Long-Acting BMS-378806 Analogues Stabilize the State-1 Conformation of the Human Immunodeficiency Virus (HIV-1) Envelope Glycoproteins

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ABSTRACT

During human immunodeficiency virus (HIV-1) entry into cells, the viral envelope glycoprotein (Env) trimer $((gp120/gp41)_3)$ binds receptors, CD4 and CCR5, and fuses the viral and cell membranes. CD4 binding changes Env from a pre-triggered (State-1) conformation to more "open" downstream conformations. BMS-806 blocks CD4-induced conformational changes in Env important for entry and is hypothesized to stabilize a State-1-like Env conformation, a key vaccine target. Here, we evaluate the effects of BMS-806 on the conformation of Env on the surface of cells and virus-like particles. BMS-806 strengthened the labile, non-covalent interaction of gp120 with the Env trimer, enhanced or maintained the binding of most broadly neutralizing antibodies and decreased the binding of poorly neutralizing antibodies. Thus, in the presence of BMS-806, the cleaved Env on the surface of cells and virus-like particles exhibits an antigenic profile consistent with a State-1 conformation. We designed novel BMS-806 analogues that stabilized Env conformation for several weeks after a single application. These long-acting BMS-806 analogues may facilitate enrichment of the metastable State-1 Env conformation for structural characterization and presentation to the immune system.

IMPORTANCE

The envelope glycoprotein (Env) spike on the surface of the human immunodeficiency virus (HIV-1) mediates the entry of the virus into host cells and is also the target for antibodies. During virus entry, Env needs to change shape. Env flexibility also contributes to the ability of HIV-1 to evade the host immune response; many shapes of

Env raise antibodies that cannot recognize the functional Env and therefore do not block virus infection. We found that an HIV-1 entry inhibitor, BMS-806, stabilizes the functional shape of Env. We developed new variants of BMS-806 that stabilize Env in its natural state for long periods of time. The availability of such long-acting stabilizers of Env shape will allow the natural Env conformation to be characterized and tested for efficacy as a vaccine.

INTRODUCTION

The entry of human immunodeficiency virus (HIV-1) into target cells is mediated by envelope glycoprotein (Env) spikes on the viral membrane (1). HIV-1 Env trimers consist of three gp120 exterior glycoproteins and three gp41 transmembrane glycoproteins. The gp120 subunits of Env bind the target cell receptors, CD4 and either CCR5 or CXCR4 (2-5). CD4 binding drives Env from its pre-triggered (State-1) conformation to an obligate intermediate (State 2) and then to the full CD4-bound (State-3) conformation (6-9). In the State-3 Env, the gp41 heptad repeat (HR1) region is formed and exposed (10). As a result of receptor-induced conformational changes in Env, the hydrophobic fusion peptide at the gp41 N-terminus is thought to interact with the target cell membrane (11). Binding of gp120 to the CCR5 or CXCR4 chemokine receptor leads to formation of a highly stable gp41 six-helix bundle, in which the HR2 helix near the viral membrane binds in an antiparallel manner to the HR1 coiled coil (12-14). The favorable energy change associated with six-helix bundle formation is used to fuse the viral and target cell membranes (15).

BMS-378806 (herein called BMS-806) and its analogues are potent inhibitors of HIV-1 entry (16,17). BMS-663068 (Fostemsavir), the prodrug of the potent analogue BMS-626529 (Temsavir) (herein called BMS-529), exhibits favorable antiviral and pharmacokinetic properties and is being evaluated as an anti-HIV-1 therapy in clinical trials (18,19). BMS-806, BMS-529 and related HIV-1 entry inhibitors bind gp120 in a hydrophobic pocket located immediately adjacent to the CD4-binding site, between the

β20-β21 loop and the α1 helix (20). At concentrations in the range where potent antiviral effects are seen, BMS-806 analogues block CD4-induced conformational changes in Env that lead to the formation/exposure of the gp41 HR1 coiled coil (10,21,22). At higher concentrations, BMS-806 can impede CD4 binding (16-18,20,21,23). However, BMS-806 inhibits CD4-independent HIV-1 infection as efficiently as CD4-dependent infection (10,24), indicating an antiviral mechanism that does not necessarily involve CD4 (25).

During natural HIV-1 infection, antibodies are elicited to many distinct conformations of Env. The majority of these antibodies recognize Env conformations other than State 1; most of these antibodies are sterically blocked from accessing their epitopes after Env engages CD4 on the target cell, and thus have little or no ability to neutralize primary HIV-1 strains (26-28). We refer to these antibodies as poorly neutralizing antibodies. In a minority of individuals infected by HIV-1 for several years, antibodies capable of neutralizing a wide range of HIV-1 strains are elicited (29-32); we refer to these antibodies as broadly neutralizing antibodies (bNAbs). Most bNAbs recognize conserved epitopes on the surface of the State-1 Env conformation (6). Broadly neutralizing antibodies have not been elicited in animals or humans immunized with HIV-1 Env preparations, including stabilized soluble Env trimers (33-37). Conformational differences between these soluble trimers and State-1 membrane Envs have been documented (9,38), and could explain the difficulty of eliciting broadly neutralizing antibodies capable of recognizing the State-1 Env on infectious virions.

Efforts to characterize the structure of the State-1 Env conformation on the cell or viral surface and to present this conformation to the immune system might benefit from small-molecule ligands like BMS-806 that block Env transitions from State 1. Here, we evaluate the effects of BMS-806 and BMS-529 treatment on the conformation of HIV-1 Env on the surface of cells and virus-like particles. We found that these effects are consistent with stabilization of a State-1-like conformation and are reversible upon removal of the compounds from Env. We also developed novel BMS-806 analogues that are long-acting and stabilize a State-1-like conformation of membrane Env for at least 21 days after a single application. Such long-acting, State-1-stabilizing compounds should greatly assist efforts to characterize the metastable pre-triggered conformation of Env and allow its presentation to the immune system.

RESULTS

Effects of BMS-806 on the conformation of cell-surface Env

The full-length HIV-1_{AD8} Env was inducibly expressed in A549 cells (herein designated A549-Env cells). The efficiency with which Env is proteolytically processed in these cells allows an evaluation of the conformation of both the mature (cleaved) Env and the uncleaved Env. The cell-surface HIV-1_{AD8} Env was precipitated by a panel of Env ligands in the presence of BMS-806 or the DMSO vehicle control (Fig. 1, A and B). Consistent with previous observations (39-41), recognition of the mature HIV-1_{AD8} gp120 glycoprotein on the cell-surface Env trimer was highly correlated with the ability of gp120 ligands to neutralize HIV-1_{AD8} (Fig. 1A, left panel); in the absence of BMS-806,

149 broadly neutralizing antibodies exhibited significantly better gp120 recognition than
150 poorly neutralizing antibodies ($P < 0.0008$, two-tailed unpaired t test). This observation
151 suggests that the mature cell-surface Env conformationally resembles the functional
152 virion Env. In the absence of BMS-806, most broadly neutralizing antibodies (bNAbs)
153 recognized the cleaved Env. Some bNAbs (PG9, PG16, PGT145, PGT151 and 35O22)
154 directed against quaternary Env epitopes (42-45) recognized both uncleaved and
155 mature Envs, but exhibited preferential recognition of the cleaved Env (Fig. 1B). The
156 2F5, 4E10 and 10E8 bNAbs against the gp41 membrane-proximal external region
157 (MPER) (46,47) only inefficiently precipitated the gp120 glycoprotein in the absence of
158 BMS-806, although these MPER bNAbs recognized gp41 and the uncleaved gp160 Env
159 efficiently. Some MPER-directed bNAbs have been shown to recognize the pre-
160 triggered Env even though they exhibit higher affinity for Env conformations induced by
161 CD4 binding (48,49). Poorly neutralizing antibodies preferentially precipitated the
162 uncleaved Env, which samples non-State-1 conformations more readily than the mature
163 Env (50,51).
164
165 BMS-806 treatment altered Env recognition by several ligands (Fig. 1, A and B).
166 BMS-806 increased the recognition of the mature Env by some bNAbs, particularly
167 those against V2 quaternary epitopes (PG9, PG16 and PGT145) and the gp120-gp41
168 interface (PGT151 and 35O22). The binding of bNAbs (2G12 and PGT121) to glycan-
169 dependent gp120 outer domain epitopes (43,52) was not affected by incubation with
170 BMS-806. The binding of bNAbs (VRC01, VRC03 and 3BNC117) against the gp120
171 CD4-binding site (CD4BS) (53,54) was either unchanged or slightly decreased by BMS-

806. BMS-806 decreased the recognition of the uncleaved Env by the poorly neutralizing antibodies (17b, 19b, 902090 and F105) (55-59).

