Supporting Information

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SI Discussion

Molecular Shape and Amphipathicity Determine the Ability of Different Rigid Amphipathic Fusion Inhibitors to Inhibit Viral Infectivity. Hydrophilic nucleosides are well characterized as antiviral scaffolds and are readily derivatized with hydrophobic substituents (1) to produce amphiphiles with hydrophilic heads of larger diameter than their hydrophobic tails. We tested the effects of amphipathicity, rigidity, and molecular shape of such amphiphiles on the infectivity of HSV-1, a most useful model for antiviral discovery (2, 3). The hydrophilic moieties, 2'-deoxyuridine, 2',3′-dideoxyuridine, or arabinouridine mimic the shape of the polar headgroups in membrane phospholipids. Different hydrophobic arylalkynyl moieties were joined to the base at position 5 by either nonpolar rigid ethynyl or polar flexible propargyloxymethyl linkers (Fig. S1 and Table S1).

Vero cells were infected with 200 infectious HSV-1 virions preexposed to test compounds for 5 min at 37 to 40 °C. Infectivity was monitored by plaquing efficiency.

We first applied structure-activity relationship (SAR) to test whether the molecular shape or amphipathicity of the compounds affected antiviral activity. Among compounds with rigid planar hydrophobic moieties of smaller cross sections than their hydrophilic heads, decreases in size of the hydrophobic moiety resulted in ≈1,000-fold increases in IC_{50} (dUY2 and dUY3 compared with dUY11) (Fig. S1 and Table S1). The IC_{50} of compounds with similarly sized hydrophobic derivatives was increased (by ≈120- to 700-fold) by introducing rotational flexibility or nonplanarity on the hydrophobic moiety, modifications that alter the molecular shape (dUY4, dUY6, dUY8, and dUY5, compared with dUY11) (Fig. S1 and Table S1). Introduction of polar groups in the linker or core hydrophobic moiety, which decrease amphipathicity, resulted in large increases in IC_{50}, or loss of antiviral activity (dUY9, dUY1, aUY1, dUY7, aUY12) (Fig. S1 and Table S1), although increasing the polarity of the hydrophilic moiety could partially rescue the antiviral activity of one of these compounds (compare aUY1 with dUY1) (Fig. S1 and Table S1). As discussed in the main text, introducing or removing one or two hydroxyl groups in the hydrophobic moiety resulted in less than threefold changes in IC_{50} (aUY11, dUY11, ddUY11) (Fig. S1 and Table S1). Even the introduction of larger modifications to the polar moiety, but which maintained overall molecular shape and amphipathicity, resulted in only twofold changes in IC_{50} (Pv-ddUY11) (Fig. S1 and Table S1). In contrast, introduction of slightly larger modifications to the polar moiety, but which change molecular shape and amphipathicity, resulted in an ≈30,000-fold increase in IC_{50} (Mk-dUY11) (Fig. S1). Amphipathicity and appropriate shape of the compound, with a hydrophobic moiety of smaller cross section than its hydrophilic head, and planarity and rigidity of the hydrophobic moiety, are therefore important for antiviral activity.

dUY11 Does not Lyse Virion Envelopes. Although dUY11 does not inhibit virion binding, we tested whether any of its effects could have resulted from envelope lysis. HSV-1 virions were exposed to 7 μM dUY11 (140-fold above IC_{50}, to maximize sensitivity to detect even mild lytic effects) or 1.5% Igepal CA-630 which lyses HSV-1 envelopes. Exposed virions were purified through a 20% sucrose cushion, such that virions and naked capsids pellet through the cushion, whereas proteins or DNA released by lysis remain in the supernatant. As expected, both dUY11 and Igepal CA-630 inhibited the infectivity of the treated virions without affecting capsid integrity (as evaluated by recovery of HSV-1 DNA in the virion pellet) (Fig. S3 *A* and *B*). Furthermore, as expected from envelope destabilization, Igepal CA-630 virion exposure led to loss of membrane glycoproteins from the virion pellets (Fig. S3*C*). In contrast, dUY11 had no effect on the recovery of virion envelope glycoproteins in the virion pellet (Fig. S3*C*). As a control, membranes were reprobed with a poly-specific antibody that recognizes multiple HSV-1 envelope and capsid proteins. Equivalent amounts of capsid proteins had been loaded for all treatments. Therefore, dUY11 does not lyse virions.

SI Methods

Cells and Viruses. Vero, human foreskin fibroblast (HFF), transformed HEK293, and HeLa cells were maintained in DMEM supplemented with 5% FBS. Huh7.5 cells were maintained in DMEM supplemented with 10% FBS. Stocks of low-passage wild-type HSV-1 strain KOS, or HSV-2 strains 333 and 186, Sindbis virus (SIN), or vesicular stomatitis virus (VSV) were prepared and titrated by standard techniques. Poliovirus (PV) Mahoney strain cDNA (4) was obtained from J. Yin and M. James (University of Alberta, Edmonton, AB, Canada). Linearized cDNA was transcribed in vitro and RNA was transfected into HeLa cells to produce the seed.

Infections. Vero cell monolayers were incubated with three plaque-forming units per cell of the indicated virus for 1 h at 37 °C, unless otherwise indicated. Huh7.5 monolayers were infected with three hepatitis C virus (HCV) genome copy equivalents per cell for 4 h at 37 °C. Inocula were removed, cells were washed twice or thrice, and overlaid with complete media alone or supplemented with 2% methylcellulose or different drugs. When indicated, cells and supernatants were harvested at selected times postinfection and infectious virions titrated by standard plaque assays in the absence of drug (5). HCV infectivity was evaluated by Western blot analyses of nonstructural proteins in infected cells.

Compounds. Rigid amphipathic nucleoside derivatives were synthesized at the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (Russian Academy of Sciences, Moscow, Russia).

3-O-(Trifluoromethylsulfonyl)-estra-1,3,5(10)-trien-17-one. The compound 3-*O*-(trifluoromethylsulfonyl)-estra-1,3,5(10)-trien-17-one (Fig S4*A*) was prepared from estrone following published procedures (6). ^{1}H NMR (500 MHz, $CDCl_3$): 0.91 (s, 3H, H-18), 1.42 to 1.55 (m, 3H, H-7, H-11, H-12), 1.56 to 1.70 (m, 3H, H-8, H-14, H-15), 1.95 to 2.01 (m, ^{1}H, H-12), 2.02 to 2.09 (m, 2H, H-7, H-15), 2.14 (m, ^{1}H, H-16), 2.29 (m, ^{1}H, H-9), 2.40 (m, ^{1}H, H-11), 2.52 (dd, ^{1}H, ^2J = 19.2 Hz, J15,16 = 8.5 Hz, H-16), 2.94 (m, 2H, H-6), 6.99 (d, ^{1}H, $^4J_{2,4}$ = 2.5 Hz, H-4), 7.03 (dd, ^{1}H, $^4J_{2,4}$ = 2.5 Hz, $J_{1,2}$ = 8.7 Hz, H-2), and 7.34 (d, ^{1}H, $J_{1,2}$ = 8.7 Hz, ^{1}H).

