitochondrial membrane potential (TMRM) using fluorescent microscopy.

TMRM Stain
Neuronal cultures were incubated for 6 days with antiretroviral compounds and then loaded with 100 nM Tetramethylrhodamine, methyl ester (TMRM) solution. Cultures were then incubated for 10 minutes at 37 ˚C and imaged using the MetaMorph™ System.

MAP-2 Immunostaining for Assessment of Neuronal Loss and Damage
Neurons were identified by microtubule-associated protein-2 (MAP-2) immunostaining. Cells were fixed in ice cold 97% methanol, 3% acetic for 10 min at room temperature and washed in 0.01 M phosphate-buffered saline (PBS, 3 x 5 min). Cells were incubated in blocking buffer containing 3% normal goat serum in 0.01 PBS for 60 min at room temp. Polyclonal rabbit anti-MAP-2 (Chemicon/Millipore, Bilerica, MA) was then applied at a dilution of 1:500 in blocking buffer and incubated overnight at 4° C. The cells were washed three times in PBS and incubated in biotinylated goat anti-rabbit at 1:500 for 1 h at room temp. The cells were washed three times in PBS and incubated for 1 h at room temp in avidin-Alexa488 (1:500, Molecular Probes/Invitrogen, Carlsbad, CA) or goat anti-rabbit Alexa488 (1:500) or mouse anti-rabbit Alexa568 (1:500). Cells were then washed 3 x 5 min, counterstained with bisbenzimide (0.5 μM, Sigma Corp, St Louis, MO) for 20 min in PBS, washed 2 X 5 min and mounted onto slides with Fluoromount (Southern Biotech, Birmingham AL).

MAP-2 Data Analysis
Images were captured in MetaMorph™ System by selecting three to four representative areas of each well. The images were then thresholded to highlight the neuronal cell bodies and proximal dendrites while limiting background. Percent threshold measurements as well as an observer rating of neuronal health on a scale of 1 to 10 (10 being very healthy and 1 representing an almost complete loss of neurons), were recorded for each image taken. Damage seen included beading of dendrites, pruning of dendrites and loss of neurons. These values were then averaged for each well within a 96-well plate and then across four replicate experiments.

Correction for DMSO damage.
Several of the drugs used were not soluble in aqueous solutions and were therefore dissolved in DMSO in the stock solutions. To correct for toxicity of the DMSO a dose response curve was run on each plate. The dose-response curve for both MAP-2 intensity measurements (% threshold) and damage assessments are illustrated in Figure 1. Values represent mean ± sem. There was no consistent DMSO toxicity until concentrations exceeded 0.44%. By 4% there was almost a complete loss of neurons. Toxicity due to DMSO was subtracted from each compound as appropriate to provide a clean estimate of the toxicity of the compound. Concentrations with DMSO greater than 1% were not used for IC50 calculations.



ARVs, with the exception of DDI, decrease mitochondrial membrane potential as assessed in live neurons with TMRM