To help interpret the observed BMS-806 effects on ligand binding to cell-surface Env trimers, monomeric soluble HIV-1_{AD8} gp120 was precipitated by Env ligands in the absence and presence of BMS-806. With the exception of the PG9, PG16 and PGT145 bNAbs, which recognize quaternary V2 epitopes at the Env trimer apex (42,43), and the PGT151 and 35O22 bNAbs, which recognize epitopes at the gp120-gp41 interface (44,45), all of the Env ligands tested efficiently precipitated the monomeric gp120 glycoprotein (Fig. 1C and data not shown). The very weak binding of the PG9 antibody against a V2 quaternary gp120 epitope to monomeric gp120 was slightly increased by BMS-806 (Fig. 1C). BMS-806 mildly decreased gp120 binding of several CD4BS antibodies, the 17b antibody against a CD4-induced (CD4i) epitope (55), and a soluble CD4-immunoglobulin fusion protein (CD4-Ig) (Fig. 1C and data not shown). These observations indicate that some of the consequences of BMS-806 on cell-surface Env recognition by CD4BS and CD4i antibodies involve local effects within a gp120 subunit; this interpretation is consistent with the proximity of the BMS-806 binding site to these gp120 epitopes (20). Many of the observed effects of BMS-806 on the binding of antibodies, particularly those (PG16, PGT145, PGT151 and 35O22) recognizing epitopes dependent on Env quaternary structure (42-45), likely involve interactions that occur in the context of the Env trimer.

194 To summarize, in the presence of BMS-806, the mature cell-surface Env
195 maintained a high level of recognition by bNAbs and a low level of recognition by poorly
196 neutralizing antibodies, consistent with the pattern expected for a functional State-1 Env
197 trimer (Fig. 1A). These results corroborate single-molecule fluorescence resonance
198 energy transfer (smFRET) data indicating that HIV-1 membrane Envs incubated with
199 BMS-806 analogues maintain a State-1-like conformation of gp120 (references 6,9 and
200 see below). Based on diminished recognition by poorly neutralizing antibodies, the
201 sampling of non-State-1 conformations by the uncleaved Env on the cell surface
202 appears to be decreased in the presence of BMS-806.
203
204 BMS-806 inhibits CD4-induced conformational changes in Env and, at higher
205 concentrations, decreases Env-CD4 binding (10,16-18,20-23). We evaluated the effect
206 of BMS-806 on the recognition of the cell-surface HIV-1_{AD8} Env by CD4-Ig and C34-Ig.
207 CD4-Ig is a soluble CD4-immunoglobulin fusion protein, and C34-Ig contains the gp41
208 HR2 region fused to an immunoglobulin Fc (10). C34-Ig efficiently recognized the cell-
209 surface Env only after incubation with soluble CD4 (sCD4), as expected from the
210 dependence of gp41 HR1 formation/exposure on CD4 binding (Fig. 1D) (10). BMS-806
211 blocked C34-Ig recognition of Env in the presence of sCD4. At a high concentration (10
212 μM), BMS-806 inhibited CD4-Ig recognition of the gp120 glycoprotein on the mature
213 Env, but not the uncleaved gp160 Env. Titration of different concentrations of BMS-806
214 suggested that C34-Ig binding to Env was inhibited almost completely at 500 nM; at this
215 concentration, CD4-Ig binding to the gp120 and gp160 Envs was still detected. These
216 results verify that BMS-806 blocks CD4-induced conformational changes in Env; at

higher concentrations, BMS-806 decreases CD4 binding, moreso for the cleaved Env than the uncleaved Env.

Effects of BMS-806 on the conformation of Env on virus-like particles

To produce virus-like particles (VLPs), the A549-Env cells expressing HIV-1_{AD8} Env were transduced with a lentivirus vector encoding an HIV-1 Gag-mCherry fusion protein. The Gag-mCherry protein produces non-infectious VLPs with an immature core morphology; filtration and low-speed centrifugation of the A549-Gag/Env cell supernatants minimized the levels of extracellular vesicles in the VLP preparations (H. Ding, S. Zhang, H. Nguyen, S. Zou, J. Sodroski and J.D. Kappes, manuscript in preparation). Both mature and uncleaved Envs were incorporated into VLPs (see Input in Fig. 2, A and C).

The recognition of the VLP HIV-1_{AD8} Env by a panel of Env ligands was evaluated in the presence of BMS-806 or the control DMSO. In one assay format, the VLPs were solubilized in 1.5% Triton X-100 detergent before incubation with Env ligands (Fig. 2, A and B). In a second assay format, the VLPs were pre-incubated with Env ligands in the absence of detergent; the VLPs were then pelleted and washed prior to solubilization in detergent and precipitation of the bound Envs with Protein A-Sepharose beads (Fig. 2, C and D). The results of the two assays were similar except that Env recognition by the quaternary V2 bNAbs (PG9, PG16 and PGT145) was less efficient for the detergent-solubilized VLPs; moreover, without BMS-806 treatment, the recognition of the mature Env on the detergent-solubilized VLPs was slightly greater for

the poorly neutralizing antibodies (the 19b anti-V3 antibody, the 17b antibody against a CD4i gp120 epitope and the 902090 antibody against a linear V2 gp120 epitope). The VRC01 and VRC03 bNAbs against the CD4 binding site also precipitated the uncleaved and mature Envs more efficiently in the presence of Triton X-100. The recognition of the VLPs solubilized in 1.5% Cymal-5 detergent by the panel of antibodies was similar to that of the Triton X-100-solubilized VLPs (data not shown).

In the absence of BMS-806, all of the bNAbs recognized the mature Env on intact, detergent-free VLPs to varying degrees (Fig. 2C). BMS-806 enhanced the recognition of the mature gp120 Env by bNAbs (PG9, PG16, PGT145, PGT151 and 35O22) directed against Env epitopes dependent on quaternary conformation. In the presence of BMS-806, coprecipitation of gp120 by the bNAbs (2F5, 4E10 and 10E8) directed against the gp41 MPER was more efficient. BMS-806 reduced recognition of the uncleaved Env by the poorly neutralizing antibodies, and nearly eliminated the binding of the 19b and 17b antibodies to the mature gp120 Env. The effects of BMS-806 on antibody recognition of gp120 on both solubilized and intact VLPs directly correlate with the effects of BMS-806 on antibody recognition of cell-surface gp120 Env (Fig. 2, B and D). As was suggested by the analysis of cell-surface Env, BMS-806 binding appears to be compatible with maintenance of a State-1-like conformation of the mature VLP-associated Env.

Effect of BMS-806 on gp120 association with Env complexes

In the cell-surface and VLP Env recognition assays, we noted several instances where BMS-806 enhanced the coprecipitation of gp41 by an anti-gp120 antibody or the coprecipitation of gp120 by an anti-gp41 antibody. For example, the precipitation of cell-surface Env by the PGT121, VRC01 and VRC03 bNAbs yielded more gp41 in the presence of BMS-806, even though recognition of gp120 was not increased (Fig. 1B). The increased coprecipitation of gp41 by some anti-gp120 bNAbs in the cell-surface Env immunoprecipitation assay was observed when BMS-806 was present throughout the procedure (as in Fig. 1B) or only prior to cell lysis (data not shown). Conversely, in the presence of BMS-806, more gp120 was precipitated by the 2F5, 4E10 and 10E8 bNAbs against the gp41 MPER, even though BMS-806 did not increase the amount of precipitated gp41 (Fig. 2, A and C). These observations led us to hypothesize that BMS-806 stabilizes the non-covalent association of gp120 with the Env trimer. To test this hypothesis, we used the His_6 tag on the gp41 C-terminus to precipitate Env from detergent lysates of VLPs or cells expressing Env (Fig. 3A). The precipitates were then Western blotted with antibodies against gp120 and gp41. When the DMSO control was added to the lysates of VLPs prepared from A549 cells expressing HIV-1_{AD8} Env and Gag-mCherry, both gp160 and gp41 were efficiently precipitated by the Ni-NTA beads; however, little gp120 was coprecipitated with gp41 under these conditions (Fig. 3B). Addition of BMS-806 to the VLP lysates increased the amount of coprecipitated gp120. Addition of sCD4 or the small-molecule CD4-mimetic compound, BNM-III-170 (60), decreased even the small amount of gp120 coprecipitated in the presence of DMSO, suggesting that these ligands induced shedding of gp120, as expected (61,62). In

assays examining Envs from different HIV-1 strains in cell lysates, BMS-806 increased the amount of coprecipitated gp120, whereas sCD4 and BNM-III-170 decreased the amount of coprecipitated gp120 (Fig. 3C). Consistent with the ability of BMS-806 to interfere with CD4 binding and CD4-induced Env conformational changes (see above), the sCD4-induced shedding of gp120 from the Env complexes could be blocked by the addition of 10 μM BMS-806 to the cell lysates (data not shown). We conclude that BMS-806 stabilizes gp120 association with detergent-solubilized Env complexes.