3-(Trimethylsilylethynyl)-estra-1,3,5(10)-trien-17-one. Under Ar, CuI (76 mg, 0.40 mmol), and triethylamine (0.56 mL, 4.0 mmol) were added successively to a solution of 3-*O*-(trifluoromethylsulfonyl)-estra-1,3,5(10)-trien-17-one (2.012 g, 5.0 mmol) and trimethylsilylacetylene (1.41 mL, 10.0 mmol) in dry DMF (30 mL) tetrakis(triphenylphosphine)palladium(0) (232 mg, 0.20 mmol) (Fig. S4*A*). The mixture was stirred for 20 h at room temperature, then diluted with EtOAc (200 mL), and washed with 3% aq. EDTA (4 × 100 mL) and water (4 × 100 mL), dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography on silica gel, eluting with 1% and then 2% EtOAc in toluene to give the title compound (1.462 g, 83%) as colorless amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$): 0.24 (s, 9H, Me_3Si), 0.91 (s, 3H, H-18), 1.38 to 1.66 (m, 6H, H-7, H-8, H-11, H-12, H-14, H-15), 1.95 to 2.08 (m, 3H, H-7, H-12, H-15), 2.14 (m, ^{1}H, H-16), 2.29 (m, ^{1}H, H-9), 2.39

(m, ^{1}H, H-11), 2.50 (dd, ^{1}H, ^2J = 19.0 Hz, $J_{15,16}$ = 8.7 Hz, H-16), 2.88 (m, 2H, H-6), and 7.18 to 7.24 (m, 3H, ^{1}H, H-2, H-4).

3-Ethynyl-estra-1,3,5(10)-trien-17-one. Tetrabutylamine fluoride trihydrate (1.577 g, 5.0 mmol) was added to a solution of 3-(trimethylsilylethynyl)-estra-1,3,5(10)-trien-17-one (1.400 g, 4.0 mmol) in THF (20 mL), and the mixture was incubated for 16 h at room temperature (Fig. S4*A*). The mixture was diluted with toluene (50 mL), washed with and water (2 × 100 mL), dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography on silica gel, eluting with 5%→7% EtOAc in toluene to give the title compound (839 mg, 75%) as colorless solid. ^{1}H NMR (500 MHz, $CDCl_3$): 0.91 (s, 3H, H-18), 1.40 to 1.66 (m, 6H, H-7, H-8, H-11, H-12, H-14, H-15), 1.95 to 2.08 (m, 3H, H-7, H-12, H-15), 2.14 (m, ^{1}H, H-16), 2.29 (m, ^{1}H, H-9), 2.41 (m, ^{1}H, H-11), 2.51 (dd, ^{1}H, ^2J = 19.1 Hz, $J_{15,16}$ = 8.5 Hz, H-16), 2.89 (m, 2H, H-6), 3.01 (s, ^{1}H, ≡CH), and 7.22 to 7.28 (m, 3H, ^{1}H, H-2, H-4).

General procedure for the preparation of protected nucleosides. A solution of 3′,5′-O-silyl protected 5-iodonucleoside (1, X = H or OH) (1 mmol), or 5′-pivaloyl-5-iododeoxyuridine and corresponding acetylene (1.5 mmol) in DMF (15 mL) was degassed three times by alternating between vacuum and argon (Fig. S4*B*). Tetrakis(triphenylphosphine)palladium(0) (116 mg, 0.10 mmol), CuI (38 mg, 0.20 mmol), and triethylamine (0.28 mL, 2 mmol) were successively added, the mixture was purged with argon, and then stirred for 40 h at room temperature. The mixture was then diluted with EtOAc (200 mL), washed with 3% aq. EDTA (4 × 100 mL) and water (4 × 100 mL), dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography on silica gel in appropriate solvent.

5-[(Estra-1,3,5(10)-trien-17-one-3-yl)ethynyl]-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (Markiewicz-dUY1). As illustrated in Fig. S5, this compound was prepared from 5-iodo-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine and 3-ethynyl-estra-1,3,5(10)-trien-17-one. Isolated by chromatography in 10% Me_2CO in toluene. White foam. Yield 402 mg (54%). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.84 (s, 3H, H-18′′), 0.95 to 1.07 (m, 28H, i-Pr), 1.30 to 1.61 (m, 6H, H-7′′, H-8′′, H-11′′, H-12′′, H-14′′, H-15′′), 1.77 (m, ^{1}H, H-12′′), 1.93 to 2.00 (m, 2H, H-7′′, H-15′′), 2.09 (m, ^{1}H, H-16′′), 2.29 to 2.44 (m, 4H, H-2′, H-9′′, H-11′′), 2.84 (m, 2H, H-6′′), 3.67 to 4.38 (m, 3H, H-3′, H-4′, H-5′), 6.06 (m, ^{1}H, ^{1}H′), 7.12 (m, ^{1}H, H-4′′), 7.18 (m, ^{1}H, H-2′′), 7.32 (m, ^{1}H, ^{1}H′′), 7.67 (s, ^{1}H, H-6), and 11.73 (br s, ^{1}H, NH).

5-[(Estra-1,3,5(10)-triene-17-one-3-yl)ethynyl]-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-arabino-uridine (Markiewicz-aUY1). As illustrated in Fig. S5, this compound was prepared from 5-iodo-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-arabino-uridine and 3-ethynyl-estra-1,3,5(10)-triene-17-one. Isolated by chromatography in 20% Me_2CO in $CHCl_3$. White foam. Yield 431 mg (57%). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.83 (s, 3H, H-18′′), 0.91 to 1.09 (m, 28H, i-Pr), 1.31 to 1.62 (m, 6H, H-7′′, H-8′′, H-11′′, H-12′′, H-14′′, H-15′′), 1.79 (m, ^{1}H, H-12′′), 1.92 to 2.01 (m, 2H, H-7′′, H-15′′), 2.10 (m, ^{1}H, H-16′′), 2.29 to 2.44 (m, 4H, H-2′, H-9′′, H-11′′), 2.84 (m, 2H, H-6′′), 3.77 (d, ^{1}H, H-4′, J = 8.3 Hz), 3.92 to 3.96 (m, ^{1}H, 5′-HA), 4.05 to 4.10 (m, ^{1}H, 5′-HB), 4.12 to 4.17 (app. t, ^{1}H, H-3′, *J* = 8.2 Hz), 4.36–4.43 (m, ^{1}H, H-2′), 5.96 (d, ^{1}H, OH, *J* = 5.1 Hz), 6.12 (d, ^{1}H, ^{1}H′, *J* = 6.4 Hz), 7.16 (s, ^{1}H, H-4′′), 7.19 (d, ^{1}H, *J* = 8.0 Hz, H-2′′), 7.36 (d, ^{1}H, ^{1}H′′, *J* = 8.0 Hz), 7.81 (s, ^{1}H, H-6), and 11.9 (br s, ^{1}H, NH).

5-(Pyren-2-yl)ethynyl-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (Markiewicz-dUY3). This compound was prepared from 5-iodo-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine and 2-ethynylpyrene (Fig. S5) (7). Isolated by chromatography in 0 to 5% EtOAc in $CHCl_3$. Yellowish foam. Yield 466 mg (67%). ^{1}H NMR (500 MHz, DMSO-d_6): 11.80 (br s, ^{1}H, NH), 8.59 (s, ^{1}H, H-6), 8.31 (s, 2H, ^{1}H", 3"), 8.20 (d, 2H, $J_{6''7''} = J_{7'',8''}$ = 7.7 Hz, H-6", 8"), 8.11 (m, 2H, H-5", 9"), 8.05 (m, 3H, H-4", 7", 10"), 5.98 (app. t, $J_{1',2\alpha'} = J_{1',2\beta'}$ = 6.3 Hz, ^{1}H′), 4.51 (m, ^{1}H, H-3′), 4.05 (m, ^{1}H, H-5′α), 3.97 (m, ^{1}H, H-5′ β), 3.75 (m, ^{1}H, H-4′), 2.50 (m, ^{1}H, H-2′ α), 2.32 (m, ^{1}H, H-2′ β), and 1.05 to 0.94 (m, 28H, Pr^i).