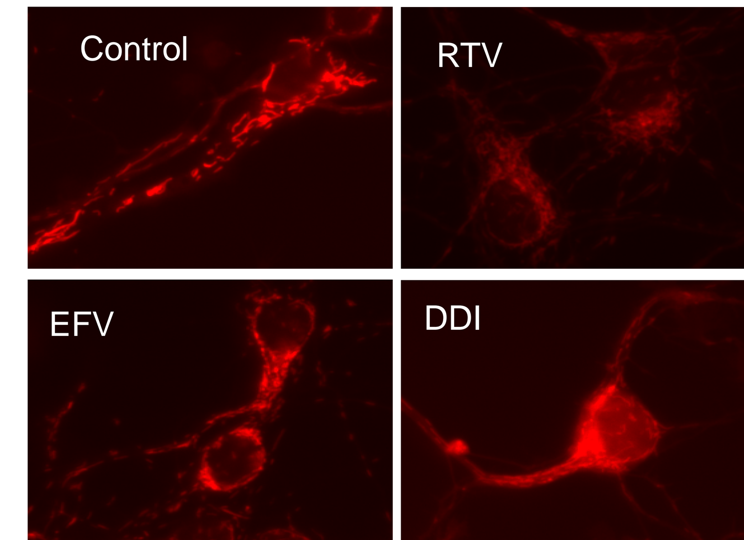


Figure 1. Tetramethylrhodamine methyl ester stain of mitochondria in neurons exposed to antiretroviral compounds (ARVs). Intensity of the red stain is directly proportional to the mitochondrial membrane potential providing an indication of the effects of each ARV. Neurons were stained and imaged live after 2 days exposure to the indicated compound.

