An Antibody-Recruiting Small Molecule That Targets HIV gp120

Christopher G. Parker,† Robert A. Domaoal,‡ Karen S. Anderson,‡ and David A. Spiegel*,†,‡

Department of Chemistry, Yale University, 225 Prospect Street, P.O. Box 208107, New Haven, Connecticut 06520-8107, and Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, SHM B350B, New Haven, Connecticut 06520

Received July 12, 2009; E-mail: david.spiegel@yale.edu

In recent years, antibody-based therapeutics have become important instruments in treating human disease.1 These approaches suffer from certain limitations, including severe side effects, lack of oral bioavailability, and high cost.2 Thus, alternative small-molecule-based methods that exploit the powerful cytolytic potential of antibodies already present in the human bloodstream could address many of these disadvantages.

Here we report that a rationally designed bifunctional small molecule,3 called “antibody-recruiting molecule targeting HIV” (ARM-H), is capable of redirecting anti-dinitrophenyl (anti-DNP) antibodies, a population of antibodies present in high concentrations in the human bloodstream,4 to the HIV gp120 env gene product (Figure 1). The Env glycoprotein, a complex between gp120 and membrane-bound gp41, is expressed on both the surface of the HIV virus and on virus-infected cells.5 The gp120 component of Env mediates the first step in viral entry into human cells by binding the protein CD4. We demonstrate here that a ternary complex formed between anti-DNP antibodies, ARM-H, and Env-expressing cells can mediate the complement-dependent destruction of these cells. Further, since ARM-H binds gp120 competitively with CD4, it also inhibits the entry of live HIV virus into human T-cells. Thus, ARM-H has the potential to interfere with the survival of HIV through multiple complementary mechanisms.

Our design of ARM-H began with the small molecule BMS-378806 (1, Figure 1b), a known inhibitor of the CD4—gp120 interaction.6 On the basis of previously reported structure—activity relationships in which the carbon atom of the C4 methoxy group could be replaced with various bulky substituents,7 we hypothesized that it might be possible to derivatize 1 at this site with a linker attached to DNP without sacrificing the compound’s ability to inhibit viral entry. This hypothesis was supported by an analysis of a published computational docking model suggesting that the C4 methoxy group in 1 points toward the solvent in the complex.8 Thus, we reasoned that BMS-378806 could be re-engineered to recruit anti-DNP antibodies to gp120-expressing particles (infected cells or viruses), increasing their “visibility” to the human immune system. On the basis of this strategy, we prepared ARM-H (4) in high yield (38% overall) via azide—alkyne cycloaddition9,10 of 2 and 3, which were derived in turn from known intermediates (Schemes S2 and S3 in the Supporting Information).6

The ability of ARM-H to inhibit CD4 binding to HIV-1 gp120 was assessed first in an enzyme-linked immunosorbtent assay (ELISA) (Figure 2a).11 Here ARM-H was able to out-compete the CD4—gp120 interaction with a mean inhibitory concentration (IC50) of ~8.7 µM, and it was only slightly less potent than the parent compound BMS-378806 (IC50 = 1.3 µM). On the basis of this observation, we then investigated ARM-H’s ability to inhibit the entry of live HIV-1 virus into the human MT-2 T-cell line (Figure 2b, IC50 = 6.4 µM). Although in this assay ARM-H once again proved to be less potent than BMS-378806 (IC50 = 0.32 µM; Figure S1), it demonstrated potency equivalent to that of d4T, which is currently a mainstay in HIV pharmacotherapy (IC50 = 4.2 µM; Figure S1). Notably, ARM-H demonstrated no observable cytotoxicity in control MT-2 cultures lacking HIV virus (Figure 2b, white circles).

Figure 1. (A) Schematic depiction of the reported approach to HIV targeting. The synthetic bifunctional small molecule ARM-H is designed to function through two autonomous mechanisms: (1) by binding the viral glycoprotein gp120, inhibiting its interaction with CD4, and (2) by recruiting antibodies to the HIV virus, and to HIV-infected cells, for destruction by the human immune system. (B) Chemical structures of small molecules employed in these studies.

Figure 2. ARM-H outcompetes the gp120—CD4 interaction in vitro and with a live HIV-1 virus assay. (A) Competition ELISA monitoring the binding of sCD4 to immobilized gp120. (B) HIV-1 viral replication assay. UV absorption at 595 nm, increased by the metabolic action of live MT-2 cells on an assay reagent,13 was monitored as a surrogate for cell viability in the presence of increasing concentrations of ARM-H alone (white circles) or ARM-H plus live HIV-1 virus (black circles).

1 Yale University.
2 Yale University School of Medicine.
We next investigated the ability of ARM-H to recruit antibodies to gp120 both in vitro and in tissue culture. To this end, initial ELISA experiments (Figure S2) demonstrated a concentration-dependent increase in anti-DNP antibody binding to the ARM-H–gp120 complex but not to gp120 alone. Thus, ARM-H is capable of templating a ternary complex that also includes gp120 and anti-DNP antibody.

To demonstrate that this ternary association could form in a complex cellular milieu, we evaluated the ability of ARM-H to recruit Alexa Fluor 488-labeled anti-DNP antibodies to HIV-Env-expressing Chinese hamster ovary cells (CHO-gp120 cells) by immunofluorescence microscopy. As depicted in Figure 3, a strong fluorescence signal was observed when CHO-gp120 cells were incubated with ARM-H and labeled anti-DNP antibodies (Figure 3a,b). This fluorescence was absent from both CHO-gp120 not treated with ARM-H (Figure 3c,d) and CHO cells not coding for HIV-env gene expression (CHO-WT cells, Figure 3e,f). Furthermore, the intense fluorescence observed in Figure 3b was considerably diminished in the presence of the competing ligands soluble CD4 (sCD4, Figure 3g,h), BMS-378806 (Figure 3i,j), and a DNP-containing alkyne that lacks the gp120-binding motif (2, Figure 3k,l). Taken together, these results provide strong evidence that ARM-H is capable of recruiting anti-DNP antibodies to cells expressing the Env glycoprotein in a fashion that depends upon its simultaneous binding to both gp120 and anti-DNP antibodies.