BMS-806 analogues

The available crystal structures of BMS-806 and BMS-529 complexed with a soluble gp140 SOSIP.664 Env trimer (PDB 5U7O) indicate that the benzoyl ring of these compounds projects into a pocket on Env (20). A photoactivatable diazirine group was added to the benzoyl ring of BMS-529 to attempt to stabilize compound-Env interaction. However, this analogue (MF463) failed to inhibit HIV-1 infection and was not evaluated further (data not shown). Instead, photoactivatable diazirine and azide groups were added to the other end of the compound (see AEG-II-159 and AEG-III-087 (with diazirine groups) and AEG-II-168 and AEG-III-095 (with azide groups) in Table 1). (See Supplemental Material for details of the synthesis of these compounds.) Modeling studies suggested that these photoactivatable groups could be accommodated in complexes of the AEG compounds and soluble gp140 SOSIP.664 Env trimers (PDB 5U7O) and could make additional favorable contacts. Binding energy scores for the best poses of AEG-II-159 and AEG-II-168 were more favorable than that of the cognate docked pose of BMS-529: -13.3 and -14.5 kcal/mol,

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307 respectively, versus -12.0 kcal/mol. A representative pose of AEG-II-168 is shown in
308 Figure 4A and a corresponding interaction map in Figure 4B. All four AEG analogues
309 inhibited HIV-1 infection with potencies comparable to that of BMS-806 (Table 1). The
310 observed inhibition was completely abolished by the S375W change in Env, which fills
311 the Phe 43 cavity that accommodates the benzoyl ring of BMS-806 analogues (20,67).
312 Thus, the antiviral activity of AEG-II-159, AEG-II-168, AEG-III-087 and AEG-III-095
313 depends upon the availability of the Phe 43 cavity, a gp120 feature also required for
314 BMS-806 and BMS-529 binding.
315
316 To determine if the photoactivatable azide or diazirine groups are required for the
317 observed anti-HIV-1 activity of the AEG compounds, analogues lacking these groups
318 (AEG-III-032 and AEG-III-096) were synthesized and tested. AEG-III-032 and AEG-III-
319 096 inhibited HIV-1 infection specifically, with potencies comparable to that of BMS-806
320 (Table 1). Therefore, the photoactivatable azide or diazirine groups of AEG-II-159,
321 AEG-II-168, AEG-III-087 and AEG-III-095 are not required for anti-HIV-1-activity.
322
323 Effects of the AEG compounds on Env conformation
324 The effects of AEG-II-159, AEG-II-168, AEG-III-087 and AEG-III-095 on HIV-1_{AD8}
325 Env conformation were evaluated. The effects of the AEG compounds on the antigenic
326 profile of the HIV-1_{AD8} Env on cell surfaces or VLPs were generally similar to those of
327 BMS-806 (Fig. 5 and data not shown). Incubation with the AEG compounds resulted in
328 decreased recognition by poorly neutralizing antibodies, whereas Env recognition by
329 bNAbs was maintained or even increased. One exception was Env recognition by

CD4BS bNAbs (VRC01, VRC03), whose binding was moderately decreased by the AEG compounds; although BMS-806 also exhibited a similar effect, the additional moieties of the AEG compounds presumably add to this competitive inhibition.

The effects of BMS-806 and AEG-II-168 on antibody recognition of the cell-surface Env from the transmitted/founder (T/F) HIV-1_{CH848} strain were evaluated. As was seen for the HIV-1_{AD8} Env (Fig. 1, A and B; Fig. 5B, left panel), bNAbs generally maintained their binding to the mature HIV-1_{CH848} Env in the presence of these compounds (Fig. 5B, right panel). The mature HIV-1_{CH848} Env was recognized inefficiently by the poorly neutralizing antibodies in both the presence and absence of these compounds (data not shown). These results are consistent with the maintenance of a pre-triggered (State 1) Env conformation in the presence of BMS-806 and AEG-II-168.

We also examined the effects of AEG-II-168 on the conformation of the HIV-1_{JR-FL} Env on virions by smFRET. For these studies, smFRET probes were situated in the gp120 V1 and V4 variable regions and the ratio of labeled to unlabeled Envs was kept low so that a single protomer of the Env trimer could be evaluated (6,8,9). In the presence of saturating concentrations of AEG-II-168, the HIV-1_{JR-FL} virion Env maintained a State-1-dominant conformation (Fig. 6). These results are consistent with those of previous smFRET studies of the effect of BMS-806 and BMS-529 on HIV-1 Env conformation (6,9), and also are consistent with the above comparisons of Env antigenicity in the presence of the different BMS-806 analogues.

353

354 Effect of AEG compounds on gp120 association with the Env trimer

355 All four photoactivatable AEG compounds, as well as AEG-III-032 and AEG-III-
356 096 lacking photoactivatable groups, stabilized the association of gp120 with detergent-
357 solubilized HIV-1_{AD8} Env trimers (Fig. 7A and data not shown). AEG-II-168 also
358 stabilized gp120 association with HIV-1_{CH848} Env complexes solubilized in NP-40 lysis
359 buffer (data not shown).

360

361 We evaluated the effect of BMS-806 and analogues on the spontaneous
362 shedding of gp120 from HIV-1_{AD8} VLPs at 4°C and 37°C in the absence of detergent.
363 BMS-806, AEG-II-168, AEG-III-087 and AEG-III-095 decreased the shedding of gp120
364 into the VLP supernatant at 4°C and 37°C (Fig. 7, B and C). We conclude that these
365 compounds stabilize the interaction of gp120 with the native membrane Env trimer
366 under physiologic conditions.

367

368 Reversibility of BMS-806 and BMS-529 binding to VLP Env

369 The BMS-806-induced decrease in binding of the 19b anti-V3 antibody can be
370 used as an indicator of BMS-806-Env binding, allowing estimation of the stability of the
371 Env-compound complex. BMS-806 and BMS-529 were incubated with the HIV-1_{AD8}
372 Env on VLPs. The VLPs were then washed and incubated at room temperature for
373 various lengths of time in a buffer with (+/+) or without (+/-) compound. After this
374 incubation, Env conformation was assessed using the 19b anti-V3 antibody, the 2G12
375 antibody (the binding of which is not affected by BMS-806), and a negative control

human immunoglobulin (hIgG) preparation. We compared 19b recognition of Env in the washed samples (+/-) with those in the samples continuously incubated with the compound (+/+) and the untreated samples (-) (Fig. 8A). By twenty-four hours, the conformational effects of BMS-806 on 19b binding were much less evident. The effects of the more potent analogue BMS-529 were still apparent at twenty-four hours, but disappeared by 2-4 days. These results agree with a previous study suggesting that the binding of BMS-806 analogues to HIV-1 Env occurs with a slow off-rate (18); despite this slow rate of dissociation of BMS-806 analogues from Env, the binding and the consequent effects of these compounds on Env conformation appear to be reversible.

Long-term effects of AEG compounds on Env conformation

We used the decrease in 19b antibody recognition to evaluate the stability of the association of the AEG compounds with HIV-1_{AD8} Env on VLPs. Pilot experiments established ultraviolet radiation doses that were compatible with our assay (data not shown). Surprisingly, even without ultraviolet (UV) irradiation, the effects of AEG-II-159, AEG-II-168, AEG-III-087 and AEG-III-095 on 19b recognition persisted for at least 3 weeks after washing (Fig. 8B and data not shown). The time-dependent association of the compounds with HIV-1_{AD8} Env on VLPs is shown in Figure 8C. We note that the effects of AEG-III-032, which lacks a photoactivatable group, on Env conformation were significantly reduced by 7 days following removal of the VLPs from the compound solution (Fig. 8C). Likewise, AEG-III-096, another analogue lacking a photoactivatable group, exhibited much faster reversibility than the corresponding compounds (AEG-III-087 and AEG-III-095) with diazirine and azide groups, respectively (Fig. 8C).

