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BRIEF REPORT

Protection against HIV-envelope-induced neuronal cell destruction by HIV attachment inhibitors

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Abstract We demonstrate that HIV attachment inhibitors (AIs) prevent HIV envelope-induced destruction of two neuronal cell lines (SH-SY5Y and BE(2)-M17) at low nanomolar concentrations. The fusion inhibitor enfuvirtide and the CCR5 inhibitors UK427,857 and TAK779 do not display protection activity, suggesting the involvement of Env/cell interaction site(s) distinct from the sites involved in the viral entry process. We surmise that by inducing conformation changes in the envelope, AIs likely obstruct novel interactions with a neuronal cell factor(s) required for induction of apoptosis. This antiretroviral class may therefore have the potential to inhibit HIV-induced neuron damage, thereby curtailing the increasing incidence of HIV-associated cognitive impairment.

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HIV enters the central nervous system (CNS) early in infection, likely via infected macrophages/microglia [3, 6]. CNS invasion by HIV is associated with several abnormalities, including encephalomyelitis, radiculopathy, and a number of opportunistic infections of the CNS [8, 14, 15]. Inflammation in the peripheral nervous system causing painful neuropathy is also common. Such insults to the CNS can lead to HIV-associated dementia (HAD), which has displayed a decreasing incidence rate with the advent of antiretroviral therapy (ART) [20]. However, a less severe but clinically relevant neurocognitive abnormality termed HIV-associated mild neurocognitive disorder is actually increasing in prevalence among ART-experienced patients who do not display additional clinical manifestations of ongoing HIV infection [17]. Moreover, although as a subcortical disorder, HAD is distinct from Alzheimer's disease, it has been postulated that the neurotoxic as well as ancillary effects of HIV infection (such as increasing amyloid levels) could predispose infected individuals to Alzheimer's disease pathogenesis [5].

It is thought that human neurons are not productively infected by HIV [11]. However, the HIV coat protein gp120 has been implicated in neuronal cell death, either through alterations of microglia and astrocyte physiology or through direct neuronal contact and induction of pro-apoptotic signaling [2, 4, 24]. Accordingly, a need for novel approaches to prevent HIV-associated CNS pathologies, including those that would inhibit the direct killing effects of HIV gp120 on neurons is indicated.

HIV attachment inhibitors (AIs) are small molecules that prevent the initial stages of HIV infection by binding to gp120, inducing conformational changes in both its CD4− and coreceptor-binding regions [10, 13]. These effects are sufficient to inhibit infection of both CD4-dependent and CD4-independent HIV strains [22], and we

therefore postulated that AIs might prevent gp120-mediated destruction of CD4-negative neuronal cells.

To test this hypothesis, we employed the neuronal cell lines SH-SY5Y and BE(2)M-17 (ATCC, Manassas, VA). Twenty thousand of these cells were seeded into 96-well plates and were differentiated using a 3 day treatment with 20 μM retinoic acid (RA, Sigma, St. Louis, MO), followed by a 5 day treatment with 50 ng/ml brain-derived growth factor (BDNF, Invitrogen, Carlsbad, CA) to induce the formation of neurite outgrowths reminiscent of those of primary human neurons [4, 24]. Immunofluorescence was employed to characterize the SH-SY5Y surface pattern of proteins that facilitate HIV entry, which revealed the expression of the HIV coreceptors CXCR4 and CCR5 and the lack of expression of the primary HIV receptor CD4 (data not shown), consistent with previous observations [9].

To study the effects of native trimeric HIV Env on these cells, a replication-defective virus-like particle (VLP) was engineered in which a portion of the *polymerase* gene (of the NL4-3 genome) containing *reverse transcriptase* and *integrase*, as well as the *vif* and *vpr* genes were deleted as described previously [1]. A stop codon was also introduced into the *env* gene of this VLP genome, allowing NL4-3 cores to be coated with exogenous Envs. VLPs were produced by cotransfection of DNAs containing selected *env* sequences and the NL4-3 backbone into 293T cells using Lipofectamine and Plus reagents (Invitrogen). The supernatants from these cultures were harvested, and the particles were concentrated by ultracentrifugation through a 20% sucrose cushion. These particles were examined for envelope protein content by western blot and ELISA and were found to have a coat density consistent with that of infectious particles, as described previously [1]. For studies of clinical isolates, infectious HIV was inactivated with Aldrithiol-2 as described previously [21].