Finally, we explored whether the ternary complex formed from anti-DNP antibody, ARM-H, and a live Env-expressing cell could activate complement proteins and mediate cellular death. Complement proteins are known to lyse cells by forming pores in lipid membranes and have been shown to play a critical role in inactivating HIV in humans. Thus, rabbit complement proteins were added to CHO-gp120 cells in the presence of ARM-H and a fixed concentration anti-DNP antibodies (Figure 4). Substantial cell killing that exhibited a significant dependence on the ARM-H concentration (data in red) was observed. Notably, in the absence of anti-DNP antibody and complement-preserved serum (data in green), in cells lacking the Env glycoprotein (CHO-WT, data in black), or in the presence of 3, which lacks the DNP group (data in blue), no cell death was observed, suggesting that ternolecular complex formation is necessary for complement-dependent cytotoxicity (CDC) and that ARM-H itself is not toxic to cells. Positive control experiments in which cells were directly labeled with 2,4-dinitrobenzenesulfonic acid (Figure S4) were found to yield levels of CDC comparable to those observed for ARM-H, providing a benchmark for the assay results depicted in Figure 4. Thus, ARM-H is capable of recruiting a functional complement-dependent cytotoxic response against Env-expressing cells.

Thus, we have shown that the bifunctional small molecule ARM-H can both recruit anti-DNP antibodies to gp120-expressing cells and inhibit the gp120–CD4 interaction. Data supporting these conclusions include the following: (1) ARM-H binds to gp120 competitively with CD4 and decreases viral infectivity in an MT-2 cell assay. (2) The small molecule can guide the formation of a ternary complex that includes anti-DNP antibodies and Env-expressing cells. (3) Antibodies present in this ternary complex can promote the complement-mediated killing of Env-expressing cells. Critically, no nonspecific cytotoxicity was observed in either MT-2 or CHO cell lines in response to ARM-H. Also, ARM-H-mediated inhibition of HIV entry and CDC activity were both operative at concentrations ranging from 6 to 30 μM, confirming that ARM-H could function simultaneously through dual mechanisms under therapeutic conditions.

While a few methods for recruiting naturally occurring antibodies to cancer cells have appeared in the literature, this area remains underexplored. In the HIV realm, all such approaches have relied upon protein- or peptide-based antibody targeting constructs. For example, Shokat and Schultz first demonstrated that anti-DNP antibodies could be redirected to immobilized protein targets (gp120 and streptavidin) as a therapeutic strategy toward HIV. More recent work in this vein has employed peptide-α-Gal conjugates to target human anti-Gal antibodies to HIV-infected cells. These peptide conjugates were shown to be effective in killing Env-expressing cells but were also found to exhibit some nonspecific cytotoxicity. ARM-H is unique in that it represents a small-molecule-based anti-HIV strategy and targets the virus lifecycle through mutually reinforcing molecular mechanisms: it both prevents virus entry and targets Env-expressing cells for immune recognition and clearance. In general, small molecules have certain advantages over proteins from a therapeutic standpoint.
because of their low propensity for immunogenicity, high metabolic stability, ready large-scale production, and relatively low cost. Small-molecule antibody-recruiting therapeutics such as ARM-H would have additional benefits over available treatment approaches to HIV. For example, directing HIV-infected cells and virus particles to Fcγ receptors on antigen-presenting cells could enhance the presentation of viral antigens on MHC proteins and contribute to long-lasting anti-HIV immunity.26,35 Furthermore, because anti-DNP antibodies are already present in the human bloodstream, no pre-vaccination would be necessary for ARM-H activity. Also, the binding of bifunctional small-molecule targeting agents to antibodies should prolong their plasma half-life, thus increasing their effectiveness.27 Elucidation of the molecular details governing the interactions among ARM-H, gp120, and anti-DNP antibodies will assist in optimization efforts as well as in the evaluation of this strategy in more complex biological models for HIV infection. These and other investigations are currently ongoing in our laboratories.

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Supporting Information Available: Detailed experimental procedures, compound characterization, and complete author lists for refs 6 and 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) More details regarding assay conditions and troubleshooting can be found in the Supporting Information.
(15) Because it was necessary to permeabilize the cells prior to labeling, we were unable to demonstrate any binding of ARM-H to cells (see ref 14). Also, because of assay incompatibilities, CDC data corresponding to ARM-H concentrations greater than 30 μM could not be obtained. More details can be found in the Supporting Information.
(16) Characteristic autoinhibitory behavior in ternary complex formation at high levels of bifunctional molecule, arising from excess free bifunctional material that drives the equilibrium toward formation of binary complexes, was not reliably observed in these assays (see the Supporting Information for more details). For more information on autoinhibitory behavior in ternary complexes, see: Mack, E. T.; Perez-Castillejos, R.; Suo, Z.; Whitesides, G. M. Anal. Chem. 2008, 80, 5550–5555, and references contained therein.
(17) More details regarding assay conditions and troubleshooting can be found in the Supporting Information.
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¹ Department of Chemistry, Yale University, 225 Prospect Street, PO Box 208107, New Haven, CT 06520-8107. ² Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, SHM B350B, New Haven, CT 06520.

Table of Contents

General Information ........................................................................................................................................ S1
1-azido-3,6,9,12-tetraoxapentadec-14-yne (6) .......................................................................................... S2
3,6,9,12-tetraoxapentadec-14-yne-1-amine (7) ......................................................................................... S3
N-(2,4-dinitrophenyl)-3,6,9,12-tetraoxapentadec-14-yne-1-amine (2) .......................................................... S3
4-Benzoyl-2-(R)-methyl-1-[(4-nitro-7-oxido-1H-pyrrolo[2,3-b]pyridin-3-yl]oxoacetyl]-piperazine (8) ........ S4
Azide (3) ..................................................................................................................................................... S4
ARM-H (4) .................................................................................................................................................. S5
MT-2 Cell Assay (Figure 2B and Supplementary Figure 1) ......................................................................... S6
CD4 Inhibition ELISA (Figure 2A) ............................................................................................................... S7
Anti-DNP IgG Recruiting ELISA’s (Supplementary Figure 2) .................................................................... S8
CHO Cell Culture ......................................................................................................................................... S10
Immunofluorescence Microscopy (Figure 3) ............................................................................................. S10
CDC Assay (Figure 4 and Supplementary Figure 3) ................................................................................... S11
CDC Positive Control (Supplementary Figure 4) ......................................................................................... S13
Flow Cytometry Detection of DNP Labeling (Supplementary Figure 5) .................................................. S15
Spectra of Compounds ............................................................................................................................... S16-S20
Complete References 6 and 11 ................................................................................................................ S21