399 Apparently, the durability of the effects of AEG-II-159, AEG-II-168, AEG-III-087 and
400 AEG-III-095 on HIV-1 Env conformation is enhanced by the presence of the
401 photoactivatable groups, but is not dependent on UV crosslinking.
402
403 Long-acting BMS-806 analogues potentially could be used to stabilize a State-1
404 conformation in Env immunogens. We compared the antigenic profile of HIV-1_{AD8} Env
405 in VLPs two weeks after each of the following treatments: buffer/DMSO, UV irradiation,
406 and AEG compounds (AEG-II-168, AEG-III-087 and AEG-III-095) plus UV irradiation.
407 Following these treatments, the VLPs were pelleted, washed and resuspended in buffer
408 without compound at 4°C for two weeks, at which time a panel of antibodies was used
409 to evaluate the Env conformation. All three AEG compounds lowered Env recognition
410 by poorly neutralizing antibodies and, with the exception of the CD4BS antibodies,
411 maintained the integrity of the bNAb epitopes (Fig. 9 and data not shown). Treatment of
412 the VLPs with AEG-II-168 and UV irradiation resulted in increased recognition by
413 several bNAbs (PG9, PGT145, PGT151, 35O22, 2F5, 4E10 and 10E8); this increase
414 was not seen for AEG-III-087 or AEG-III-095. UV irradiation of VLP Env in the presence
415 of AEG-II-168 also led to an intensification of the gp160 band and the appearance of
416 two new bands of approximately 240 and 280 kD. We speculate that these represent
417 gp120-gp41, gp120-gp120 and gp120-gp120-gp41 crosslinked products, respectively;
418 the long extension (furan ring-amide linkage-tetrafluorobenzene ring) between the
419 gp120-docking portion and the photoactivable azide of AEG-II-168 apparently allows
420 these crosslinks to form more readily than in AEG-III-087 and AEG-III-095, which have
421 shorter spacers. Mass spectrometric analysis of UV-irradiated Env preparations

422 incubated with AEG-II-168 failed to identify specific crosslinks (data not shown). In
423 summary, the above results indicate that several of the AEG compounds can exert long-
424 term stabilizing effects on State-1-like Env conformations.
425

426 **DISCUSSION**

427

428 In cells expressing HIV-1 Envs, both mature (cleaved) and uncleaved Envs are
429 transported to the cell surface. The mature, functional Env, which largely resides in a
430 State-1 conformation (6-9), is able to be recognized by most bNAbs but not by poorly
431 neutralizing antibodies (38-41). The uncleaved Env has been suggested to assume a
432 number of conformations and therefore binds both bNAbs and poorly neutralizing
433 antibodies (50,51). In general, we found that the binding of most bNAbs to the mature
434 Env trimer is maintained or increased in the presence of BMS-806, whereas BMS-806
435 inhibits the binding of poorly neutralizing antibodies to the uncleaved Env. The effects
436 of BMS-806 on the antigenicities of the cleaved cell-surface Env and the cleaved VLP
437 Env strongly correlated. The differential effects of BMS-806 on the binding of bNAbs
438 and poorly neutralizing antibodies are consistent with the compound stabilizing a State-
439 1-like, pre-triggered conformation of the membrane Env trimer. Several smFRET
440 studies of HIV-1 virion Env, including this study (Fig. 6), corroborate a model in which
441 BMS-806 analogues bind and maintain a functional State-1 Env conformation (6,9).

442

443 The gp120 exterior Env associates non-covalently with the mature Env trimer,
444 creating the potential for dissociation (shedding) of gp120 from the Env complex
445 (61,62). The shedding of gp120 occurs spontaneously, but is enhanced by the binding
446 of CD4 (61,62); apparently, the CD4-induced opening of the Env trimer to the State-3
447 conformation destabilizes the association of the gp120 subunits with the membrane-
448 anchored gp41 subunits. We found that BMS-806 and its analogues strengthened

449 gp120:Env association on the viral surface under physiologic conditions, as well as in
450 detergent lysates. BMS-806-induced enrichment of a pretriggered, State-1-like Env
451 conformation and the consequent decrease of open State-3 Env conformations likely
452 relates to the observed stabilization of gp120:trimer association. This assertion is
453 supported by the observed BMS-806-induced increases in the binding of several bNAbs
454 to quaternary epitopes that span the subunits of the State-1 Env trimer. As one
455 example, BMS-806 enhanced the binding of the V2 quaternary bNAbs (PG9, PG16,
456 PGT145), which recognize carbohydrate-dependent epitopes at the membrane-distal
457 apex of the Env trimer formed by interactions of the gp120 protomers (42,43,63). HIV-1
458 mutants with Env changes that destabilize the State-1 conformation exhibit decreased
459 sensitivity to the V2 quaternary antibodies, suggesting that these antibodies
460 preferentially, although not exclusively, recognize a State-1 Env conformation (7,64).
461 BMS-806 induced smaller enhancements of the binding of the PGT151 and 35O22
462 bNAbs, which recognize quaternary structures spanning the gp120-gp41 interface
463 (44,45).
464
465 What is the mechanism by which the binding of a small molecule like BMS-806
466 stabilizes a State-1-like Env trimer structure? Recent data suggest that current high-
467 resolution structures of HIV-1 Env trimers more closely represent State 2 than State 1
468 (9). In the absence of a detailed State-1 Env trimer structure, it is difficult to address the
469 question above with precision. Nonetheless, crystal structures of BMS-806 and its
470 analogues have been solved in complexes with soluble gp140 SOSIP.664 trimers (20),
471 which are in a State-2-like conformation (9). These observed BMS-806 and BMS-529

472 binding sites are consistent with data on HIV-1 escape mutants and are likely relevant
473 to functional Envs (16,20,23,65). In these structures, BMS-806 and its analogues bind
474 in a hydrophobic pocket between the gp120 inner domain α1 helix and the β20-β21 loop
475 (20). This pocket is adjacent to both the CD4 binding site and the trimer association
476 domain, which governs the quaternary interactions among gp120 protomers. Thus,
477 BMS-806 may be well-positioned to influence the adjacent trimer association domain,
478 which includes the V1/V2 and V3 variable regions. The trimer association domains of
479 gp120 are thought to "open" as State 1 makes transitions to downstream conformations,
480 either spontaneously or as a result of CD4 binding (6-9,66-68). BMS-806 may limit the
481 flexibility of the trimer association domain, predisposing Env to maintain a State-1-like
482 gp120 conformation. Such a model is consistent with our observation that BMS-806
483 enhances Env recognition by V2 quaternary antibodies. Indeed, some BMS-806-like
484 HIV-1 entry inhibitors have been shown to impede the decrease in Env recognition by
485 V2 quaternary antibodies that accompanies CD4 binding (22). Although BMS-806 did
486 not exhibit this activity, BMS-806 can apparently decrease the spontaneous sampling of
487 more open State 2/3 Env conformations (6,9,22); the blockade of spontaneous
488 transitions is presumably more readily achieved by a small molecule than the blockade
489 of the changes in the gp120 trimer association domain induced by a large protein like
490 CD4.
491
492 A more universal activity observed for the BMS-806 class of HIV-1 entry
493 inhibitors is the blockade of formation/exposure of the gp41 HR1 coiled coil (10,21,22).
494 As the formation/exposure of the HR1 coiled coil is a natural consequence of CD4

binding to gp120 (10), conformational changes in gp120 that are impeded by BMS-806 may be prerequisites for these gp41 HR1 transitions. BMS-806 may also more directly influence gp41 in the as-yet-uncharacterized State-1 conformation. We observed that BMS-806 inhibited gp41 HR1 formation/exposure, as measured by C34-Ig binding, in response to sCD4 in different contexts, even in detergent lysates of VLPs.

Our assays measuring the reversibility of Env conformational effects of BMS-806 analogues indicate a remarkably slow off-rate of these compounds. Even the parental compounds, BMS-806 and BMS-529, as well as the control compounds, AEG-III-032 and AEG-III-096, which lack photoactivatable groups, required 1-4 days to reverse their conformational effects on Env. A previous study noted the slow off-rate of BMS-529 (18). Insertion of these compounds into a conserved, hydrophobic interdomain interface on Env may impose unfavorable enthalpic and entropic penalties on their extraction from Env and resolubilization. The addition of photoactivatable groups in AEG-II-159 and AEG-II-168 significantly decreased their reversibility, with maintenance of Env conformational effects for at least three weeks. Surprisingly, even without UV irradiation and even when exposure to visible light was minimized, this prolongation of Env conformational effect was observed. The antigenic profile of Env was not apparently altered by UV irradiation, leaving open the possibility of UV crosslinking these or other BMS-806 analogues in the future.

The availability of BMS analogues that can maintain State-1-like Env conformations for prolonged time periods may assist presentation of this conformation

to the immune system. Given that State 1 is targeted by many bNAbs and is intrinsically metastable, limiting the conformations of Env immunogens to State 1 may facilitate the elicitation of bNAbs. The context in which Env immunogens are formulated could influence the success of this approach; for example, BMS analogues enrich State-1-like conformations in membrane-anchored Envs better than in soluble Env trimers (9, M. Lu, X. Ma, N. Reichard, D.S. Terry, J. Arthos, A.B. Smith III, J.G. Sodroski, S.C. Blanchard and W. Mothes, submitted). The influence of the BMS analogues on the immunogenicity of bNAb epitopes near their binding site will need to be determined empirically. BMS analogues with prolonged activity may also assist structural studies of State-1-like conformations, which could benefit from the availability of Env trimer preparations that are enriched in these pre-triggered conformations.

MATERIALS AND METHODS

BMS-806 and BMS-529

BMS-378806 (herein called BMS-806) and BMS-626529 (Temsavir) (herein called BMS-529) were purchased from Selleckchem and APExBIO, respectively.

Synthetic Chemistry

The synthesis of the AEG compounds is described in the Supplemental Material for Publication.