5-(Perylen-3-yl)ethynyl-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (Markiewicz-dUY11). This compound was prepared from 5-iodo-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine and 3-ethynylperylene (Fig S5). Isolated by chromatography in 20 to 40% Me_2CO in PhMe. Orange foam. Yield 640 mg (81%). ^{1}H NMR (500 MHz, DMSO-d_6): 2.28 (m, ^{1}H), 2.54 (m, ^{1}H) (H-2′), 3.42–3.59 (m, 3H, H-4′, H-5′), 3.99 to 4.08 (m, ^{1}H, H-3′), 6.28 (app. t, ^{1}H, *J* = 6.5 Hz, ^{1}H′), 7.52 to 7.64 (m, 2H), 7.63 to 7.71 (m, 2H), 7.80 to 7.86 (m, 2H), 8.16 (d, ^{1}H, *J* = 8.0 Hz), 8.47 to 8.37 (m, 4H) (perylene), 8.67 (s, ^{1}H, H-6), and 11.70 (br s, ^{1}H, NH).

5-(Perylen-3-yl)ethynyl-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-arabino-uridine (Markiewicz-aUY11). This compound was prepared from 5-iodo-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-*arabino*-uridine and 3-ethynylperylene. Isolated by chromatography in 0 to 3% EtOH in $CHCl_3$. Orange foam. Yield 245 mg (32%). ^{1}H NMR (600 MHz, DMSO-d_6): 11.89 (br s, ^{1}H, N*H*), 8.45 (d, ^{1}H, *J* = 7.3), 8.42 to 8.35 (m, 3H) (^{1}H", 6", 7", 12"), 8.25 (d, ^{1}H, *J* = 8.3, H-4"), 7.83 (m, 2H, H-9", 10"), 7.80 (s, ^{1}H, H-6), 7.65 (m, 2H, H-2", 5"), 7.56 (m, 2H, H-8", 11"), 6.11 (d, ^{1}H, *J* = 6.4, ^{1}H′), 5.96 (d, ^{1}H, *J* = 5.9, O*H*), 4.38 (m, ^{1}H, H-4′), 4.13 (m, ^{1}H, H-3′), 4.07 (m, ^{1}H, H5′a); 3.93 (m, ^{1}H, H-5′b); 3.75 (m, ^{1}H, H-2′α), 1.05 to 0.94 (m, 28H, Pr^i). ^{13}C NMR (150 MHz, DMSO-d_6): 161.46 (C4), 149.41 (C2), 143.64 (C6), 134.20, 133.88, 131.33, 131.02, 130.47, 130.10, 129.82, 128.72, 128.39, 127.80 (2C), 127.62, 127.04, 126.99, 125.72, 121.71, 121.43, 121.34, 120.31, 119.35 (perylene), 97.82 (C5), 90.86 (Ca), 88.14 (Cb), 82.99, 79.15, 74.30, 74.25, 59.97 (C1′, 2′, 3′, 4′, 5′), 17.27 (2C), 17.23, 17.09, 16.93, 16.82 (2C), 16.76 (*C*H_3), 12.90, 12.40, 12.09, and 11.89 (Si*C*).

5-(Perylen-3-ylmethyloxymethyl)ethynyl-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-arabino-uridine (Markiewicz-aUY12). This compound was prepared from 5-iodo-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-*arabino*-uridine and 3-ethynylmethyloxymethylperylene. Isolated by chromatography in 0 to 2% MeOH in $CHCl_3$. Orange foam. Yield 428 mg (55%). ^{1}H NMR (700 MHz, DMSO-d_6): 11.74 (br s, ^{1}H, N*H*), 8.39 (d, ^{1}H, *J* = 7.7), 8.36 (m, 2H), 8.32 (d, ^{1}H, *J* = 7.7) (^{1}H", 6", 7", 12"), 7.98 (d, ^{1}H, *J* = 8.3, H-4"), 7.80 (m, 2H, H-9", 10"), 7.65 (s, ^{1}H, H-6), 7.58 (m, 2H, H-2", 5"), 7.54 (m, 2H, H-8", 11"), 6.05 (d, ^{1}H, *J* = 6.3, ^{1}H′), 5.89 (d, ^{1}H, *J* = 5.7, O*H*), 4.95 (s, 2H, ArCH_2), 4.44 (s, 2H,≡CCH_2), 4.33 (m, ^{1}H, H-4′), 4.06 (m, ^{1}H, H-3′), 4.01 (m, ^{1}H, $^2J_{5'a,5'b}$ = 11.6, H-5′a), 3.88 (m, ^{1}H,
$^2J_{5'a,5'b}$= 11.6, H-5′b), 3.70 (m, ^{1}H, H-2′α), 1.03 to 0.94 (m, 28H, Pr^i). ^{13}C NMR (175 MHz, DMSO-d_6): 161.47 (C4), 149.38 (C2), 143.38 (C6), 134.22 (C9a"), 133.01 (C3"), 132.62 (C3a"), 130.84 (C6a"), 130.46 (C6b"), 130.30 (C12a"), 128.83, 128.74, 128.23, 128.06, 127.72 (C3a1", C6b1", C9", C10", C12b"), 127.10, 126.95 (3C) (C2", C5", C8", ^{11}C"), 124.18 (C4"), 120.91 (2C), 120.79 (C6", C7", C12"), 120.07 (C1"), 97.11 (C5), 88.94 (CH_2C≡*C*), 84.36 (C4′, 84.18 (C1′), 78.98 (CH_2*C*≡), 69.25 (C3′), 68.86 (ArCH_2), 60.81 (C5′), 57.71 (*C*H_2C≡), 38.60 (C2′), 17.33, 17.18 (3C), 17.07, 16.89 (2C), 16.81 (*C*H_3), 12.76, 12.45, 12.02, and 11.94 (Si*C*).

5-(Perylen-3-ylmethyloxymethyl)ethynyl-5′-O-pivaloyl-2′,3′-dideoxyuridine (Pv-ddUY11). This compound was prepared from 5-iodo-5′-*O*-pivaloyl-2′,3′-dideoxyuridine and 3-ethynylperylene. Isolated by chromatography in 0 to 2% MeOH in $CHCl_3$. Orange foam. Yield 545 mg (96%). ^{1}H NMR (600 MHz, DMSO-d_6): 11.85 (br s, ^{1}H, N*H*), 8.43 (d, ^{1}H, *J* = 7.8), 8.40 to 8.34 (m, 3H) (^{1}H", 6", 7", 12"), 8.31 (d, ^{1}H, *J* = 8.2, H-4"), 8.06 (s, ^{1}H, H-6), 7.82 (m, 2H, H-9", 10"), 7.67 (m, 2H, H-2", 5"), 7.55 (m, 2H, H-8", 11"), 6.00 (m, ^{1}H, ^{1}H′), 4.35 (m, ^{1}H, $^2J_{5'a,5'b}$ = 11.5, $J_{4',5'a}$ = 4.6, H-5′a), 4.31 to 4.24 (m, 2H, H-4′,5′b), 2.38 (m, ^{1}H), 2.14 (m, ^{1}H), 2.02 (m, ^{1}H), 1.86 (m, ^{1}H) (H-2′,3′), 1.17 (s, 9H, CH_3). ^{13}C NMR (150 MHz, DMSO-d_6): 177.45 (COO), 161.65 (C4), 149.41 (C2), 142.68 (C6), 134.20, 133.81, 131.20, 130.98, 130.42, 130.11, 129.83, 128.65, 128.35, 127.79 (2C), 127.62, 127.02, 126.98, 125.89, 121.62, 121.43, 121.29, 120.32, 119.51 (perylene), 98.39 (C5), 91.03 (Ca), 88.65 (Cb), 86.25, 78.35, 64.72 (C1′, 4′, 5′), 31.26, 30.69, 26.97 (3C, CCH_3), and 25.23 (C2′, 3′, CCH_3).