The capacity of HIV to induce neuronal cell destruction was examined employing a CCR5-utilizing Env clone from a brain isolate (B12) derived from a patient who died with dementia and florid giant-cell encephalitis [19]. Neuronal cell apoptosis was assayed 4 days after removal of BDNF and initiation of VLP treatment by phosphatidylserine exposure at the extracellular face of the plasma membrane as detected by annexin V staining [23] (Vybrant Apoptosis Assay Kit, Invitrogen), for which the fluorescent signal was measured using an ArrayScan HCS Reader (Cellomics, Pittsburgh, PA). Neuronal cell cytotoxicity was shown via adenylate kinase release using a Bioluminescence Cytotoxicity Assay Kit (BioVision Research Products, Mountain View, CA). To determine the effect of HIV entry inhibitors neuronal cells were treated with serial dilutions of these drugs, which were added at the time of initiation of VLP treatment.

To determine anti-entry potencies of HIV entry inhibitors MT-2 cells, were centrifuged at 1,200 rpm in a Beckman Allegra centrifuge for 10 min. HIV was added to the pellet in a volume of 0.5 ml at an MOI of 0.01. The cell/virus mixture was resuspended in RPMI 1640 plus 10% FBS to a final density of 1.0×10^5 cells/ml containing serial dilutions of anti-entry test compounds. The cultures were incubated for 5 days, and the level of HIV production and inhibition was quantified by p24 ELISA (Perkin Elmer Life Sciences, Carlsbad, CA) following the manufacturer's recommendations. For CC_{50} determinations, MT-2 cells were treated with a series of fivefold dilutions of AI for 5 days. At that time, XTT was added, and the level of cytotoxicity was assessed following the manufacturer's protocol (Sigma).

BDNF can protect neuronal cells from apoptosis by inducing the expression of anti-apoptosis factors such a BCL-2 [12], and its removal via washing induced apoptosis without a requirement of VLPs. These observations led us to conclude that a trace amount of this factor was required to detect VLP-induced apoptosis. However, the trade-off for this requirement was that the advent of measurable apoptosis was delayed when VLPs were applied, with the strongest signal being observed 4 days after VLP exposure (data not shown). Thus, VLP treatment immediately after BDNF removal and cytotoxic signal measurements 4 days thereafter became our standard experimental procedure.

Having established the study conditions, we determined the effect of HIV entry inhibitors on differentiated SH-SY5Y cells. These experiments revealed that azaindole attachment inhibitors (BMS-585248 and BMS-488043) decrease annexin V fluorescence in a dose-dependent manner (Fig. 1). These data were used to determine potencies of neuronal cell protection from the VLP, and they revealed that this activity (EC_{50} = 22.6 nM; Fig. 1g) had a potency comparable to the anti-entry activity of BMS-585248 (EC_{50} = 5.9 nM; Table 1). Similar neural cell protection activity was observed for BMS-488043 (EC_{50} = 23.8 nM; Fig. 1h). Our analysis also showed that the CCR5 inhibitor UK427,857 and the fusion inhibitor enfuvirtide do not possess detectable neuronal cell protection activity (EC_{50}s > 1,000 nM; Table 1) despite displaying anti-entry activity comparable to that of the AIs (EC_{50}s of 17.0 and 20.0 nM, respectively; Table 1).

To expand from these proof-of-concept studies to provide further evidence of the protective effects of AIs against B12/VLP-mediated SH-SY5Y cell death, we employed an independent high-throughput endpoint that measures cytotoxicity via adenylate kinase release, utilizing the standard VLP/cell/compound conditions described above. These experiments revealed potent neuroprotective activity for BMS-488043 and BMS-585248 but not for UK427,857 and enfuvirtide (Table 1). Moreover,