Neuronal damage produced by antiretrovirals

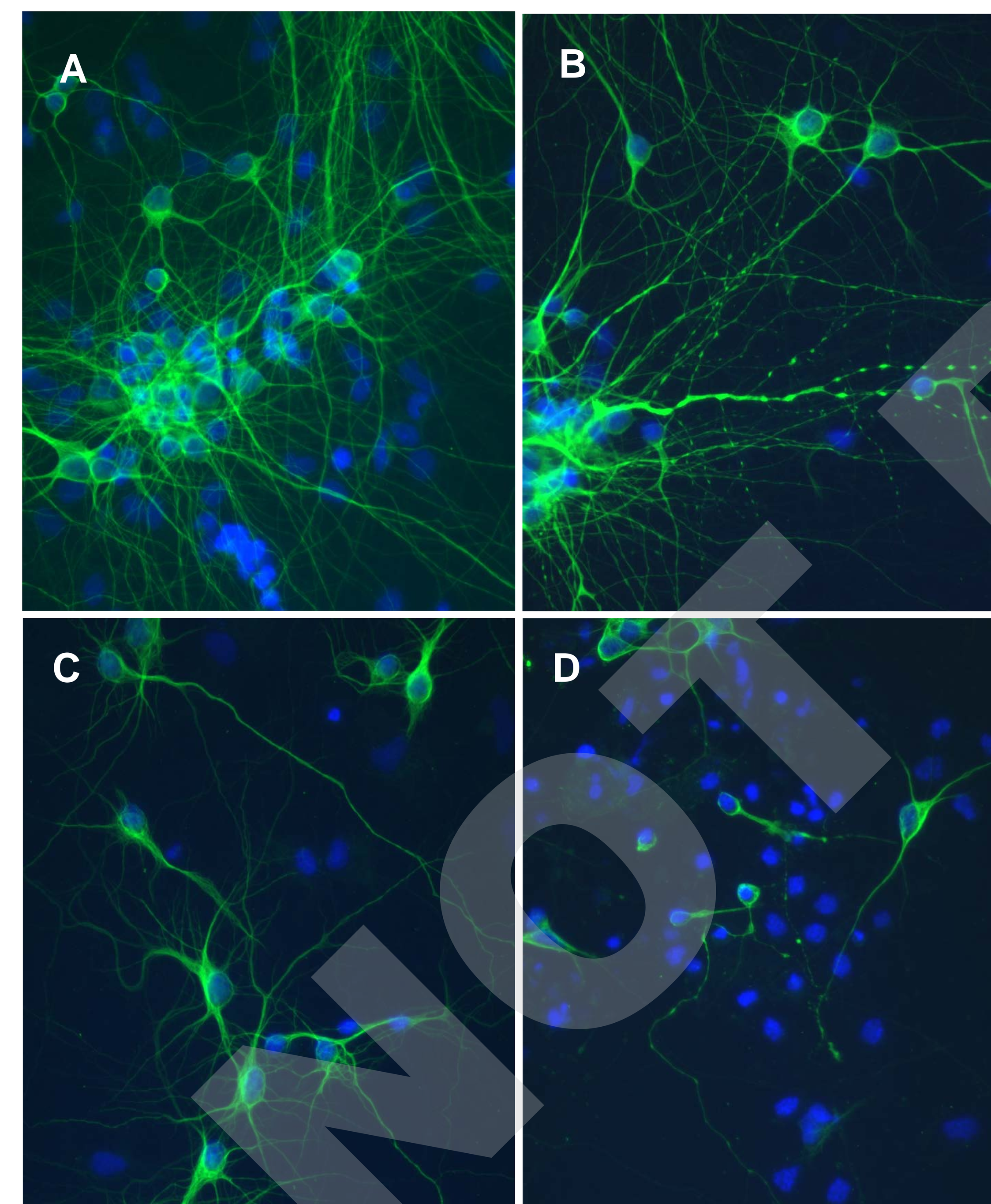


Figure 4. Neurons stained for microtubule-associated protein-2 (MAP-2) illustrating normal untreated cultures (A) and cultures that showed dendritic beading (B), pruning of dendrites (C) and loss of neuron density (D) following 6 days exposure to ARVs at reported plasma concentrations. Greater loss of neurons and damage were seen with increasing concentrations of ARVs. Figures B and C were taken from cultures treated with Atazanavir and figure D represents maximal damage seen in a culture treated with Efavarenz.