General procedure for the preparation of 5-arylethynylated 2′-deoxyuridines and arabino-uridines. Triethylamine trihydrofluoride (245 μL, 1.5 mmol) was added to a solution of the corresponding 5-arylethynyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (0.5 mmol) in THF (3 mL). After 12 h at room temperature, the product was precipitated with ether. The precipitate was filtered, washed with ether (2 × 10 mL), and dried in vacuum (Fig. S4*B*).

5-(Estra-1,3,5(10)-triene-17-one-3-yl)ethynyl-2′-deoxyuridine (dUY1). This compound was prepared from 5-[(estra-1,3,5(10)-triene-17-one-3-yl) ethynyl]-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (Markiewicz-dUY1) (Fig. S5). White solid. Yield 182 mg (72%). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.83 (s, 3H, H-18′′), 1.33 to 1.63 (m, 6H, H-7′′, H-8′′, H-11′′, H-12′′, H-14′′, H-15′′), 1.76 to 1.81 (m, ^{1}H, H-12′′), 1.93 to 2.02 (m, 2H, H-7′′, H-15′′), 2.03 to 2.12 (m, ^{1}H, H-16′′), 2.25 to 2.50 (m, 5H, H-2′, H-9′′, H-11′′, H-16′′), 2.82 to 2.88 (m, 2H, H-6′′), 3.68 to 3.84 (m, 3H, H-4′, H-5′), 4.11 (m, ^{1}H, H-3′), 5.14 (t, ^{1}H, 5′-OH, J = 5.0 Hz), 5.22 (d, ^{1}H, 3′-OH, J = 4.3 Hz), 6.22 (app. t, ^{1}H, J = 6.4, ^{1}H′), 7.17 (s, ^{1}H, H-4′′), 7.20 (d, ^{1}H, J = 8.0 Hz, H-2′′), 7.33 (d, ^{1}H, J = 8.0 Hz, ^{1}H′′), 8.11 (s, ^{1}H, H-6), and 11.6 (br s, ^{1}H, NH).

5-[(Estra-1,3,5(10)-triene-17-one-3-yl)ethynyl]-arabino-uridine (aUY1). This compound was prepared from 5-[(estra-1,3,5(10)-triene-17-one-3-yl)ethynyl]-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-*arabino*-uridine (Markiewicz-aUY1) (Fig. S5). White solid. Yield 211 mg (81%). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.83 (s, 3H, H-18′′), 1.30 to 1.62 (m, 6H, H-7′′, H-8′′, H-11′′, H-12′′, H-14′′, H-15′′), 1.77 to 1.84 (m, ^{1}H, H-12′′), 1.93 to 2.02 (m, 2H, H-7′′, H-15′′), 2.03 to 2.14 (m, ^{1}H, H-16′′), 2.34 to 2.53 (m, 4H, H-9′′, H-11′′, H-16′′), 2.82 to 2.86 (m, 2H, H-6′′), 3.67 to 3.76 (m, 2H), 3.80 to 3.82 (m, ^{1}H), 3.98 to 4.05 (m, ^{1}H), 4.08 to 4.14 (m, ^{1}H) (H-2′, H-3′, H-4′,H-5′), 5.20 to 5.30 (br s, ^{1}H), 5.50 to 5.66 (br s, ^{1}H), 5.72 to 5.81 (br s, ^{1}H) (2′-OH, 3′-OH, 5′-OH), 6.07 (d, ^{1}H, ^{1}H′, J = 6.4), 7.14 (s, ^{1}H, H-4′′), 7.18 (d, ^{1}H, J = 8.0, H-2′′), 7.38 (d, ^{1}H, ^{1}H′′, J = 8.0), 8.26 (s, ^{1}H, H-6), and 11.3 to 11.8 (br s, ^{1}H, NH).

5-(Pyren-2-yl)ethynyl-2′-deoxyuridine (dUY3). This compound was prepared from 5-(pyren-2-yl)ethynyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (Markiewicz-dUY3) (Fig. S5). Yellowish solid. Yield 213 mg (94%). ^{1}H NMR (500 MHz, DMSO-d_6): 11.79 (br s, ^{1}H, N*H*), 8.61 (s, ^{1}H, H-6), 8.32 (s, 2H, ^{1}H”,3′′), 8.20 (d, 2H, $J_{6'',7''} = J_{7'',8''}$ = 7.7 Hz, H-6”,8”), 8.10 (m, 2H, H-5′′, 9”), 8.04 (m, 3H, H-4”,7”,10”), 6.19 (app.t, $J_{1',2\alpha'} = J_{1',2\beta'}$ = 6.4 Hz, ^{1}H′), 5.30 (d, ^{1}H, J = 4.3 Hz, 3′-O*H*), 5.27 (t, ^{1}H, J = 5.0 Hz, 5′-O*H*), 4.36 (m, ^{1}H, H-3′), 3.90 (m, ^{1}H, H-4′), 3.80 to3.75 (m, ^{1}H, H-5′ α), 3.71 to 3.66 (m, ^{1}H, H-5′ β), and 2.30 to 2.17 (m, 2H, H-2’).

5-(Perylen-3-yl)ethynyl-2′-deoxyuridine (dUY11). This compound was prepared from 5-(perylen-3-yl)ethynyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine (Markiewicz-dUY11). Analytical characteristics identical to material obtained earlier (Fig. S5) (8).

5-(Perylen-3-yl)ethynyl-arabino-uridine (aUY11). This compound was prepared from 5-(Perylen-3-yl)ethynyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-*arabino*-uridine (Markiewicz-aUY11) (Fig. S5). Orange solid. Yield 173 mg (67%). ^{1}H NMR (700 MHz, DMSO-d_6): 11.74 (br s, ^{1}H, N*H*), 8.45 (d, ^{1}H, J = 7.7), 8.45 (d, ^{1}H, J = 7.6), 8.39 (m, 2H), 8.35 (d, ^{1}H, J = 7.6) (^{1}H”, 6”, 7”, 12”), 8.29 (d, ^{1}H, J = 8.3, H-4”), 8.25 (s, ^{1}H, H-6), 7.83 (m, 2H, H-9”, 10”), 7.71 (d, ^{1}H, J = 7.6, H-2”), 7.68 (m, 2H, H-5”), 7.57 (m, 2H, H-8”, 11”), 6.06 (d, ^{1}H, J = 3.8, ^{1}H′), 5.71 (br s, ^{1}H), 5.53 (br s, ^{1}H), 5.26 (br s, ^{1}H) (2’, 3′, 5′-O*H*), 4.10 (m, ^{1}H, H-3′), 3.99 (m, ^{1}H, H-2′), 3.81 (m, ^{1}H, H-4′), 3.70 (m, 2H, H-5′). ^{13}C NMR (175 MHz, DMSO-d_6): 161.67 (C4), 149.42 (C2), 145.02 (C6), 134.21, 133.76, 131.10, 130.99, 130.45, 130.13, 129.85, 128.63, 128.36, 127.90, 127.79, 127.62, 127.03, 126.99, 125.79, 121.58, 121.39, 121.26, 120.33, 119.54 (perylene), 97.01 (C5), 90.51 (Ca), 88.98 (Cb), 85.60, 84.81, 75.35, 74.95, and 60.33 (C1’, 2’, 3’, 4’, 5’).