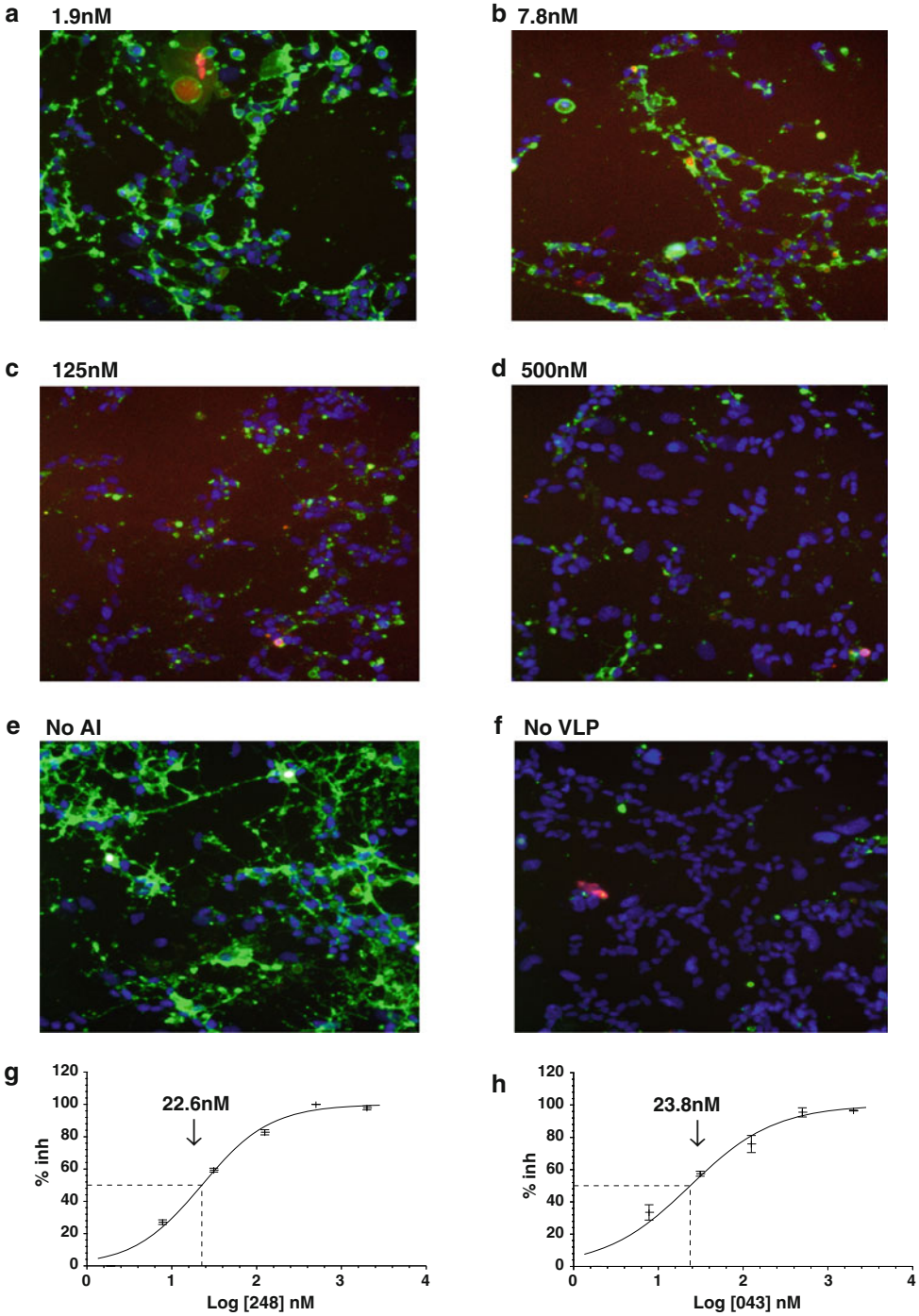


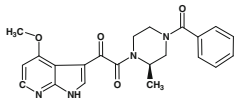
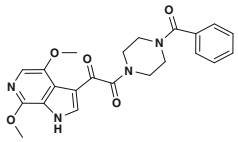
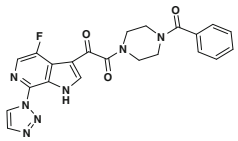
Fig. 1 **a–d** Annexin V staining (*green*) of SH-SY5Y cells infected with VLPs coated with the B12 envelope and treated with the indicated doses of BMS-585248. The nuclear stain (Hoechst) is shown in *blue*. **e–f** Infected/untreated and uninfected/untreated cells controls, respectively. **g–h** Protection activity curves (with *error bars*) of BMS-585248 (**g**) and BMS-488043 (**h**) from two independent experiments based on data such as shown in **a–d**. The *arrows* indicate the EC_{50} values

neurocytotoxicity protection was seen with a third azaindole AI (BMS-378806; Table 1) but not with a second CCR5 blocker (TAK779; Table 1). All of the entry inhibitors used in this study displayed anti-entry activity in the low nanomolar range (Table 1).