General Information

Starting materials were used as received unless otherwise noted. All moisture sensitive reactions were performed in an inert, dry atmosphere of nitrogen in flame dried glassware. Reagent grade solvents were used for extractions and flash chromatography. Reaction progress was checked by analytical thin-layer chromatography (TLC, Merck silica gel 60 F-254 plates). The plates were monitored either with UV illumination, or by charring with anisaldehyde (2.5% p-anisaldehyde, 1% AcOH, 3.5% H₂SO₄(conc.) in 95% EtOH) or ninhydrin (0.3%
ninhydrin (w/v), 97:3 EtOH-AcOH) stains. Flash column chromatography was performed using silica gel (230-400 mesh). The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. Infrared (IR) spectra were recorded on a Thermo Nicolet 6700 FT-IR Spectrometer. $^1$H-NMR spectra were recorded at either 400 or 500 MHz and are reported in parts per million (ppm) on the δ scale relative to CDCl$_3$ (δ 7.26) as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration. $^{13}$C-NMR spectra were recorded at either 100 or 125 MHz and are reported in parts per million (ppm) on the δ scale relative to CDCl$_3$ (δ 77.00). High resolution mass spectra (HRMS) were recorded on a 9.4T Bruker Qe FT-ICR MS (W.M. Keck Facility, Yale University). Unless otherwise noted, all micro-plate based assays were quantitated using a BioTek Synergy 3 Microplate reader and data was fitted and graphed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) or KaleidaGraph (Synergy Software). ELISA and CDC experiments were performed in triplicate and repeated at least three times unless otherwise noted. Immunofluorescence (IF) experiments were performed in triplicate and repeated at least two times.

**Supplementary Scheme 1.** Synthesis of Alkyne-PEG-DNP (2)

1-azido-3,6,9,12-tetraoxapentadec-14-yne (6)

2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanol$^1$ (7.55 g, 34 mmol, 1.0 equiv., 5) was dissolved in DMF (150mL), and sodium hydride (989 mg, 40.8 mmol, 1.2 equiv.) was added, followed by propargyl bromide (80% in PhMe, 7.4 mL, 68 mmol, 2.0 equiv.). The reaction ran for 3 h at rt, at which time it was found complete by thin layer chromatography, concentrated on a rotary evaporator, and chromatographed on silica gel (30% EtOAc in hexanes) to yield 6 as a clear oil (7.55 g, 86% yield). IR (thin film/NaCl) 3252 (w), 2869 (s), 2110 (s), 1460 (w), 1349 (m), 1103 (s), 943 (w), 848 (w), 663 (w) cm$^{-1}$; $^1$HNMR

(400 MHz, CDCl₃) δ 4.13-4.23 (t, J = 2.4 Hz, 1H), 3.71 – 3.59 (m, 14H), 3.41 – 3.30 (t, J=5.1 Hz, 2H), 2.41 (t, J = 2.4 Hz, 1H); ¹³CNMR (125 MHz, CDCl₃) δ 79.55, 74.47, 70.47, 70.45, 70.43, 70.42, 70.20, 69.84, 68.91, 58.18, 50.50; HRMS (ES+) calc’d for C₁₁H₁₀N₂O₂ (M+H) m/z 258.14483.  Found 258.14496.

3,6,9,12-tetraoxapentadec-14-yn-1-amine (7)

![Chemical structure](image)

1-azido-3,6,9,12-tetraoxapentadec-14-yne (6, 2.45 g, 9.53 mmol, 1 equiv.), triphenylphosphine (3.00 g, 11.4 mmol, 1.2 equiv.), and water (172 µL, 9.53 mmol, 1.0 equiv.) were dissolved in THF (30 mL) and stirred for 12 h at which point TLC (95:5 CH₂Cl₂/ CH₃OH) indicated completion. The reaction was then concentrated under reduced pressure, and chromatographed on silica gel (100% CH₂Cl₂ to 80:20:1 CH₂Cl₂/MeOH/Et₃N) to yield 7 as a clear oil (1.88 g, 85% yield). IR (thin film, NaCl) 3372 (br), 3251 (s), 2868 (s), 2112 (w), 1652 (m), 1596 (m), 1459 (m), 1350 (m), 1301 (m), 1249 (m), 1100 (s), 946 (m), 681 (m) cm⁻¹. ¹H-NMR (500 MHz, CDCl₃) δ 4.16 (m, 2H), 3.66-3.57 (m, 12H), 3.49 (t, 2H, J= 5.3 Hz), 2.85 (t, 2H, J= 5.0 Hz), 2.56 (bs, 2H), 2.41 (m, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 79.52, 74.63, 73.06, 70.45, 70.41, 70.24, 70.11, 68.96, 58.26, 41.50. HRMS (ES+) calc’d for C₁₁H₂₁NO₄ (M+H) m/z 232.154335.  Found 232.15402.