5-(Perylen-3-ylmethyloxymethyl)ethynyl-arabino-uridine (aUY12). This compound was prepared from 5-(Perylen-3-ylmethyloxymethyl) ethynyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-*arabino*-uridine (Markiewicz-aUY12). Orange solid. Yield 228 mg (82%). ^{1}H NMR (500 MHz, DMSO-d_6): 11.66 (s, ^{1}H, NH), 8.43 to 8.32 (m, 4H, ^{1}H”, H-6”, H-7”, H-12”), 8.35 (s, ^{1}H, H-6), 8.01 (d, ^{1}H, $J_{4'',5''}$=8.2, H-4”), 7.81 (d, 2H, $J_{8'',9''} = J_{10'',11''}$ = 8.2, H-9”, H-10”), 7.65 to 7.53 (m, 4H, H-2”, H-5”, H-8”, H-11”), 6.13 (app. t, ^{1}H, $J_{1',2'\alpha}=J_{1',2'\beta}$ = 6.4, H-10), 5.67 (m, ^{1}H), 5.20 (br s, ^{1}H), 5.18 (br s, ^{1}H) (2′,3′,5′-O*H*), 4.92 (s, 2H, $ArCH_2$), 4.47 (s, 2H, $\equiv CCH_2$), 4.25 (m, ^{1}H, H-3′), 3.81 (m, ^{1}H, H-4’), 3.67 to 3.55 (s, 2H, H-5′), 2.20 to 2.10 (m, 2H, H-2’). ^{13}C NMR (125.7 MHz, DMSO-d_6): 161.69 (C4), 149.51 (C2), 144.12 (C6), 134.29 (C9a”), 133.13 (C300), 132.73 (C3a”), 130.88 (C6a”), 130.52 (C6b”), 130.36 (C12a”), 128.22, 128.13, 128.08, 128.00, 127.72 (C12b”, C3a1, C6b1”, C9”, C10”), 127.17, 126.98 (3C) (C2”, C5”, C8”, ^{11}C”), 124.34 (C4”), 120.91 (2C), 120.84 (C6”, C7”, C12”), 120.14 (C1”), 97.84 (C5), 88.76 ($CH_2C\equiv C$), 87.73 (C4’), 84.92 (C1’), 79.23 ($CH_2C\equiv$), 74.95,70.15 (C2′, C3′), 69.18 ($ArCH_2$), 61.01 (C5′), 57.67 ($CH_2C\equiv$).

5-Perylenylethynyl-2′,3′-dideoxyuridine (ddUY11). Solid KOH (310 mg, 5.52 mmol was added to a suspension of 5′-*O*-pivaloyl-5-perylenylethynyl-2′,3′-dideoxyuridine (350 mg, 0.613 mmol) in MeOH (80 mL). The mixture was stirred overnight, quenched with acetic acid, concentrated in vacuum, re-evaporated twice with methanol, and subjected to column chromatography in in 0 to 3% EtOH in $CHCl_3$. Orange crystals; m.p. above 300 °C (MeOH/$CHCl_3$). Yield 299 mg (99%). ^{1}H NMR (700 MHz, DMSO-d_6): 11.74 (br s, ^{1}H, N*H*), 8.75 (s, ^{1}H, H-6), 8.45 (d, ^{1}H, J = 7.6), 8.39 (m, 2H), 8.35 (d, ^{1}H, J = 7.7) (^{1}H”, 6”, 7”, 12”), 8.29 (d, ^{1}H, J = 8.3, H-4”), 7.82 (m, 2H, H-9”, 10”), 7.68 (m, 2H, H-2”, 5”), 7.56 (m, 2H, H-8”, 11”), 5.98 (m, ^{1}H, ^{1}H′), 5.34 (br s, ^{1}H, O*H*), 4.12 (m, ^{1}H, H-4′), 3.85 (m, ^{1}H, $^{2}J_{5'a,5'b}$ = 11.6, H-5′a), 3.63 (m, ^{1}H, $^{2}J_{5'a,5'b}$ = 11.6, H-5′b), 2.34 (m, ^{1}H), 2.14 (m, ^{1}H), 1.96 (m, ^{1}H), 1.88 (m, ^{1}H) (H-2′,3′). ^{13}C NMR (175 MHz, DMSO-d_6): 161.73 (C4), 149.43 (C2), 144.08 (C6), 134.21, 133.72, 131.01, 130.95, 130.34, 130.14, 129.87, 128.60, 128.33, 127.89, 127.78, 127.62, 127.02, 126.97, 125.86, 121.44, 121.38, 121.24, 120.30, 119.67 (perylene), 97.42 (C5), 90.37 (Ca), 89.24 (Cb), 86.19, 82.46, 61.20 (C1′, 4′, 5′), 32.59, and 23.82 (C2′, 3′).

Other Compounds. The synthesis of all other compounds is described elsewhere (8–12).

Compound and Drug Use. Test compounds were prepared in DMSO as 10-mM stocks, and resuspended to indicated concentrations in DMEM supplemented with or without 5% FBS just before use. For cytotoxicity assays at high concentrations, dUY11, ddUY11, and aUY11 were prepared as 30- or 100-mM stocks (aUY11 in 1:1 DMSO:ethanol). Stocks of phosphonoacetic acid were prepared at 100 mg/mL in DMEM, neutralized with NaOH and stored frozen. Stocks were diluted to the desired concentrations in DMEM supplemented with 5% FBS just before use. Stocks of heparin were prepared at 100 mg/mL in DMEM and used at concentrations from 30 to 1,000 μg/mL.

Cytotoxicity Assays. Vero cell monolayers (4 to 6 × 10^4 cells/well in 24-well plates) were incubated with phenol red-free DMEM supplemented with 5% FBS and the required concentrations of each rigid amphipathic fusion inhibitor (RAFI). For incubations longer than 48 h, media were replaced at 24, 48, and 72 h with fresh phenol red-free DMEM supplemented with 5% FBS and the same concentration of the same RAFI. Cell number and viability were analyzed by [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction (MTT) assays at 24, 48, 72, or 96 h of treatment. Absorbance was read at 570 nm and background at 650 nm. Cell numbers are expressed as fold-change from before adding the compounds.

Infectivity Assays. One volume containing 200 infectious virions (HSV-1, HSV-2, VSV, SIN, or PV) was mixed with one volume of DMEM supplemented with twice the final concentration of test compound, or sufficient 10 to 1,000 μM dUY11 in DMSO was

added to 3×10^6 HCV genome copy equivalents/mL in 500 μL to achieve the desired final concentrations, and incubated at 37 to 40 °C for 5 min. Control treatments contained the highest concentration of DMSO necessary to achieve the highest concentration of RAFI tested. Cell monolayers (typically 2.5 to 6×10^5 cells per well in 6-well plates) were incubated with 500 (HCV) or 200 μL (all other viruses) of the exposed virions at 37 °C for 4 (HCV) or 1 h (all other viruses). Cells were then washed twice with ice-cold DMEM and overlaid with drug-free DMEM supplemented with 10% FBS (HCV) or 5% FBS and 2% methylcellulose (all other viruses). For HCV, cells were passed as required and proteins were extracted 4 d after infection. For all other viruses, cells were fixed and stained with crystal violet in 17% methanol in water when well-defined plaques had developed (typically, 2–3 d postinfection).