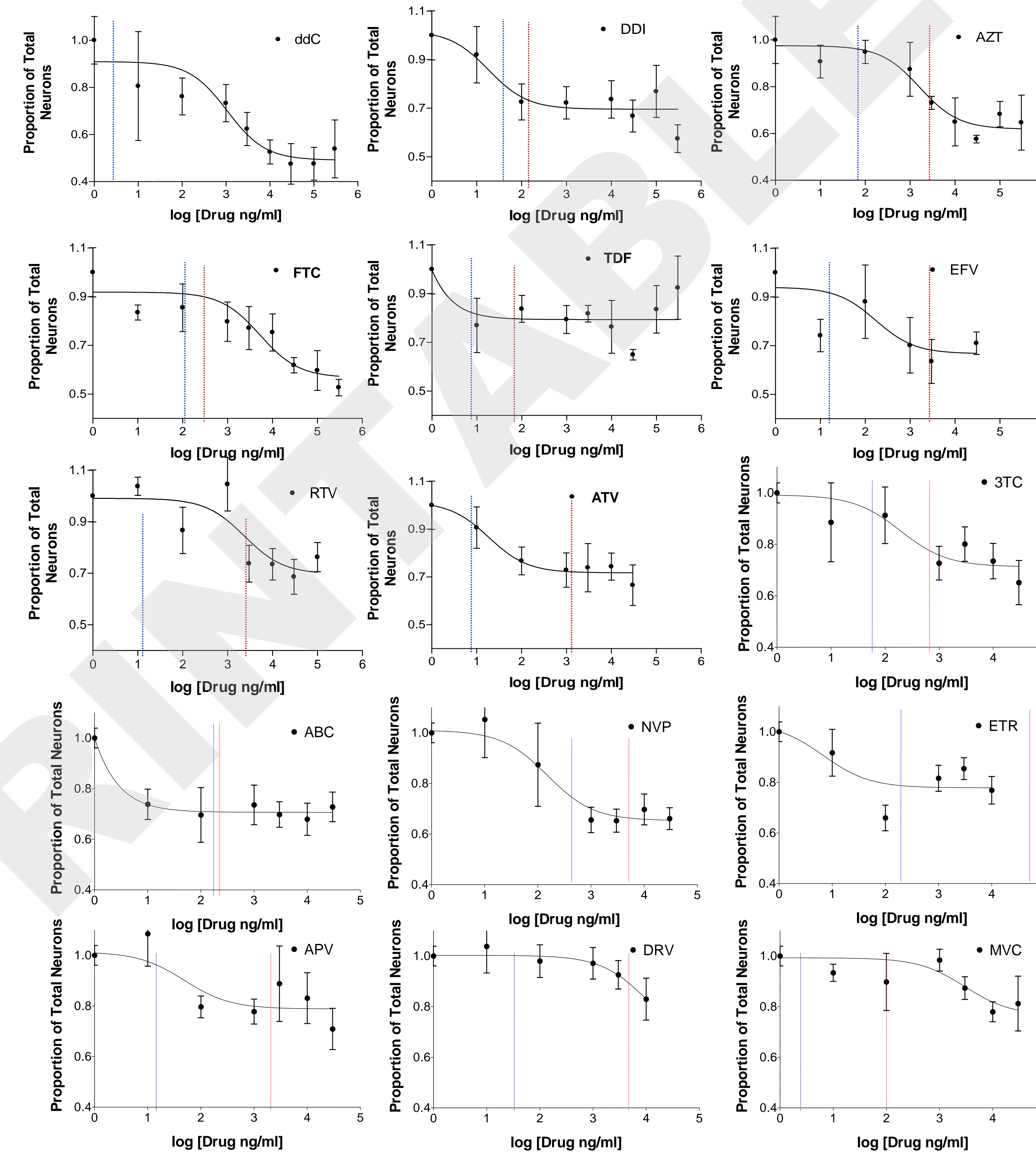
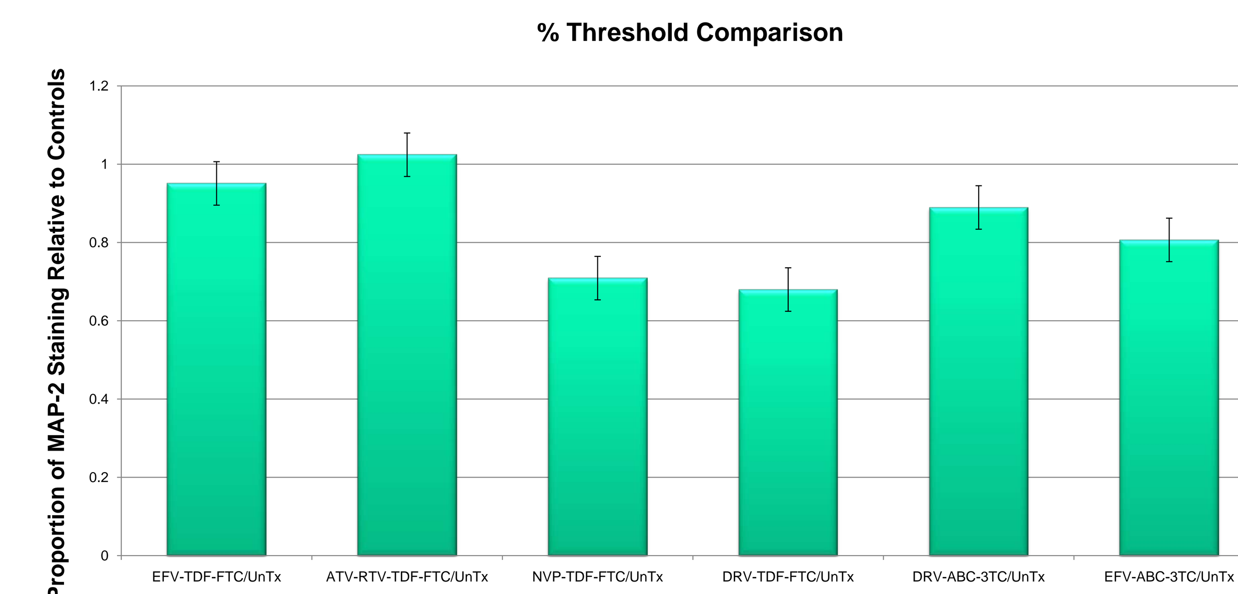


Figure 5. Concentration-effect curves showing the loss of MAP-2 staining (neurons and/or processes) in response to fifteen antiretroviral compounds. Neurons were treated from day 6 to day 12 in culture. Values represent the mean ± sem of four determinations. Drug effects were corrected for damage due to vehicle. Dashed red line represents the concentration found in the plasma of patients on therapy and the dashed blue line represents published concentrations in the CSF of patients on therapy.