N-(2,4-dinitrophenyl)-3,6,9,12-tetraoxapentadec-14-yn-1-amine (2)

To a solution of 3,6,9,12-tetraoxapentadec-14-yn-1-amine 7 (1.0 g, 4.3 mmol, 1 equiv.) dissolved in EtOH (18mL), was added triethylamine (1.68mL, 8.6mmol, 2 equiv.) and 1-chloro-2,4-dinitrobenzene (876 mg, 4.3 mmol, 1 equiv.) via syringe. The reaction flask was then fitted with a reflux condenser and the reaction was heated to reflux for 2 h, cooled, and concentrated to provide a crude yellow oil. The crude mixture was re-dissolved in H₂O (25 mL) and extracted with CH₂Cl₂ (5 x 10 mL). The organic layers were dried over Na₂SO₄ and concentrated to a yellow oil that was further purified by flash chromatography (CombiFlash Automated Chromatographer, 25g column, dryloaded with 25g pre-packed dry loading column; gradient elution ranging from 10% EtOAc:Hexanes to 50% EtOAc:Hexanes was performed over 30 column volumes, followed by an EtOAc flush) to yield 2 as a yellow solid (1.70g, >98% yield). IR (thin film/NaCl) 3360(m), 3290 (m), 3110 (w), 2873 (m), 2114(w), 1621 (s), 1588 (m), 1336 (s), 1134 (m) cm⁻¹; ¹H-NMR (500 MHz, CDCl₃) δ 9.13 (d, 1H, J = 2.6 Hz), 8.80 (bs, 1H), 8.25 (dd, 1H, J =
2.6, \( J = 9.5 \text{ Hz} \), 6.94 (d, 1H, \( J = 9.5 \text{ Hz} \)), 4.18 (m, 2H), 3.83 (t, 2H, \( J = 5.2 \text{ Hz} \)), 3.67 (m, 12H), 3.60 (q, 2H), 2.41 (m, 1H); \(^{13}\text{C-NMR (125 MHz, CDCl}_3\) \( \delta \) 148.6, 136.2, 130.4, 124.4, 114.3, 79.8, 74.7, 70.8, 70.7, 70.5, 69.2, 68.7, 58.5, 43.4; \text{HRMS (EI) calc'd for C}_{17}H_{23}N_3O_8 (MH^+) m/z 398.1558. Found 398.1557.

Supplementary Scheme 2. Synthesis of 4 (ARM-H)

4-Benzoyl-2-(R)-methyl-1-[(4-nitro-7-oxido-1H-pyrrolo[2,3-b]pyridin-3-yl)oxoacetyl]-piperazine (8)

This compound was synthesized as previously described\(^2\). All synthetic intermediates as well as the final product analytical data were in agreement with that reported.

Azide (3)

To a stirred mixture of 255 mg NaH (95%, 9.88 mmol, 7.2 equiv, Aldrich), anhydrous dimethoxyethane (21 mL) was added followed by hexaethylene azido glycol\(^3\) (1.512g, 4.93 mmol, 7.2 equiv) in anhydrous dimethoxyethane (21 mL). The resulting yellow solution was stirred for 2 hrs before 8 (300 mg, 0.686 mmol), then added as a solution in

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2. See Manuscript Reference 6.
dimethoxyethane (7.5 mL) via cannula at room temperature. The resulting copper-colored mixture was heated to 45 °C and monitored by TLC (9:1 CH₂Cl₂/CH₃OH). After approximately 2 hrs, the mixture (now brown in color) was allowed to cool to room temperature, a saturated aqueous solution of NH₄Cl (15 mL) was slowly added, and the organic layer was extracted with CH₂Cl₂ (5 x 80 mL). The organic phases were combined, dried over anhydrous MgSO₄, filtered, and all solvents were evaporated under reduced pressure. The resulting crude brown residue, containing compound 9, was purified chromatographically on silica (0% to 20% CH₃OH in CH₂Cl₂) to remove unreacted azido alcohol, and provided a yellow oil (650 mg), which was then dissolved in 40 mL EtOAc. To this solution was added 1.10 mL PCl₃ (6.92 mmol, 10 equiv), resulting in a heterogeneous orange mixture, and allowed to stir at r.t. After 2 hrs, the reaction was cooled to 0 °C, and quenched by the careful addition of saturated aqueous NaHCO₃ until pH of 6 was reached. The mixture was extracted with EtOAc (5 x 50 mL), the combined organic layers were dried over anhydrous Na₂SO₄, filtered, and all solvents were evaporated by rotary evaporation. Crude isolates were then purified by column chromatography (0% to 8% CH₃OH in CH₂Cl₂), to yield 3 as a clear solid (421 mg, 0.618 mmol, 45% over 2 steps). IR (thin film/NaCl) 3095 (w), 2872 (s), 2107 (s), 1635 (s), 1433 (m), 1097 (m) cm⁻¹. ¹H-NMR (500 MHz, CDCl₃, rt) δ 12.5 (s, 1H), 8.28 (d, J = 5.6 Hz, 1H), 8.03 (d, J = 18.3 Hz, 1H) 7.41 (broad peak, 5H), 6.76 (d, J = 3.8 Hz, 1H), 5.05-4.45 (broad peak, 2H), 4.38 (broad peak, 2H), 4.02 (broad peak, 2H), 3.78 (broad peak, 2H) 3.68-3.58 (m, 18H), 3.48 (m, 1H), 3.37 (m, 2H), 3.15 (broad peak, 2H), 1.30 (bs, 3H) ¹³C-NMR (75 MHz, CD₂OD, rt) δ 184.8, 167.2, 161.4, 152.1, 146.8, 135.5, 130.5, 129.1, 127.5, 114.5, 108.4, 102.3, 71.5, 71.1, 71.0, 70.4, 69.7, 69.00, 68.9, 51.1, 45.2, 16.6, 15.5. HRMS (EI) m/z (%) for C₃₅H₄₃N₇O₉ (MH⁺) calc’d 682.3195, found 682.3205; for (M+Na)+ calc’d 704.3014, found 704.3017.

**ARM-H (4)**

To a solution of 2 (45.0 mg, 0.066 mmol) dissolved in t-BuOH (1.7 mL) and H₂O (1.6 mL) was added 3 (51 mg, 0.125 mmol, 1.9 equiv). The mixture was stirred for 5 min before aqueous CuSO₄·5H₂O (0.1M, 33 µL, 0.05 equiv) and aqueous sodium ascorbate (0.1M, 66 µL, 0.1 equiv) were added. The reaction vessel was then capped and heated in a microwave reactor for 20 min at 125 °C at which time TLC (9:1 CH₂Cl₂/CH₃OH)
indicated reaction completion. The golden yellow solution was transferred to a flask using CH$_3$OH, and solvents were evaporated, providing 35 mg of crude 4 as a golden yellow solid, which was purified by flash chromatography (0% to 10% CH$_3$OH in CH$_2$Cl$_2$) to deliver 57 mg (0.053 mmol, 80%) of 2 as a golden yellow solid. IR (thin film/NaCl) 3356 (w), 3111 (w), 2931 (m), 2876 (s), 1620 (s), 1515 (m), 1433 (m), 1334 (m), 1297 (m), 1123 (m) cm$^{-1}$.  