To extract proteins from HCV-infected cells, cell monolayers were washed once with PBS and lysed using radioimmuno precipitation assay buffer (10 mM Tris-HCL, 140 mM NaCl, 0.02% sodium azide, 1% Triton ×100, 0.1% SDS, 1% deoxycholic acid) supplemented with a mixture of protease inhibitors (Roche). Samples were vortexed, sonicated, and incubated on ice for 30 min. Afterward, cell extracts were cleared at 14,000 rpm (in a FA45-30–11 rotor in an Eppendorf 5810R centrifuge; Eppendorff, Mississagua, ON, Canada) for 10 min at 4 °C. Protein concentration in the supernatant was determined using Biorad D_C Protein Assay (Bio-Rad Laboratories).

HSV-1 and adenovirus expressing GFP reporter genes [GFP HSV-1 (13) or GFP AdV, respectively] were exposed to dUY11 as described above. HEK293 cell monolayers were incubated for 1 h with the exposed virions, washed, and overlaid with drug-free DMEM supplemented with 5% FBS. GFP expression was evaluated by fluorescence microscopy (Leica DM IRB) and documented using a digital camera (QIMAGING Retiga 1300).

To evaluate effects on progeny virions, Vero cell monolayers infected with 3 pfu of HSV-1 KOS or Sindbis per cell were treated with DMEM supplemented with 5% FBS and increasing concentration of test compounds, either continuously until harvesting or for only 1 h (from 1 to 2 h after infection). In the latter case, drug-containing medium was removed, cells were washed twice with DMEM, and overlaid with drug-free DMEM supplemented with 5% FBS. At 24 h after infection, supernatants were collected from HSV-1 or Sindbis infected cells. HSV-1 infected cells were then washed with 500 μL DMEM, scraped, transferred to tubes, and freeze-thawed three-times by alternating in an ethanol-dry ice bath and a 37 °C water bath. Cellular debris from the cell lysates and supernatants was pelleted by centrifugation at 4,000 rpm at 4 °C for 30 min in a SW A-4–62 rotor in an Eppendorf 5810R centrifuge (Eppendorff, Mississagua, ON, Canada). Equal volumes of supernatants or cell lysates, containing 200 infectious units for the untreated cells, were titrated onto Vero cells in the absence of drug. Alternatively, virions were purified from the entire supernatants by high-speed centrifugation followed by a sucrose cushion. Virion budding was then determined by viral DNA levels in the purified virions, expressed as percentage of HSV-1 DNA in cells plus virions.

Binding Assays. HSV-1 virions were metabolically labeled with ^{35}S methionine to ≈0.5 cpm/μL (~1.2 cpm/pfu). Labeled virions were exposed for 5 min at 37 °C to 0 or 7 μM dUY11 (140-fold above IC_{50}, to maximize sensitivity for even weak binding inhibition), or 100 μg/mL heparin. Samples were cooled on ice and diluted in ice-cold DMEM supplemented with no drug, 7 μM dUY11, or 100 μg/mL heparin. Vero cell monolayers precooled to 4 °C were then incubated with ≈1.6×10^5 cpm of radiolabeled HSV-1 virions preexposed to dUY11 or vehicle. Following 1 h adsorption at 4 °C, supernatants were recovered and cells were washed three times with ice-cold DMEM. Radioactivity was evaluated by β-scintillation counting. Equal amounts of unlabeled HSV-1 virions were processed following the same steps in parallel and at the same time, except that cells were overlaid with DMEM supplemented with 2% methylcellulose and 5% FBS after the 1 h adsorption and then transferred to 37 °C CO_2 incubators until plaques developed. Cells were then fixed and stained with crystal violet as described above.

For secondary gD-mediated binding, radiolabeled HSV-1 virions preexposed to dUY11 or vehicle were adsorbed onto precooled Vero cell monolayers for 15 min at 4 °C. Cells were then washed twice with ice-cold DMEM. One set of cells infected with virions exposed to 0 or 7 μM dUY11 was lysed to assess total binding. All other infected cells were washed twice more with ice-cold DMEM and then once for 1 h with DMEM supplemented with 0, 30, 100, 300, or 1,000 μg/mL heparin. Cells were then washed twice with ice-cold DMEM supplemented with 0, 30, 100, 300, or 1,000 μg/mL heparin. One-hundred percent virion binding was defined as cell-bound cpm before the heparin washes. High-affinity binding was defined as percentage cpm bound to cells after all heparin washes.

Entry Assays. HSV-1 KOS was centrifuged at 10,000 × g for 2 h and resuspended in PBS. Aliquots of 100 μL were subjected to UV (3,000 mJ) in open tissue culture dishes (Stratalinker 2400; Stratagene) for increasing times (30-s intervals from 0 to 5 min). Original and UV-inactivated virus suspensions were titrated by standard plaque assays on Vero and Vero clone 57 cells. Only viral stocks inactivated by at least 10,000-fold but that still fully induced RFP expression were used. UV-inactivated virions were exposed to 0, 0.05, 0.2, 0.65, or 2 μM dUY11 (the highest being 3.5-fold below the concentrations that did not inhibit binding, to ensure that any observed inhibition of entry was not secondary to inhibition of binding) before infecting Vero clone 57 cells. RFP expression was evaluated 24 h later by fluorescence microscopy (Leica DM IRB) and documented at 400 magnifications using a digital camera (QIMAGING Retiga 1300).

DNA Analyses. Vero cell monolayers were mock or HSV-1 infected for 1 h at 37 °C, washed twice with PBS, and overlaid with DMEM supplemented with 5% FBS and no drug, 2 μM dUY11 (40-fold above IC_{50}), or 400 μg/mL phosphonoacetic acid. Mock- or HSV-1-infected cells were harvested at 1, 5, 18, or 24 h. Supernatants were harvested at 24 h and virions were purified through a 20% sucrose cushion at 100,000 × *g* for 1 h at 4 °C. Total cellular and viral DNA was extracted from HSV-1-infected cells. Virion HSV-1 DNA was extracted from the purified virions or from virions preexposed to dUY11 or Igepal CA-630. DNA was purified by digesting proteins for 5 h at 55 °C in STE buffer containing 5% SDS and 200 μg/mL proteinase K. DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by an equal volume of chloroform:isoamyl alcohol (24:1), and precipitated overnight with 2.5 volumes of 100% ethanol at −20 °C. The DNA pellet was then rinsed with 70% ethanol, resuspended in TE (10 mM Tris-Cl, 1 mM EDTA; pH 7.5), resolved in 0.7% agarose gel electrophoresis, and transferred onto BiodyneB nylon membranes (Pall Corporation), following standard protocols. Dried membranes were prehybridized with rapid hybrid buffer (Amersham Sciences) at 80 °C, and then hybridized with ^{32}P-dCTP labeled EcoRI JK HSV-1 fragment for 3 h at 80 °C. Membranes were rinsed at room temperature twice for 15 min each in 300 mM NaCl, 30 mM sodium citrate, and 0.1% SDS, then rinsed as necessary for 5 min at 80 °C in 75 mM NaCl, 7.5 mM sodium citrate (0.5 × SSC), and 0.5% SDS. Washed membranes were exposed to Kodak PhosphorImager screens and scanned in a Molecular Imager FX (BIO-RAD).