Envelope glycoprotein constructs

The HIV-1_{JR-FL} Env and HIV-1_{AD8} mutant Envs were coexpressed in HOS cells with the Rev protein by the pSVIIIenv expression vector, using the natural HIV-1 *env* and *rev* sequences. The plasmids for the expression of HIV-1 virions with the HIV-1_{JR-FL} Env used for single-molecule FRET have been previously described (6). The wild-type HIV-1_{AD8} and HIV-1_{CH848} Envs were expressed in A549 cells using a lentivirus vector, as described below.

Antibodies

Antibodies against HIV-1 Env were kindly supplied by Dr. Dennis Burton (Scripps), Drs. Peter Kwong and John Mascola (Vaccine Research Center, NIH), Dr. Barton Haynes (Duke), Dr. Michel Nussenzweig (Rockefeller), Dr. Hermann Katinger (Polymun), Dr. James Robinson (Tulane) and Dr. Marshall Posner (Mount Sinai Medical Center). In some cases, anti-Env antibodies were obtained through the NIH AIDS Reagent Program. Antibodies for Western blotting include goat anti-gp120 polyclonal antibody (ThermoFisher), the 4E10 anti-gp41 antibody (Polymun), and anti-Gag p55/p24/p17 (Abcam). An HRP-conjugated rabbit anti-goat IgG antibody (ThermoFisher) or HRP-conjugated goat anti-human IgG (Santa Cruz) were used as secondary antibodies for Western blotting.

Cell lines

293T cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml of penicillin-streptomycin. Cf2Th-CD4/CCR5 cells stably expressing the human CD4 and CCR5 coreceptors for HIV-1 were grown in the same

564 medium supplemented with 0.4 mg/ml of G418 and 0.2 mg/ml of hygromycin. HOS
565 cells (ATCC) were grown in DMEM with 5% FBS and 100 µg/ml of penicillin-
566 streptomycin. The A549 lung epithelial cells (ATCC) were grown in DMEM/F12, 10%
567 FBS supplemented with L-glutamine and penicillin-streptomycin. All cell culture
568 reagents are from Life Technologies.
569
570 Human A549 cells inducibly expressing Env or Env on virus-like particles (VLPs)
571 were established. A549 cells constitutively expressing the reverse tet transactivator
572 were transduced with an HIV-1-based lentivirus vector expressing Rev and Env from
573 HIV-1_{AD8}, a primary HIV-1 strain. The vector transcribes a bicistronic mRNA comprising
574 HIV-1_{AD8} *rev* and *env* and two selectable marker genes (puromycin-T2A-enhanced
575 green fluorescent protein (EGFP)) fused in-frame with a T2A peptide-coding sequence.
576 In the transduced cells, Env expression is controlled by the Tet-Responsive Element
577 (TRE) promoter and tet-on transcriptional regulatory elements. A similar strategy was
578 used to express Rev and the transmitted/founder HIV-1_{CH848} Env in A549 cells. Env-
579 expressing cells were enriched by doxycycline induction and fluorescence-activated cell
580 sorting for the co-expressed EGFP marker. Approximately 72 hours after treatment of
581 these cells with 2 µg/ml doxycycline, the HIV-1_{AD8} and HIV-1_{CH848} gp160 Env precursor
582 and the mature gp120 and gp41 glycoproteins were expressed. Herein, we designate
583 these cells A549-Env.
584
585 To produce cells expressing Env and VLPs, the A549-Env cells expressing the
586 HIV-1_{AD8} Env were transduced with a lentivirus vector expressing the HIV-1 Gag

precursor fused with mCherry. The doxycycline-regulated expression of the Gag-mCherry fusion protein resulted in the release of Env-containing VLPs into the medium. Herein, we designate these cells A549-Gag/Env.

Immunoprecipitation of cell-surface Env

Doxycycline-induced A549-Env cells were washed twice with washing buffer (1 x PBS + 5% FBS), with or without 10 μM BMS-806 or an analogue. The cells were then incubated with 5 μg/ml antibody for one hour at 4°C in the continued presence or absence of the BMS-806 analogue. After washing four times in washing buffer, the cells were lysed in NP-40 lysis buffer (0.5% NP-40, 0.5 M NaCl, 10 mM Tris, pH 7.5) for five minutes at 4°C with gentle agitation. For the samples with a BMS-806 analogue, the compound was added to the NP-40 lysis buffer at a 10 μM concentration. The lysates were cleared by centrifugation at 13,200 x g for 20 minutes at 4°C, and the clarified supernatants were incubated with Protein A-Sepharose beads (50 μl of 25 mg/ml in PBS per sample) for one hour at room temperature. The beads were pelleted (1000 rpm x 1 min) and washed three times with final wash buffer (200 mM Tris-HCl, pH 8.0, 100 mM $(NH_4)_2SO_4$, 1 M NaCl and 0.1% NP-40). The beads were suspended in LDS sample buffer, boiled and analyzed by Western blotting using 1:2000 goat anti-gp120 polyclonal antibody (ThermoFisher) and 1:2000 HRP-conjugated rabbit anti-goat IgG (ThermoFisher). The transmembrane Env was analyzed by Western blot with the 4E10 anti-gp41 antibody and HRP-conjugated goat anti-human IgG (Santa Cruz).

609 For analysis of total Env expression in the cell, some of the clarified lysates were
610 saved before the addition of Protein A-Sepharose beads and Western blotted as
611 described above ("Input" samples). Detection of the antibody heavy and light chains in
612 the gp41 Western blots provides an indication of the amount of antibody added,
613 captured and loaded in each experiment.
614
615 **Antibody recognition of monomeric gp120**
616 To produce gp120 monomers, a stop codon was introduced into the HIV-1_{AD8} *env*
617 gene sequence encoding the gp120-gp41 junction. Transfection of 293F cells with this
618 plasmid DNA resulted in the transient expression of a secreted, monomeric gp120
619 glycoprotein into cell supernatants. The supernatants were clarified by low-speed
620 centrifugation and 0.45-μ filtration, and then used for precipitation by antibodies in the
621 absence or presence of 10 μM BMS-806. Precipitates were Western blotted with a goat
622 anti-gp120 polyclonal antibody (ThermoFisher) and 1:3000 HRP-conjugated rabbit anti-
623 goat IgG antibody (ThermoFisher).
624
625 **Characterization of VLP-associated Env**
626 To prepare VLPs with HIV-1_{AD8} Env, 150-mm dishes of 30-40% confluent A549-
627 Gag/Env cells were seeded and, on the following day, treated with 2 μg/ml doxycycline.
628 Approximately 72 hours after induction, cell supernatants were harvested and cleared
629 by low-speed centrifugation (500 x g for 15 minutes at 4°C) and 0.45-μ filtration. VLPs
630 were pelleted by centrifugation at 100,000 x g for one hour at 4°C. The resuspended
631 VLP preparation was clarified by low-speed centrifugation. BMS-806 and its analogues

were added to the clarified VLP-containing suspensions. In some cases, the VLP-compound mixtures were irradiated with a 100-watt, 365-nm ultraviolet (UV) lamp for 10 minutes at room temperature. For all UV studies, exposure of the samples to visible light was minimized.

For studies of the reversibility of BMS-806 analogue effects, the compounds were added to the VLPs and mixed for several seconds at room temperature. Then the VLPs were pelleted (20,000 x g for 30 minutes at 4°C) twice, washing with 1.5 ml PBS with 2% DMSO. The pelleted VLPs were resuspended in 1 ml PBS with 2% DMSO and incubated at room temperature for various lengths of time (20 minutes – 3 weeks). Control VLPs were incubated in the continued presence of the BMS-806 analogues at room temperature for the same length of time. Then the VLPs were pelleted (20,000 x g for 30 minutes at 4°C) and the pellet incubated with 1.5% Triton X-100 for 30 minutes at 4°C. The VLP lysates were centrifuged (20,000 x g for 30 minutes at 4°C) and the supernatants were incubated with antibodies (10 µg/ml) in a 50-µl volume with 25 mg/ml of Protein A-Sepharose beads for one hour at 4°C. The beads were pelleted (1000 rpm for 1 minute at room temperature) and washed three times with 1 ml wash buffer with 1% Triton X-100. The beads were suspended in NuPAGE LDS Sample Buffer (ThermoFisher), boiled and analyzed by Western blot, as described above.

For studies of the binding of ligands (antibodies, CD4-Ig and C34-Ig) to detergent-solubilized VLP Env, VLPs prepared as described above were pelleted (20,000 x g for 30 minutes at 4°C). The pellet was incubated with 1.5% Triton X-100 for

30 minutes at 4°C. The VLP lysates were centrifuged (20,000 x g for 30 minutes at 4°C) and the supernatants were incubated with ligands (10 µg/ml) in a 50-µl volume with 25 mg/ml of Protein A-Sepharose beads for one hour at 4°C. The beads were pelleted (1000 rpm for 1 minute at room temperature), washed three times with 1 ml wash buffer with 1% Triton X-100, suspended in LDS Sample Buffer and analyzed by Western blot, as described above.