H-NMR (500 MHz, CDCl$_3$, rt) $\delta$ 12.74, (b, 1H), 9.08 (d, $J = 2.5$ Hz, 1H) 8.76 (s, 1H), 8.26 (d, $J = 4.7$ Hz, 1H), 8.22 (dd, $J = 2.6$, 9.5 Hz, 1H), 7.70 (s, 1H), 7.40 (broad peak, 5H), 6.93 (bs, 1H), 5.1-4.7 (broad peak, 1H) 4.4 (bs, 2H), 4.49 (t, $J = 5.1$ Hz), 4.36 (bs, 2H), 4.01 (bs, 2H), 3.95-3.31 (m, 38H), 3.29-2.9 (broad peak, 2H) 1.30 (bs, 3H).

NOTE: When sample is heated in DMSO-$d_6$ to 100 °C, broadened proton signals coalesce, suggesting the presence of multiple rotameric conformations.  

C-NMR (75 MHz, CDCl$_3$, rt) $\delta$ 184.9, 167.1, 161.2, 151.9, 148.8, 147.2, 145.2, 136.5, 135.6, 130.9, 130.6, 130.5, 130.4, 129.1, 127.5, 124.6, 124.1, 114.6, 114.5, 108.1, 102.3, 102.3, 71.5, 71.1, 71.1, 71.0, 70.9, 70.8, 70.0, 69.9, 69.8, 69.7, 69.0, 64.9, 50.6, 50.3, 45.2, 43.7, 16.6, 15.5.  

HRMS (EI) $m/z$ (%) for C$_{50}$H$_{66}$N$_{10}$O$_{17}$ (MH$^+$) calc’d 1079.4680, found 1079.4663; for (M+Na)$^+$ calc’d 1101.4500, found 1101.4458.

MT-2 Cell Assay (Figure 2B and Supplementary Figure 1)

Antiviral activity and cellular toxicity were determined using the MTT colorimetric method$^{4,5}$. MT-2 cells$^{6,7}$ at a concentration of 1 x 10$^5$ cells per mL were infected with wild-type HIV IIIB$^{8,9,10}$ at a multiplicity of infection (MOI) of 0.1. Infected and mock-infected cells were incubated in growth medium (RPMI 1640, 10% δFBS, kanamycin) for 5 days with varying concentrations of each compound being tested in triplicate in a 96-well plate. MTT (thiazolyl blue tetrazolium bromide), a cell-permeable tetrazolium dye, was then added to each well. Active mitochondria reduce the yellow tetrazolium salt to a blue formazan precipitate. After 5 h, “stop solution” (86% isopropanol, 4% NP-40, 10% H$_2$O, and 0.3% concentrated HCl) was added to lyse the cells and stop the reaction. The plates were gently shaken gently overnight on a horizontal rotator, and quantitated the following morning. The amount of purple precipitate, which serves as a surrogate for cell viability, was measured via determining the absorbance at 595 nm.

using a Multiskan Plus from Labsystems (Helsinki, Finland) microplate reader. The average of triplicate samples were then plotted as a function of inhibitor concentration to generate dose–response curves. The 50% effective concentration (EC\textsubscript{50}) and 50% cytotoxic concentration (CC\textsubscript{50}) of the compounds were defined as the concentrations required to inhibit viral replication and to reduce the number of viable cells by 50%, respectively. Positive controls were performed during each set of experiments using d4T and the appropriate parent NNRTI (HI-236 or TMC-derivative). Data were analyzed using the KaleidaGraph software package (Synergy Software).

**Supplementary Figure 1.** BMS-377806 (A) (IC\textsubscript{50} = 320 nM) and d4T (B) (IC\textsubscript{50} = 4.2 µM) HIV-1 inhibition in MT-2 cell assay. Raw absorbance data reported ± SD.

**CD4 Inhibition ELISA (Figure 2A)**

This procedure was adapted from the protocol reported by Lin, et al.\textsuperscript{11} 96 well plates (Nunc; Immuno) were coated overnight at 4 °C with soluble recombinant HIV-1 gp120\textsubscript{JRFL} (Immune Technology; Yonkers, NY) at 1 µg/ml in 0.05M Buffer A (carbonate/bicarbonate, pH =9.6, Aldrich). Plates were washed with PBS (Aldrich, 1x100µL) and then blocked with 3% nonfat milk in phosphate buffered saline solution (PBS, Aldrich) for 1 hr at room temperature. After washing with Buffer B (20 mM Tris HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.4), varying concentrations of the inhibitor were added along with recombinant human T-cell CD4 (ImmunoDiagnostics, Inc; Woburn, MA) in Buffer C (50 mM Tris HCl, 100 mM NaCl, 1% BSA, pH 7.4) to bring the final concentration of CD4 in each well to 0.1 µg/mL. Plates were then incubated for 1 hr at room temperature, washed with Buffer B (3x100µL), and incubated with mouse OKT4 anti-CD4 IgG antibody (Biolegend; San Diego, CA) at 0.36 µg/ml in Buffer C at RT for 1 hr. Following washes with Buffer B, plates were incubated with horse radish peroxidase (HRP)-conjugated

goat anti-mouse antibody (1:2500; Biolegend; San Diego, CA). Bound antibody was detected with the chromogenic HRP substrate 3,3,5,5-tetramethylbenzidine (TMB, Pierce Protein Research Products), and absorbance was read at 450 nm. The means (±SD) of these triplicate samples were then plotted versus inhibitor concentration, and a non-linear fit curve was generated using GraphPad Prism. The 50% inhibitory concentration (IC$_{50}$) was defined as the concentration of inhibitor to reduce the maximal amount of bound CD4 to gp120 by 50%.