Western Blot Analyses. Twenty micrograms of protein extracted from HCV or mock-infected Huh7.5 cells, or lysates from 1×10^8 HSV-1 virions, were resolved by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blot with mouse monoclonal anti-HCV NS3 helicase domain antibody (Chemicon International) diluted 1:2,000, or anti-actin (Millipore Bioscience Research Reagents) diluted 1:10,000, or with rabbit polyclonal anti-gD or anti-gH/gL antibodies (kind gifts from Gary Cohen,

University of Pennsylvania) diluted 1:1,000 or 1:2,000, respectively, or with rabbit polyclonal anti-HSV-1 antibody (catalog #B0114 DakoCytomation Canada, Inc.) diluted 1:2,000. Membranes were incubated for 1 h in 1:1 PBS:Odyssey blocking buffer (LI-COR Biosciences) before the 2-h incubation at room temperature with the respective primary antibodies. Membranes were then washed at room temperature thrice in PBS-Tween 0.1% for 5 min each, followed by one wash in PBS before incubation at room temperature with IRDye 800-labeled goat anti-mouse or anti-rabbit secondary antibodies diluted 1:20,000 (Rockland). Membranes were washed in the dark four times as described above, scanned and quantitated in an Odyssey Infrared Imaging System (LI-COR Biosciences).

Cellular Localization of dUY11. Near-confluent Vero cell monolayers seeded onto coverslips were treated with 600 nM dUY11 (12-fold above IC_{50}) for 5 min at 37 °C, washed twice with DMEM and incubated with 250 nM PKH26 membrane dye (Sigma-Aldrich) for 5 min at 37 °C. Cells were then washed twice with DMEM, mounted onto coverslips in 90% glycerol in PBS, and evaluated in a Zeiss LSM510 laser scanning confocal microscope with a 63 × Plan-Apochromat oil immersion DIC objective lens (numerical aperture, 1.4; working diameter, 0.09 mm) using a 25-mW Argon laser (458 nm) and a 1 mW HeNe laser (543 nm) with a band-pass filter of 470 to 600 nm. The pinhole aperture was 1.0 airy unit for each channel. The images were collected as 12-bit images using Zen 2009 software (Zeiss) with zoom factor of 1 (top pictures) or 1.3 (bottom pictures). Contrast and brightness were finely adjusted in Microsoft PowerPoint.

Temperature-Dependence Assays. HSV-1 KOS at $\approx 1 \times 10^7$ pfu/mL was mixed with an equal volume of 4 μM dUY11 (final concentration, 2 μM, 40-fold above IC_{50}, to maximize the inhibition of infectivity) for 5 min at 4 or 37 °C. Exposures were terminated by the addition of 900 μL of ice-cold DMEM. Virions were serially diluted 10-fold in ice-cold DMEM. Two hundred microliters of each dilution were adsorbed onto Vero cell monolayers for 1 h at 4 or 37 °C. Inocula were removed, and cells were washed twice with ice-cold DMEM before overlaying with DMEM supplemented with 5% FBS and 2% methylcellulose.

Virion Integrity Analysis. Infectious HSV-1 virions (2.25×10^8 virions per treatment) were exposed to 0 or 7 μM dUY11 for 5 min at 37 °C, or incubated with 1.5% Igepal CA-630 for 30 min at 4 °C. Equal aliquots from each treatment, containing 200 infectious units for the virions exposed to 0 μM dUY11, were tested for virion infectivity.

The remaining aliquots were layered onto 20% sucrose cushions and ultracentrifuged at $200{,}000 \times g$ for 3 h at 4 °C in a Beckmann SW40 rotor. The supernatant was then carefully decanted, and the virion pellets were resuspended in STE buffer (1 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA) and lysed by addition of SDS (0.5%). Samples were further subdivided in two equal aliquots. One was analyzed by Southern blot and the other by Western blot.

Spectra. The RAFI dUY11 was added to 2.5 mL of 180 mM Na_2HPO_4, 10 mM citric acid (pH 7.4) (fusion buffer), or 2.5 mL of 1-octanol, to a final concentration of 48 or 0.48 nM, respectively. Alternately, dUY11 was added to 25 μg VSV or 2 nmol L-α-phosphatidylcholine β-oleoyl-γ-palmitoyl liposomes (Sigma) in 2.5 mL of fusion buffer (pH 7.4), to a final concentration of 48 nM. Spectra were collected at 37 °C. Fluorescence was excited at 455 nm using a QuantaMaster 40 scanning spectrofluorometer (Photon Technology International) equipped with a 75W xenon lamp. Emitted fluorescence was detected using a model 814 switchable photon-counting/analog photomultiplier detector with an R1527 photomultiplier tube. Data were collected using FeliX32 software (Photon Technology International). Spectra were normalized to the highest fluorescence signal intensity obtained for all conditions (which was in octanol).

Fusion Assays. VSV virions (21.6 μg VSV protein; 5.18×10^7 infectious units) were labeled in the dark with 0.59 μM octadecyl rhodamine B chloride (R18, Invitrogen) in fusion buffer (pH 7.4) for 1 h at room temperature. Labeled VSV was purified through a Sephadex G-100 column. Purified labeled VSV (0.60 μg viral protein; 1×10^4 infectious units) was exposed to 0.1% DMSO or 15 nM dUY11 for 10 min at 39 °C, and then incubated on ice for 3 min, before mixing with 1×10^6 prechilled Vero cells in 220 μL of fusion buffer (pH 7.4) and incubated on ice for 30 min (to allow VSV binding but not fusion). Virus-cell complexes were washed with fusion buffer (pH 7.4), resuspended in 2.5 mL of the same buffer, warmed at 37 °C for 5 min, and added to a prewarmed cuvette. After 10 min, the pH of one duplicate such sample was adjusted to 5.5 by adding 0.31 mL of 500 mM citric acid, to trigger fusion, whereas the other duplicate was maintained at pH 7.4 by adding 0.31 mL of fusion buffer (pH 7.4). Excitation, 560 nm; detection, 590 nm. Data were collected using FeliX32 software (Photon Technology International). Percent-dequenching was calculated according to:

$$\%\text{dequenching} = (((F_{5.5} - F_0)/(F_{max} - F_0)) - ((F_{7.4} - F_0)/(F_{max} - F_0)))\text{x}100$$

where $F_{5.5}$ is the fluorescence at pH 5.5 at each time point, $F_{7.4}$ is the fluorescence at pH 7.4 at each time point, F_0 is the fluorescence after 10-min incubation at pH 7.4, and F_{max} is the total fluorescence detected after disrupting the cells with Triton X-100 (to a final concentration of 0.1%).

Effects on Membrane Curvature. Changes in intrinsic monolayer curvature induced by dUY11 were assessed by determining its effects on the bilayer to hexagonal phase transition temperature of dielaidoylphosphatidylethanolamine (DEPE) (Avanti Polar Lipids). A solution of dUY11 was made in chloroform:methanol (2:1). The concentration of dUY11 was determined by absorbance at 467 nm of solutions in methanol:water (1:1). A stock solution of DEPE in chloroform:methanol (2:1) was also made and aliquots of the DEPE solution containing 5 mg DEPE were mixed in a glass tube with appropriate amounts of dUY11 solution to have several samples in a range of mole fractions of dUY11 from 0 to 0.025. The samples were dried under nitrogen gas, making a thin film on the walls of the glass tube and then placed in a vacuum desiccator for 3 h. The dried films were hydrated with 0.8 mL of 20 mM Pipes, 0.14 M NaCl, 1 mM EDTA pH 7.4 by extensive vortexing, degassed, and then placed in the calorimeter cell. Runs were performed at a scan rate of 1°/min, with buffer in the reference cell. Buffer vs. buffer scans were subtracted from all sample scans. The results were plotted with the program ORIGIN 7.0 and analyzed with the curve fitting program DA-2 supplied by Microcal, Inc.