In a second assay format, we examined the effect of BMS-806 analogues on the binding of antibodies to Env on intact VLPs, in the absence of detergent. In this case, VLPs in A549-Gag/Env supernatants, clarified as described above, were pelleted at 100,000 x g for one hour at 4°C. The pellet was resuspended in PBS and the VLPs pelleted at 20,000 x g for 30 minutes at 4°C. The VLPs were resuspended in PBS and incubated with antibodies (10 µg/ml) in a 100-µl volume for one hour at 4°C. The VLPs were then pelleted (20,000 x g for 30 minutes at 4°C) and washed with PBS three times. The VLP pellet was then solubilized in 1.5% Triton X-100 for 30 minutes at 4°C, after which the VLP lysates were clarified by centrifugation at 20,000 x g for 30 minutes at 4°C. The supernatants were incubated with 50 µl of Protein A-Sepharose beads for one hour at 4°C. The beads were pelleted (1000 rpm for 1 minute at room temperature), washed three times with 1 ml wash buffer with 1% Triton X-100, suspended in LDS Sample Buffer, and analyzed by Western blot, as described above.

Association of gp120 with Env complexes

The non-covalent association of gp120 with HIV-1 Env complexes was studied using carboxy-terminally His_6-tagged Envs from three different sources: 1) VLPs produced from A549-Gag/Env cells; 2) A549 cells expressing HIV-1_{AD8} and HIV-1_{CH848} Envs; and 3) HOS cells transiently expressing wild-type HIV-1_{JR-FL} Env. VLP and cell lysates from A549 cells were prepared in 1.5% Triton X-100. Cell lysates from HOS cells were prepared in 1.5% Cymal-5 (Anatrace). The VLP and cell lysates were clarified as described above. DMSO, a BMS-806 analogue, sCD4 or the CD4-mimetic compound, BNM-III-170 (60), was added to the lysate. Aliquots of the lysates were saved for Western blotting to detect the gp160, gp120 and gp41 glycoproteins in the input sample. The bulk of the lysates was incubated with nickel-nitriloacetic acid (Ni-NTA) beads (Qiagen) for 1.5 hours at 4°C. The beads were pelleted (1000 rpm for 1 minute at room temperature), washed 3 times at room temperature with wash buffer with 1% Triton X-100, boiled in LDS sample buffer, and analyzed by Western blotting as described above. The association of gp120 with the Env complex was calculated as follows: $[(gp120/gp160)_{compound} \times (Input\ gp120/Input\ gp160)_{DMSO}] \div [(gp120/gp160)_{DMSO} \times (Input\ gp120/Input\ gp160)_{compound}]$.

Shedding of gp120 from VLP Env

The effect of BMS-806 analogues on the spontaneous shedding of gp120 from VLP Env was evaluated. VLPs with the wild-type HIV-1_{AD8} Env were prepared from the supernatants of A549-Gag/Env cells, as described above. The VLPs were suspended in PBS, to which was added either DMSO or a BMS-806 analogue. An aliquot of the VLP suspension in PBS/DMSO was processed as described below to serve as a Day 0 control. The VLP suspensions were incubated for 4 days at 4°C, room temperature or

37°C (with gentle rocking). Then the VLPs were pelleted (100,000 x g for 30 min at 4°C). The pellets and supernatants were boiled in LDS sample buffer and analyzed by Western blotting with either 1:2000 goat anti-gp120 polyclonal antibody (ThermoFisher) and HRP-conjugated rabbit anti-goat IgG antibody, or with 1:5000 rabbit anti-Gag p55/p24/p17 antibody (Abcam) and HRP-conjugated goat anti-rabbit IgG antibody (Abcam).

Infection of single-round recombinant viruses

To produce single-round HIV-1 expressing luciferase, 293T human embryonic kidney cells were cotransfected with plasmids expressing the pCMVΔP1Δenv HIV-1 Gag-Pol packaging construct, the HIV-1 envelope glycoproteins or the envelope glycoprotein of the control amphotropic murine leukemia virus (AMLV), and the firefly luciferase-expressing vector at a DNA ratio of 1:1:3 μg using the Effectene transfection reagent (Qiagen) (65). The plasmids expressing the HIV-1 envelope glycoproteins and Rev protein were based on pSVIIIenv or pcDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA). Cotransfection produced recombinant, luciferase-expressing viruses capable of a single round of infection. The virus-containing supernatants were harvested between 36 and 40 h after transfection and cleared of debris by low-speed centrifugation. Aliquots of the virus preparations were frozen at -80°C until further use. The reverse transcriptase (RT) levels of all virus stocks were measured.

Cf2Th-CD4/CCR5 target cells were seeded at a density of 6 x 10^3 cells/well in 96-well luminometer-compatible tissue culture plates (PerkinElmer) 24 h before

724 infection. On the day of infection, BMS-806 analogues (0-100 nM) were incubated with
725 recombinant viruses (10,000 RT units) at 37°C for 30 min. The virus-compound
726 mixtures were added to the target cells and incubated for 48 h at 37°C. After this time,
727 the medium was removed from each well and the cells were lysed by the addition of 30
728 µl passive lysis buffer (Promega) and three freeze-thaw cycles. An EG&G Berthold LB
729 96 V microplate luminometer was used to measure the luciferase activity in each well
730 after the addition of 100 µl of luciferin buffer (15 mM $MgSO_4$, 15 mM KPO_4, pH 7.8, 1
731 mM ATP, and 1 mM dithiothreitol) and 50 µl of 1 mM 99% Firefly d-luciferin free acid
732 (Prolume).
733
734 **Single-molecule fluorescence resonance energy transfer (smFRET)**
735 Viruses with HIV-1_{JR-FL} Env that is double-tagged at V1-Q3 and V4-A1 were
736 prepared for smFRET imaging, as previously described (6). The high (40:1) ratio of
737 wild-type Env to tagged Env ensures that, on average, only one tagged protomer is
738 available for imaging on a single virus particle. The Q3 and A1 double-tagged viruses
739 allowed the incorporation of Cy3B and Cy5 fluorescent labels, respectively. Thus, the
740 relative movements of the V1 and V4 regions in one gp120 subunit of an individual Env
741 trimer could be monitored in real time. The smFRET images were acquired on an in-
742 house-built total internal reflection fluorescent microscope, as described (6). HIV-1_{JR-FL}
743 viruses incubated with saturating concentrations (100 µM) of AEG-II-168 or control HIV-
744 1_{JR-FL} viruses without compound were used for smFRET imaging. Data were analyzed,
745 as described, with the customized MATLAB (Mathworks) program SPARTAN (69).
746 FRET trajectories meeting the criteria of quality (sufficient signal:noise ratio, single dye

747 photobleaching, anti-correlated features between donor and acceptor intensity and
748 fluorescence lifetime) were compiled into FRET histograms. Hidden Markov modeling
749 was used to fit the FRET histograms with the sum of three Gaussian distributions. The
750 three-state model yielded the lowest log likelihood value in this case.
751
752 **Code availability**
753 The customized Matlab (Mathwoks) program SPARTAN for smFRET analysis is
754 publicly available at https://www.scottblanchardlab.com/software.
755
756 **Modeling BMS-806 analogue interaction with Env**
757 The HIV-1_{BG505} soluble gp140 SOSIP.664 trimer complexed with BMS-529 (PDB
758 5U7O) (20) was used as a docking target and prepared using Maestro 12.0.012
759 (Schrodinger 2019). The 20 best-ranked poses for BMS-529, AEG-II-168 and AEG-II-
760 159 were determined by Glide (Schrodinger).
761
762 **Statistical analysis**
763 Statistical analyses were performed using GraphPad Prism 6 (Graph Pad
764 Software).
765
766 The Kolmogorov-Smirnov test was used to check the data distribution in Figure
767 1. A two-tailed paired Student's t-test or Wilcoxon matched-pairs signed-rank test was
768 used to compare two groups and to determine if the data fitted a normal distribution,
769 respectively.

Pearson's correlation was used to analyze the correlation between groups in Figure 2.

In all cases we used a P value less than 0.05 as a cutoff for statistical significance.

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Table 1. Inhibition of HIV-1 infection by BMS-806 analogues.

Recombinant, luciferase-expressing single-round HIV-1 with the indicated HIV-1 Envs or the envelope glycoproteins of the amphotropic murine leukemia virus (AMLV) were incubated with different concentrations of BMS-806 analogues for 30 minutes at 37°C. The virus-compound mixtures were then incubated with Cf2Th-CD4/CCR5 target cells for 48 hours at 37°C in 5% CO_2. Then the cells were lysed and luciferase activity was measured. The 50% inhibitory concentrations (IC_{50} values in nM) were calculated from four independent experiments and are reported as means and standard errors. ND – not determined.