Anti-DNP IgG Recruiting ELISA’s (Supplementary Figure 2)

A. Varying ARM-H Concentration (Supplementary Figure 2A)

Nunc-Immuno 96-well plates were coated with soluble gp120 and blocked as described above. After washing with PBS (Aldrich), the indicated concentrations of ARM-H (4) were added, and assay plates were incubated for 1 hr at RT. After washing (3x100 µL) the plate with Buffer D (50 mM Tris HCl, 100 mM NaCl, 23 mM HEPES, 1 mM MgCl$_2$, 1 mM CaCl$_2$, pH 7.4), wells were incubated with rat monoclonal anti-dinitrophenyl (anti-DNP) IgG antibodies (Zymed; Carlsbad, CA) at 5 µg/ml in Buffer E (50 mM Tris HCl, 100 mM NaCl, 23 mM HEPES, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1% BSA, pH 7.4) at room temperature for 1 hr. Plates were then washed and incubated with HRP-conjugated goat anti-rat antibody (1:2000; Novus Biologicals; Littleton, CO). Bound antibody was detected with 3,3,5,5-tetramethylbenzidine (TMB; Pierce Protein Research Products), and the absorbance was read at 450 nm. The means (±SD) of these triplicate samples were then plotted as a function of inhibitor concentration and a non-linear fit curve was generated using GraphPad Prism. The 50% effective concentration (EC$_{50}$) was defined as the concentration of ARM-H to bind 50% of the maximum bound HRP conjugated anti-DNP in the ternary complex with sgp120.

B. Varying Anti-DNP IgG Concentration (Supplementary Figure 2B)

Nunc-Immuno 96-well plates were coated with soluble gp120 and blocked as described above. After the PBS (Aldrich) wash, 25 µM of ARM-H (4) was added, and assay plates were allow to incubate for 1 hr at RT. After washing the plate with Buffer D (3x100 µL), wells were incubated with indicated concentrations of rat monoclonal anti-dinitrophenyl (anti-DNP) IgG antibodies (Zymed; Carlsbad, CA) in Buffer E at room temperature for 1 hr. Plates were then washed and incubated with HRP-conjugated goat anti-rat antibody. Bound antibody was detected
after incubation with 3,3,5,5-tetramethylbenzidine (TMB; Pierce Protein Research Products), by measuring the absorbance at 450 nm. The means (±SD) of these triplicate samples were plotted as a function of inhibitor concentration, and a non-linear fit was generated using GraphPad Prism. The 50% effective concentration (EC₅₀) was defined as the concentration of anti-DNP antibody to bind 50% of the maximum bound HRP conjugated anti-DNP in the ternary complex with ARM-H (4).

**Supplementary Figure 2.** (A) ELISA showing ARM-H concentration dependent increase in absorbance when anti-DNP antibodies were allowed to bind to complex of ARM-H and gp120. (B) ELISA showing anti-DNP IgG concentration dependent increase in absorbance when allowed to bind to complexed ARM-H (25 µM) and gp120. Raw absorbance data reported ± SD.

*Note: The competition ELISAs were conducted following a known assay protocol containing a detergent (see Supporting Information Ref. 11), and had previously been employed to measure the IC₅₀ for BMS-378806, the parent compound in our studies. Utilizing these published conditions allowed us to calibrate our IC₅₀ values directly with literature values. However, when investigating recruitment of anti-DNP antibodies by ARM-H (Supplementary Figure 2), we utilized an ELISA buffer system that was compatible with tissue culture (i.e., one that lacks detergent) in order to model the conditions employed in subsequent CDC assays. Thus we would not expect an exact correlation between the competition ELISA IC₅₀ and the antibody recruiting ELISA EC₅₀ values. In addition, we recognize that the antibody/ARM-H/gp120 complex should exhibit auto-inhibitory behaviors consistent with ternary complex formation under certain circumstances, however, data collected in the antibody recruiting ELISA (Supp. Fig. 2) would not be expected to do so because a series of washes were performed prior to addition of anti-DNP antibody, thus removing unbound ARM-H, and preventing auto-inhibition by antibody/ARM-H complexes.
CHO Cell Culture

Wild-type HIV-1 env expressing CHO-WT (described as ‘CHO-gp120’ in the text) cells were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CHO-WT from Dr. Carol Weiss and Dr. Judith White. Cells were grown on glutamine-deficient minimal essential medium containing 400 µM methionine sulfoximine (“GMEM-S selection media”; MSX, Sigma) as described by associated NIH protocol12. CHO-K1 cells (described as “CHO-WT” in the text; ATCC) were grown in ATCC-formulated F-12K medium supplemented with 10% FBS (Gibco). Cells were detached from cell culture flasks with 2.5mM EDTA/0.5mM EGTA in DPBS (Gibco) for passage.

*Note: As described in the associated NIH culture protocol, a drop in envelope expression in CHO-WT cells was observed upon several passages and it is recommended to maintain low passage stocks of cells in liquid nitrogen.

Immunofluorescence Microcopy (Figure 3).

Confluent CHO-WT (env expressing) or CHO-K1 (non-env expressing) cells were incubated on cover slips (15CIR-1D, Fisher) over 2 nights at 37 °C in 5% CO₂. Cover slips were washed with DPBS (1 mL, Gibco) and then cells fixed with 4% paraformaldehyde in DPBS at 4 °C for 10 minutes. To demonstrate antibody recruitment, cover slips were washed with DPBS (1 x 70 µl) then with Buffer E (1 x 70 µl), and incubated with ARM-H (4) in Buffer E (70 µl) for 1 hr at 4°C. Cover slips were then washed with Buffer E (2 x 70 µl) and incubated with AlexaFluor488 rabbit anti-DNP IgG antibodies (Invitrogen) at 15 µg/ml in Buffer E (70 µl) for 1 hr at 4°C. All cover slips were washed prior to mounting onto slides using Gel Mount mounting medium (Biomeda Corp.) with Buffer E and water. Corresponding competition experiments were performed as indicated in the presence of recombinant human T-cell CD4 (ImmuNoDiagnostics, Inc; Woburn, MA), BMS-378806 (1) and DNP-PEG-alkyne (2). Micrographs were obtained using a Zeiss Axiovert 200M fluorescence microscope equipped with a GFP filter.