Figure 6. Proportion of neurons stained for MAP-2 in neural cultures treated with six different antiretroviral drug combinations. Cells were grown in a 96 well plate and challenged for 7 days with reported plasma concentrations of each drug. Drugs were refreshed with culture feedings, every 2 days. Total MAP-2 staining was ratioed to parallel, vehicle-treated controls. No damage represents a ratio of 1. Values are Mean ± sem for 16 measures each.



Estimated Median Toxic Concentration (TC_{50}) Based on MAP-2 Staining

Class	Description	Molecular Wt.	Max % Cell Loss (MAP-2)	% Threshold TC50	Median Plasma Conc (ng/ml)	Median CSF Conc (ng/ml)
NRTI	Abacavir (ABC)	286.3	27.2	2.2	139.0	128.0
NRTI	2',3'-Dideoxycytidine (ddC)	211.2	52.5	1065.0	25.3	17.9
NRTI	2',3'-Dideoxyinosine (DDI)	236.0	42.5	18.4	165.2	37.8
NRTI	Emtricitabine [(-) FTC]	247.2	47.3	5287.0	261.0	109.0
NRTI	Tenofovir (TDF)	305.2	18.0	80.9	97.0	5.5
NRTI	Lamivudine (3TC)	229.3	34.9	193.4	889.5	45.9
NRTI	Zidovudine (AZT)	267.0	42.4	1638.0	1684.8	80.1
NNRTI	Efavirenz (EFV)	315.7	36.5	199.5	2145.0	13.9
NNRTI	Etravirine (ETR)	435.3	23.1	6.8	171069.0	171.1
NNRTI	Nevirapine (NVP)	266.3	34.7	151.2	4980.0	657.8
PI	Amprenavir (APV)	505.6	29.1	49.0	2150.0	25.0
PI	Atazanavir Sulfate (ATV)	802.9	33.5	15.8	1278.0	10.3
PI	Darunavir (DRV)	593.7	17.0	10452.0	3930.0	34.2
PI	Ritonavir (RTV)	721.0	31.4	2375.0	8900.0	23.1
EI	Maraviroc (MVC)	513.7	22.0	2978.0	86.1	2.3

Table 1. Summary of the toxic effects of 15 antiretroviral compounds in primary cultures of rat neurons. The maximum % cell loss based on decreased MAP-2 stain and the median toxic concentration (TC_{50}) are shown for each compound. TC_{50} values were calculated from the plot of log P/(1-P) vs log [Drug] based on the data in Figure 5. Published values for the reported concentrations found in patients on therapy area also provided for comparison to the toxic concentrations. These reference values were graciously provided by Dr. Scott Letendre and the UCSD HIV Neurobehavioral Research Center.

Conclusions

1) All of these drugs produced neural toxicity at concentrations above 10μg/ml.

2) The median toxic dose of ABC, DDI, ETR, and NVP fell within the range of concentrations seen in both the plasma and CSF of patients on antiretroviral therapy.

3) The median toxic dose of ABC, APV, ATV, AZT, DDI, EFV, ETR, NVP, RTV, TDF and 3TC, fell within the range of concentrations seen in the plasma.

4) ddC, DRV, FTC, and MRV produced little toxicity at relevant plasma and CSF concentrations.

5) Combinations of NVP-TDF-FTC and DRV-TDF-FTC reduced neuronal density (MAP2 staining) by approximately 35%.

6) Penetration of antiretrovirals into the brain at concentrations sufficient to suppress viral synthesis will carry some risk of neuronal damage.

Supported by the NIH AIDS Research and Reference Reagent Program