FIGURE LEGENDS

Figure 1. Effect of BMS-806 on the conformation of cell-surface Env. **A.** A549-Env cells expressing HIV-1_{AD8} Env were incubated with the indicated antibodies, in the presence of 10 μM BMS-806 (+) or the DMSO vehicle control (-). The cells were then washed and lysed, and cell lysates were incubated with Protein A-Sepharose beads. For the samples with BMS-806, the compound was also added to the cell lysates at a 10 μM concentration. Precipitates were analyzed by Western blotting with a rabbit anti-gp120 antiserum or the 4E10 anti-gp41 antibody. The precipitation of the gp120 and gp160 glycoproteins was quantified from two independent experiments like that shown in **B**, and is reported relative to the input level of each glycoprotein. Recognition of the indicated Env glycoprotein by poorly neutralizing antibodies and bNAbs was compared in the absence and presence of BMS-806, using a two-tailed paired Students' t-test. $*$ $P < 0.05$; ns – not significant. The HIV-1 Env epitopes recognized by the antibodies are indicated in parentheses: CD4i – CD4-induced gp120 epitope; CD4BS – CD4-binding site gp120 epitope; V2q – V2 quaternary gp120 epitope; OD glycan – gp120 outer domain glycans; gp120-gp41 – gp120-gp41 interface; and MPER – membrane-proximal external region of gp41. **B.** A representative experiment used to produce the results in **A** is shown. The upper panels were Western blotted with a rabbit anti-gp120 antiserum, the lower panels with the 4E10 anti-gp41 antibody. hIgG – human immunoglobulin G. **C.** Monomeric soluble HIV-1_{AD8} gp120 was precipitated by the indicated antibodies in the absence or presence of 10 μM BMS-806. The precipitated proteins were Western blotted with a rabbit anti-gp120

1183 antiserum. **D.** Recognition of cell-surface Env by C34-Ig was assessed in the absence
1184 or presence of sCD4 and BMS-806. In parallel, recognition of cell-surface Env by CD4-
1185 Ig and by a negative control, human IgG (hIgG), was studied.
1186
1187 **Figure 2. Effect of BMS-806 on the conformation of VLP Env.**
1188 **A.** VLPs prepared from the supernatants of A549-Gag/Env cells expressing HIV-1_{AD8}
1189 Env and Gag-mCherry were solubilized in Triton X-100, and the VLP lysates were
1190 incubated with the indicated antibodies in the presence of BMS-806 (+) or the DMSO
1191 vehicle control (-). Precipitates were Western blotted with a rabbit anti-gp120 antibody
1192 (upper panels) or the 4E10 anti-gp41 antibody (lower panels). **B.** Correlation between
1193 the effect of BMS-806 on antibody recognition of gp120 from VLPs solubilized as in **A**
1194 and the effect of BMS-806 on recognition of cell-surface gp120 Env (as in Fig. 1A and
1195 B). In each case, the effect of BMS-806 represents gp120 precipitation by the antibody
1196 in the presence of BMS-806 divided by gp120 precipitation in the absence of BMS-806.
1197 r, Pearson correlation coefficient. **C.** VLPs from A549-Gag/Env cell supernatants were
1198 incubated with antibodies in the presence of BMS-806 (+) or the DMSO vehicle control
1199 (-), and then pelleted and washed. The repelleted VLPs were solubilized in Triton X-
1200 100 and the VLP lysates were incubated with Protein A-Sepharose beads. Precipitates
1201 were Western blotted with a rabbit anti-gp120 antibody (upper panels) or the 4E10 anti-
1202 gp41 antibody (lower panels). **D.** Correlation between the effect of BMS-806 on
1203 antibody recognition of gp120 Env on intact, detergent-free VLPs (as in **C**) and the
1204 effect of BMS-806 on recognition of cell-surface gp120 Env (as in Fig. 1A). In each
1205 case, the effect of BMS-806 represents the precipitation of gp120 by the antibody in the

presence of BMS-806 divided by gp120 precipitation in the absence of BMS-806. r, Pearson correlation coefficient.

Figure 3. BMS-806 stabilization of gp120 association with Env complexes. **A.** To evaluate the stability of the non-covalent association of gp120 with Env complexes, the detergent-solubilized, His_6-tagged Envs were captured on Ni-NTA beads and Western blotted with rabbit anti-gp120 antibody or the 4E10 anti-gp41 antibody. **B.** VLPs were prepared from the supernatants of A549-Gag/Env cells expressing Gag-mCherry and HIV-1_{AD8} Env. Triton X-100 lysates of VLPs were incubated with DMSO or the indicated molecules. Env was then captured on Ni-NTA beads and Western blotted as described in **A**. **C.** Triton X-100 lysates of A549 or HOS cells expressing Envs from different HIV-1 strains were incubated with Ni-NTA beads in the presence of the indicated molecules, and then the captured molecules were Western blotted as described in **A.** The experiments in **B** and **C** were performed at least twice, and a representative experiment is shown.

Figure 4. Computational docking of BMS-806 analogues. Maestro and Glide (Schrodinger) were used to dock BMS-529, AEG-II-159, and AEG-II-168 to the HIV-1_{BG505} soluble gp140 SOSIP.664 trimer complexed with BMS-529 (PDB 5U7O) (20). Both AEG compounds docked in the existing BMS-529 pocket in the same orientation as that exhibited by BMS-529 in the crystal structure. **A.** Rendering of the best docked pose of AEG-II-168 (pink sticks) with the crystallographic pose of BMS-529 (yellow sticks) superimposed. The gp120 subunits are depicted as dark blue ribbons,

and the gp41 subunits as light blue ribbons. **B.** Residue interaction map of the docked AEG-II-168 molecule. The gp120 residues are colored as follows: non-polar (green); polar (blue); basic (indigo); acidic (red).

Figure 5. Effect of AEG compounds on the conformation of cell-surface and VLP Env.

A. Recognition of cell-surface HIV-1_{AD8} Env by the indicated antibodies in the absence or presence of AEG-II-168 was assessed as described in the Figure 1A legend. **B.** Recognition of Env on the surface of A549 cells expressing HIV-1_{AD8} Env (left panel) or HIV-1_{CH848} Env (right panel) by the indicated antibodies was assessed as in the Figure 1A legend. The Env-expressing cells were incubated with 10 μM BMS-806, 1μM AEG-II-168 or the DMSO vehicle control. The precipitation of the gp120 glycoprotein of the mature cell-surface Env was quantified from at least two independent experiments, and the mean values calculated. The values shown on the y-axis represent these mean values relative to the input gp120, normalized to the values obtained for DMSO. Recognition of the mature cell-surface gp120 Env by the poorly neutralizing 17b, 19b and 902090 antibodies was below the level of detection in both the absence and presence of AEG-II-168 (data not shown). **C.** Recognition of HIV-1_{AD8} Env on the surface of VLPs by the indicated antibodies in the absence or presence of AEG-II-168 was assessed as described in the Figure 2C legend. **D.** Correlation between the effect of the indicated AEG compound on antibody recognition of gp120 Env on intact, detergent-free VLPs (as in **C** above) and the effect of BMS-806 on antibody recognition of VLPs (as in Fig. 2C). In each case, the effect of the compound represents the

precipitation of gp120 by the antibody in the presence of the compound divided by gp120 precipitation in the absence of the compound. r, Pearson correlation coefficient.

Figure 6. Effect of AEG-II-168 on virion Env conformational states.

The indicated number (N) of FRET traces were collected on HIV-1_{JR-FL} virus Env labeled in the gp120 V1 and V4 regions, in the absence and presence of a saturating concentration (100 μM) of BMS-806 or AEG-II-168. FRET histograms were compiled from the data and fitted for three Gaussian distributions centered at a low (0.1) FRET (State 1), intermediate (0.33) FRET (State 3) and high (0.65) FRET (State 2). Relative state occupancies are presented as means +/- standard errors of the mean.

Figure 7. Effect of BMS-806 and AEG compounds on gp120 association with solubilized Env complexes and with Env on VLPs.

A. Triton X-100 lysates of A549-Env cells expressing HIV-1_{AD8} Env were incubated with Ni-NTA beads in the presence of the indicated compounds at either 1 or 10 μM concentrations. The precipitated Envs were analyzed by Western blotting with a rabbit anti-gp120 antibody and the 4E10 anti-gp41 antibody. The gp120 association with the Env complex was calculated as described in Materials and Methods for each compound, and is shown relative to the value observed in the DMSO control. * P<0.05; ** P<0.01; *** P<0.001; ns – not significant.