CDC Assay (Figure 4 and Supplementary Figure 3)

CHO-WT or CHO-K1 cells taken from a T-75 flask (~80% confluent), were washed once with DPBS (Gibco, 5 mL), and cells were then detached with 2.5mM EDTA/0.5mM EGTA in DPBS. The resulting cell suspension was centrifuged at 900 rpm for five minutes, the pellet was then aspirated and re-suspended at a density of 9.00 X 10^5 cells/mL in Buffer E. Cell suspensions were then added to prepared dilutions of ARM-H (4) (or the indicated control molecule) in Buffer E. Resulting cell mixtures were plated in triplicate (50 µL, 22,500 cells/well) on 96 well plates (CoStar, black sides/clear bottom), covered with tin foil, and incubated at 4 °C for 1.5 hours. To each well, 50 µL of 20% rabbit complement serum (v/v, Aldrich) and 100 µg/mL rat anti-DNP IgG (Zymed; Carlsbad, CA) in Buffer E was added, resulting in a 10% (v/v) complement and 50 µg/mL antibody concentration per well. Negative control wells containing only ARM-H, and maximum cell death controls (0.15% H_2O_2) were also included. The covered plate was incubated for an additional hour at 4 °C and then for 4 hours at room temperature. Cell viability was determined using the luciferase-based CellTiter-Glo Luminescent Cell Viability Assay (Promega). Complement mediated cell death and cytotoxicity was calculated as: \([1 - ((\text{sample-max killing})/(\text{untreated-max killing}))] \times 100\) and plotted using GraphPad Prism. Raw data was subjected to Dixon Q-test analysis at the 90% confidence interval and statistical outliers were removed accordingly.\(^{13,14}\)

*Note:* When performing above CDC analyses, authors observed consistent complement-dependent cytotoxicity results over several CHO-gp120 cell passages. However, as reported in the NIH culture protocol, we observed a decrease in envelope protein expression over many passages, as well as a consequent reduction in ARM-H (4)-mediated CDC. In addition, at higher concentrations of both ARM-H and azide (3) (>50 µM), authors observed enhanced cell viability (decreased cell death) in both CHO-gp120 and CHO-WT cells, as shown in Supplementary Figure 3. *Importantly, this effect was only observed at concentrations greater than ~50 µM, which is outside the concentration range reported in Figure 3.* Furthermore, since these effects lead to an enhancement in viability, they would tend to underestimate the effect of ARM-H mediated antibody-dependent cytotoxicity.

Nevertheless, we studied this phenomenon in more detail, and based upon follow up experiments, this enhancement in viability appears to be independent of the presence of antibody/complement and gp120. It is also not due to a

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direct effect on assay reagents. Thus, following the general protocol for CDC experiments outlined above, we found that both ARM-H (4) and azide (3) exhibited some degree of viability enhancement at high concentrations in both wild-type and gp120-expressing CHO cells (Supplementary Figure 3A). However, in the absence of all cellular material (Supplementary Figure 3B), when assay reagents are combined with either 1 or 4 and ATP (50 nM), no increase in assay signal is observed. Because this assay limitation prevented us from testing ARM concentrations greater than 50 μM, auto-inhibition of ternary complex formation (and consequent reduction of CDC) associated with formation of gp120/ARM-H and ARM-H/antibody binary complexes at high ARM-H concentrations was not observed in these assays. This behavior may manifest at concentrations of ARM-H tested as shown in Supplementary Figure 3A (red dots) and may indeed explain the decrease in cell killing at concentrations of ARM-H greater than 30 μM in the presence of antibody and serum (Note: this ARM-H dependent CDC never attains “negative” values). However, this characteristic behavior cannot be confirmed, as it may be masked by the viability enhancement described above. The optimal ATP concentration was determined in Supplementary Figure 3B by generating a standard concentration-signal curve for ATP as outlined by the associated Promega protocol for CellTiter-Glo. Percent change in luminescence was calculated as: [1-(sample/untreated)] X 100.

**Supplementary Figure 3.** ARM-H-mediated killing of CHO-gp120 cells and ATP control. (A) HIV gp120-expressing CHO cells were treated in the presence or absence of antibody (rat anti-DNP IgG (50 μg/mL) and rabbit complement, plus the indicated concentrations of ARM-H or control compounds as detailed above. Decreased cell death (enhanced cell viability) is observed at concentrations greater than ~50μM. (B) In the absence of cellular material when assay reagents are combined with ARM-H or BMS-378806 (1) and ATP (50nM), no increase/decrease in assay signal is observed. Values on the Y-axes correspond to the percent of cell death (versus background) or the corresponding percent change in luminescence. Data represents the mean ± standard error. All individual experiments were performed in triplicate, and the indicated trends were reproduced on at least six separate occasions.
CDC Positive Control (Supplementary Figure 4)

A series of anti-gp120 antibodies were screened and subjected to the CDC assay conditions described above; however, none of these were capable of mediating CDC under our conditions. These results are consistent with literature reports; to our knowledge, no available monoclonal or polyclonal anti-gp120 antibodies is capable of mediating CDC of gp120-expressing cells. Additionally, there are only two examples of anti-gp120 antibodies capable of effecting virolysis.\textsuperscript{15,16} In these studies, no single antibody was capable of mediating virolysis beyond 20%. Following a rigorous search, we found one commercially available antibody that has been reported to mediate CDC of “gp120 V3-like protein”, which is expressed natively on activated T cells.\textsuperscript{17} In addition to this antibody, we screened five other antibodies, including: goat polyclonal anti-HIV-1-gp120 IgG (Abcam; Cambridge, MA; ab21179); rabbit polyclonal anti-HIV-1-gp120 IgG (Abbiotec, San Diego, CA; 250694); human anti-HIV-1-gp120 monoclonal (binds to CD4 binding region of gp120) IgG (ImmunoDiagnostics; Woburn, MA; 3501); mouse anti-HIV-1-gp120 monoclonal IgG1 (Novus; Littleton, CO; NB120-13411); rabbit polyclonal anti-HIV-1-gp120/160 IgG (Thermo; Rockford, IL; PA1-43526); anti-gp120 V3 loop (a.a.’s 308-322) monoclonal IgG (PerkinElmer; Waltham, MA; NEA9205).\textsuperscript{15} Given that none of these were effective in CDC assays, our results underscore the potential utility of these reported small molecule conjugates.