To assess if this pattern of neuroprotection was specific for the SH-SY5Y line, cytotoxicity experiments as described above were performed on the neuronal cell line BE(2)-M17. These studies revealed that the different classes of entry inhibitors behave similarly in the two lines in that AIs provide potent BE(2)-M17 cell protection against B12/VLP neurocytotoxicty, whereas fusion and CCR5 inhibitors do not display BE(2)-M17 protection activity (Table 1). These observations suggest that Env-induced neurotoxicity likely operates through a common mechanism in the two lines.

To test if protection of SH-SY5Y cells is conferred to diverse Envs by a distinct AI chemotype (indole; Table 2), we examined the effects of BMS-389176 against CCR5

Table 1 Activity of entry inhibitors against B12/VLP-induced cytotoxicity

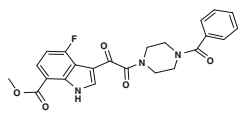
Inhibitor	Entry inhibition mechanism	EC_{50} (nM) anti-entry	EC_{50} (nM) neuronal cell protection		EC_{50} (nM) neuronal cell anti-apoptosis
			SH-SY5Y	BE(2)-M17	SH-SY5Y
BMS-378806 	gp120-CD4 interaction	2.5 ± 0.6	14.7 ± 4.0	17.5 ± 4.9	ND
BMS-488043 	gp120-CD4 interaction	4.1 ± 1.1	11.3 ± 0.9	10.6 ± 2.9	23.8 ± 2.7
BMS-585248 	gp120-CD4 interaction	5.9 ± 0.3	15.3 ± 0.7	18.6 ± 1.3	22.6 ± 2.9
Enfuvirtide	Fusion	20.0 ± 0.5	>1000	>1000	>1000
UK427,857	CCR5 block	17.0 ± 1.1	>1000	>1000	>1000
TAK779	CCR5 block	23.6 ± 0.7	>1000	>1000	ND

These data are representative of three independent experiments performed in triplicate

ND not done

Table 2 Neuronal cell protection and anti-viral activity of BMS-389176 against SH-SY5Y cell cytotoxicity induced by HIV clinical isolates

BMS-389176 (CC_{50} = 161 μM) EC_{50} (nM)



Clinical isolate	Neuronal cell protection	Anti-entry
91US005	0.7 ± 0.5	0.4
93US143	1.3 ± 0.4	0.7
ASM34	4.7 ± 1.5	6.2
91US715	115 ± 30	102
92US660	229 ± 42	270

These data are representative of three independent experiments performed in triplicate

utilizing HIV clinical isolates (NIH AIDS Research and Reference Reagent Program, Rockville, MD). These clade B isolates display a range of AI anti-entry sensitivities, although all maintain EC_{50}s in the low nM range (in contrast to its CC_{50} of 161 μM, Table 2), as has been observed previously for independent clinical samples [13]. The neuronal protection experiments revealed that this molecule provided SH-SY5Y cell protection for all of the isolates tested (Table 2). Interestingly, the sensitivity of these isolates to this molecule was comparable for both neuronal cell protection and anti-entry activity despite the observation that distinct mechanisms underlie these activities (Table 2).

Our investigations demonstrate that HIV attachment inhibitors provide potent protection against neuronal cell destruction by a variety of HIV strains. It appears that Env-mediated SH-SY5Y destruction does not operate through a cell-entry-associated mechanism, since fusion and CCR5 inhibitors do not inhibit Env-mediated neurotoxicity. Despite this apparent distinction in mechanism, the sensitivity of different HIV isolates to AI was comparable for both neuronal protection and anti-entry activities (Table 2). Since anti-entry sensitivity to AIs correlates with the affinity of these compounds for particular gp120 sequences [10], these observations suggest that both of these activities require AI binding and

probably the resulting conformational changes in gp120. For neuronal cell protection, such changes are likely to occlude the binding of a factor not required for viral entry that mediates neurocytotoxic signals. However, we cannot exclude the possibility that AI-bound gp120 behaves like platelet-derived growth factor or lithium, which can mitigate the toxic effects of the viral envelope on neuronal cells apparently by increasing anti-apoptotic signals without directly engaging gp120 [7, 18].

In conclusion, our studies illustrate the potential of AIs to prevent the loss of neurons from the direct effects of HIV if penetration of the blood–brain barrier can be achieved (a focus of ongoing research [16, 25]). The potentially unique benefits of this class of inhibitor would be increasingly relevant for an aging HIV-infected population with an increasing prevalence of CNS abnormalities and a diminished quality of life [5].

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