B, C. VLPs with the HIV-1_{AD8} Env were incubated with DMSO, BMS-806 or AEG compounds in physiologic buffer for 4 days at 4°C, room temperature (RT) or 37°C. The VLPs were then pelleted and lysed; the lysed VLPs and supernatants were

analyzed by Western blotting with a rabbit anti-gp120 antibody (upper panels) and anti-Gag p55/p24/p17 antibody (lower panels). Note that, because the amount of shed gp120 is low compared with that of the VLP-associated gp120, the level of gp120 in the VLP supernatants is a more accurate indicator of gp120 shedding. The results of typical experiments of two independent experiments are shown.

Figure 8. Duration of the effect of BMS and AEG compounds on VLP Env conformation.

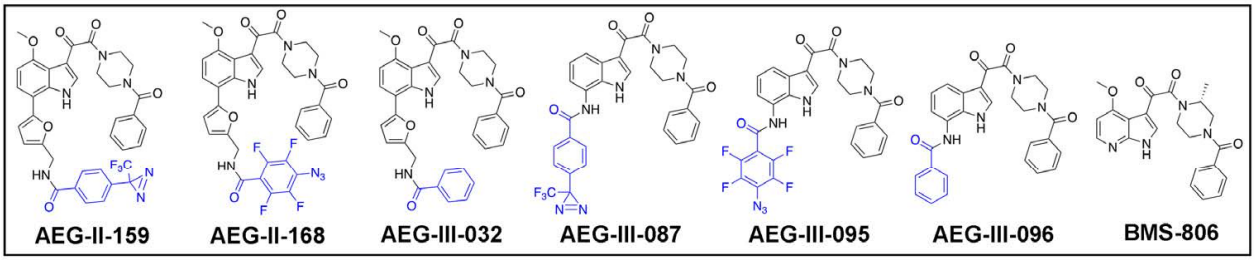
A. VLPs prepared from A549-Gag/Env cells expressing HIV-1_{AD8} Env and Gag-mCherry were incubated with DMSO or 10 μM BMS-806 or BMS-529. The VLPs were pelleted and washed twice, resuspended in PBS with 2% DMSO or fresh compound, and incubated at room temperature for the indicated times. Then the pelleted VLPs were lysed and incubated with antibodies and Protein-A-Sepharose beads. The precipitated proteins were Western blotted with a rabbit anti-gp120 antiserum. The results are shown for the absence of the compound (-), in the continuous presence of the compound (+/+), or after initial exposure to the compound, washing and incubation in PBS with 2% DMSO for the indicated times (+/-). **B.** VLPs with the HIV-1_{AD8} Env were incubated with DMSO or the indicated AEG compound and, in some cases, irradiated with UV light. The VLPs were pelleted and washed twice and then resuspended in either PBS with 2% DMSO or fresh compound at room temperature for 3 weeks. The VLPs were then pelleted and lysed. The VLP lysates were directly Western blotted (Input) or incubated with the 19b or 2G12 antibody and Protein A-Sepharose beads. The precipitated proteins were Western blotted with a rabbit anti-

gp120 antiserum. Results are shown for the absence of the compound (-), in the continuous presence of the compound (+/+), or after initial exposure to the compound, washing and incubation in PBS with 2% DMSO for 3 weeks (+/-). The results shown are typical of those obtained in two independent experiments. **C.** The binding of the BMS and AEG compounds to the VLP Env is shown as a function of the time of incubation of the virus:compound complex in PBS with 2% DMSO at room temperature. The compound:VLP Env binding index was calculated as follows:

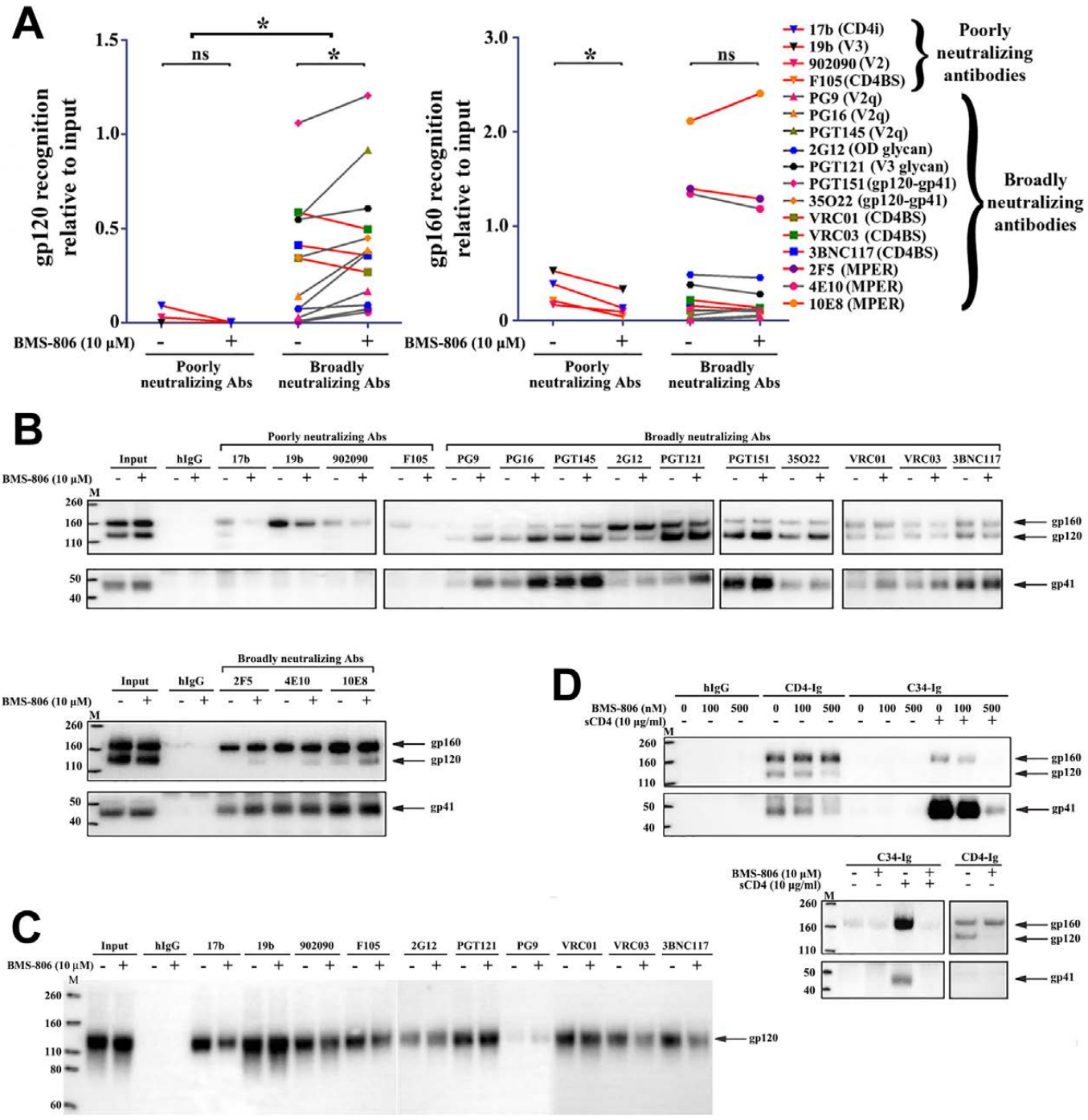
Compound:VLP Env binding index = $\left[\frac{(-)-(+/-)}{(-)-(+/+)}\right]$ x 100%, where the symbols in the parentheses represent the total amount of gp160 and gp120 recognized by the 19b antibody, as defined in **B.**

Figure 9. Long-term effects of AEG compounds on HIV-1 Env conformation.

A, B. VLPs prepared from A549 cells expressing HIV-1_{AD8} Env were incubated with DMSO or the indicated AEG compound and, in some cases, irradiated with UV light. The pelleted VLPs were washed and then resuspended in PBS with 2% DMSO. The VLPs were incubated at room temperature for 2 weeks. The VLPs were pelleted and lysed, and the cell lysates were incubated with the indicated antibodies and Protein A-Sepharose. The precipitated proteins were Western blotted with a rabbit anti-gp120 antiserum (upper panels) and with the 4E10 anti-gp41 antibody (lower panels). The results of typical experiments of two independent experiments are shown.



	AEG-II-159	AEG-II-168	AEG-III-032	AEG-III-087	AEG-III-095	AEG-III-096	BMS-806
AD8	0.2+/-0.05	2.8+/-0.1	0.36+/-0.02	0.06+/-0.02	2.2+/-0.4	2.4+/-0.1	0.4+/-0.01
BG505	0.8+/-0.05	0.22+/-0.08	1.5+/-0.8	1.9+/-0.4	ND	ND	0.8+/-0.2
JR-FL	1.6+/-0.1	1.5+/-0.2	0.6+/-0.3	0.1+/-0.01	0.7+/-0.1	0.9+/-0.1	1.6+/-0.5
JR-FL S375W	>100	>100	>100	>100	>100	>100	>100
AMLV	>100	>100	>100	>100	>100	>100	>100



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