All gp120 antibodies were tested at concentrations up to 50 µg/mL (ImmunoDiagnostics #3501 was tested up to 25 µg/mL) and were performed once in triplicate. CHO-WT cells were detached and re-suspended at a density of 9.00 X 10\textsuperscript{5} cells/mL in Buffer E as described above. Subsequently, the cell suspension was added to prepared dilutions of anti-gp120 antibody in Buffer E. Resulting mixtures were plated in triplicate (50 µL, 22,500 cells/well) onto 96 well plates (CoStar, black sides/clear bottom), covered with tin foil, and incubated at 4 °C for 1.5 hours. To each well, 50 µL of 20% rabbit complement serum (v/v, Aldrich) in Buffer E was added, resulting in a 10% (v/v) complement concentration per well. Negative control wells containing only antibody dilutions were prepared in addition to maximum cell death controls (0.15% H\textsubscript{2}O\textsubscript{2}). The tin-foil-covered plate was incubated for an additional hour at 4 °C and then for 4 hours at room temperature. Cell viability was quantitated with CellTiter-Glo Luminescent Cell Viability Assay as described above.

\textsuperscript{17} Trujillo, J. R.; Rogers, R. A.; Brain, J. D. Virology 1998, 246, 53-62.
As an alternative positive control, we labeled CHO-K1 cells with 2,4-dinitrobenzenesulfonic acid for use in CDC assays (Supplementary Figure 4). Data presented represent the means ± standard error of triplicate experiments, and the observed trends were reproduced on at least two separate occasions. This procedure was adapted from the protocol reported by Geczy and coworkers.\textsuperscript{18} CHO-K1 cells taken from a T-75 flask (~80% confluent), were washed once with DPBS (Gibco, 5 mL), and cells were then detached with 2.5mM EDTA/0.5mM EGTA in DPBS. The resulting cell suspension was centrifuged at 900 rpm for five minutes, then the pellet was aspirated and re-suspended at a density of 9.0 x 10\textsuperscript{5} cells/mL in F12-K growth medium. Cells were incubated with 2,4-dinitrobenzenesulfonic acid (TCI, 50mg/mL solution in MeOH) at a concentration of 1 mg/mL for 30 min. at room temperature. Cells were then centrifuged, washed with growth medium (x2), and with Buffer E (x1). The cell suspension was added to prepared dilutions of rat anti-DNP IgG (Zymed; Carlsbad, CA) in Buffer E and subsequently plated in triplicate (50 μL, 22,500 cells/well) onto 96 well plates, covered with tin foil and incubated at 4 °C for 1.5 hours. To each well, 50 μL of 20% rabbit complement serum (v/v, Aldrich) in Buffer E was added, resulting in a 10% (v/v) complement concentration per well. Cell viability was quantitated as described above.

![Supplementary Figure 4](image)

**Supplementary Figure 4.** Rat anti-DNP mediated CDC of dinitrobenzenesulfonic acid labeled CHO-K1 cells. DNP labeled (see below) and un-labeled CHO-K1 cells were incubated with a concentration series of rat anti-DNP IgG antibodies in the presence and absence of rabbit complement serum. DNP-labeled cells, in the presence of serum (red), demonstrated a anti-DNP concentration dependent trend of cell death whereas unlabeled CHO-K1 cells (blue) demonstrated no cell death. Complement dependence of cell death was confirmed with incubations of antibody with labeled (black) and unlabeled (green) CHO-K1 cells in the absence of complement. Data represents the mean ± standard error.

Flow Cytometry Detection of DNP Labeling (Supplementary Figure 5)

CHO-K1 cells taken from a T-75 flask (~80% confluent), were washed once with DPBS (Gibco, 5 mL), and cells were then detached with 2.5mM EDTA/0.5mM EGTA in DPBS. The resulting cell suspension was centrifuged at 900 rpm for five minutes, then the pellet was aspirated and re-suspended at a density of 1.40 X 10^6 cells/mL in F12-K growth medium. Cells were incubated with 2,4-dinitrobenzenesulfonic acid (TCI, 50mg/mL solution in MeOH) at varying concentrations for 30 min. at room temperature. Cells were centrifuged, washed with growth medium (x3), and then aliquoted (0.5ml) into Eppendorf tubes at a cell density of 1.40 X 10^6 cells/mL. Cells were incubated with AlexaFluor 488 conjugated rabbit anti-DNP IgG (Invitrogen) antibodies (20 µg/mL) for 1 hr at 4 °C. Cells were subsequently centrifuged, washed with DPBS (3x0.5mL), resuspended in 0.5mL DPBS containing 150 µg of propidium iodide (to monitor cell death) and immediately analyzed for fluorescence using a FACSCalibur flow cytometer (Becton Dickinson) monitoring at least 10,000 events/measurement. Data were analyzed using FlowJo Analysis Software (Tree Star).

Supplementary Figure 5. 2,4-Dinitrobenzenesulfonic acid labeling of CHO-K1 cells. (A) Fluorescence shift of CHO-K1 cells labeled with dinitrobenzenesulfonic acid and then stained with AlexaFluor 488 conjugated anti-DNP IgG (20 µg/mL, green) compared to unlabeled cells (red). (B) Concentration screen of dinitrobenzenesulfonic acid labeling of CHO-K1 cells as determined through flow cytometry. Note: Significant cell death detected at higher concentrations of dinitrobenzenesulfonic acid (data not shown).
Spectra of Compounds

$^1$H NMR Spectrum for 6 (CDCl$_3$)

$^{13}$C NMR Spectrum for 6 (CDCl$_3$)
$^1$H NMR Spectrum for 7 (CDCl$_3$)

$^{13}$C NMR Spectrum for 7 (CDCl$_3$)
$^{1}H$ NMR Spectrum for 2 (CDCl$_3$)

$^{13}C$ NMR Spectrum for 2 (CDCl$_3$)
$^1$H NMR Spectrum for 3 (CDCl$_3$)

$^{13}$C NMR Spectrum for 3 (CDCl$_3$)

S19
$^1$H NMR Spectrum for 4 (CDCl$_3$)

$^{13}$C NMR Spectrum for 4 (CDCl$_3$)
Complete References 6 and 11