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Fig. S1. SAR studies of the antiviral activity of RAFIs. Chemical structures of the hydrophilic (*A*) and hydrophobic (*B*) moieties and 3D models (*B*), displayed in three orthogonal perspectives, of 5-arylalkynyl nucleoside derivatives compared with (*C*) two membrane phospholipids, phosphatidylethanolamine, and sphingomyelin. The former has hydrophobic and hydrophilic moieties of similar cross-sections and is normally relatively enriched in inner membrane leaflets; the latter has a hydrophilic head larger than its hydrophobic moiety and is normally relatively enriched in the outer membrane leaflets. Gray, carbon; teal, hydrogen; pink, oxygen; blue, nitrogen; red, phosphorous. (*D*) Dose-response activity of each compound against HSV-1 infectivity; graph *I*, dUY11 (□), aUY11 (○), ddUY11 (▲), Mk-dUY11 (△), Pv-ddUY11 (●); graph *II*, dUY2 (○), dUY3 (▲); graph *III*, dUY4 (▲), dUY6 (○), dUY8 (□); graph *IV*, dUY1 (●), aUY9 (△), dUY9 (□); graph *V*, dUY5 (■), dUY7 (△), aUY12 (○). Compounds are grouped in each graph corresponding to the groups in *B*.

Fig. S2. Characterization of the antiviral and cellular activities of RAFIs. (*A*) Inhibition of HSV-1 infectivity is independent of infected cell type. Semilogarithmic line graph showing the reduction in HSV-1 infectivity plotted against dUY11 concentration. HSV-1 virions (200 infectious units) were exposed to 0, 0.002, 0.007, 0.02, 0.07, 0.2, 0.7, 2, 7, or 20 μM dUY11 for 5 min at 37 to 40 °C. HEK293 (▲), HFF (□), HeLa (△), or Vero cells (●) were infected with the HSV-1 virions preexposed to dUY11. Error bars, SD (*n* = 4, HeLa; *n* = 3, HEK293 and HFF; *n* = 22, Vero). (*B*) Inhibition of infectivity of progeny SIN virions produced by cells treated with dUY11. Semilogarithmic line graph showing the reduction in progeny virion infectivity against dUY11 concentration. Vero cells were infected with thee infectious units per cell of Sindbis virus, washed, and overlaid with media supplemented with 0, 0.002, 0.007, 0.02, 0.07, 2, 7, 20, or 70 μM dUY11 from 1 to 2 h (■) or from 1 to 24 h (□) after infection. Supernatants were harvested at 24 h. Infectivity of equal volumes of supernatant was evaluated by plaque assays. Error bars, SD (*n* = 4). (*C*) Antiviral and cytotoxic effects of different RAFIs are unrelated to each other. Line graph showing inhibition of cell replication (*C*) or of cell viability (*D*) for different RAFIs. Vero cells were treated with the indicated concentrations of each RAFI, expressed as multiples of IC_{50}, and the number of viable cells was evaluated by MTT assays at 0 and 48 h. Error bars, ranges ($n \geq 2$). The highest concentration tested for each compound was determined by solubility or by achieving ≥50% cytostaticity or cytotoxicity. (*C*) Cytostatic compounds or compounds that have no measurable effects on cell proliferation or viability. Cytostatic effects are expressed as percentage of untreated cell replication in 48 h. Green, dUY4; light blue, Pv-ddUY11; plum, dUY7; orange, dUY2; green, aUY1; red, dUY1; dark blue, Mk-dUY11; olive, dUY8; gray-blue, dUY6; pink, aUY12; black, dUY3. (*D*) Cytotoxic compounds, expressed as percentage of cell viability relative to the number of cells before adding the compound. Red, dUY5; blue, dUY9; black, dUY3. Because dUY3 is cytostatic or cytotoxic at lower or higher concentrations, respectively, it is presented in both graphs.

Fig. S3. RAFI dUY11 does not lyse HSV-1 virions. Photographic images, bar graphs, and Southern and Western blots showing virion infectivity (*A*), relative HSV-1 DNA levels (*B*), or HSV-1 structural proteins in virions (*C*). HSV-1 virions exposed to no drug (ND), 7 μM dUY11 ("11"), or 1.5% Igepal CA-630 ("30") were analyzed for infectivity by plaque assays (*A*). The wells in each panel were infected with the virions exposed to the indicated agent and undiluted (*Top Left*), or with serial 1:10 dilutions (from *Top Left* to *Bottom Right*). *0.015% Igepal CA-630 is cytotoxic. Alternatively, virions exposed to the different agents were purified through 20% sucrose cushions. HSV-1 DNA (*B*) or proteins (*C*) were then tested in the virion pellets. The top two panels in *C* were probed with antibodies raised against the envelope glycoprotein gD and the envelope glycoprotein complex gH/gL, respectively (the antibody recognizes the gH moiety of the complex). The bottom panel was probed with an antibody that recognizes multiple envelope, tegument, and capsid proteins. Only membrane proteins are absent from the virions treated with Igepal CA-630, whereas no proteins are absent from the virions exposed to dUY11.

Fig. S4. (*A* and *B*) Schemes of syntheses of RAFIs.

Fig. S5. Structures of dUY1, aUY1, dUY3, dUY11, and their Markiewicz intermediates.

Table S1. RAFIs IC_{50} and CC_{50}

		CC_{50} (μM)	
Compound	IC_{50} ± SD (μM)	Cytostatic	Cytotoxic*
dUY11	0.048 ± 0.012	>>150	—
ddUY11	0.087 ± 0.053	33	—
aUY11	0.131 ± 0.034	>>>150	—
Pv-ddUY11	0.190 ± 0.011	>>>38	—
Mk-dUY11	165 ± 26.339	>>>165	—
aUY12	14.05 ± 2.218	>>>98.35	—
dUY1	>200[†]	>>>200	—
aUY1	60.15 ± 9.062	<60.1	—
dUY2	51.10 ± 16.138	<51.1	—
dUY3	40.08 ± 14.173	43.31	<80
dUY4	7.42 ± 0.520	74.1	—
dUY5	36.97 ± 3.872	N/A	<36.9
dUY6	35.22 ± 16.954	>105.6	—
dUY7	>200[†]	>>200	—
dUY8	6.21 ± 2.035	62.1	—
dUY9	>200[†]	N/A	150

Inhibition of cell replication with respect to vehicle treated cells. >>> Less than 15% inhibition of cell replication at the highest concentration tested. >> More than 15 but less than 30% inhibition of cell replication at the highest concentration tested. > More than 30% inhibition in cell replication at the highest concentration tested. < More than 50% cytostatic or cytotoxic effect at the lowest concentration tested (for dUY3, the lowest concentration showing cytotoxic effects). N/A, not available, compound was cytotoxic at the lowest concentration tested. —, no tested concentration resulted in a lower cell numbers at 4 d than before treatment.

*Decrease in number of viable cells with respect to before starting treatment.

[†]dUY1, dUY7, and dUY9 did not achieve 50% inhibition of HSV-1 infectivity at the highest concentration tested.

Table S2. IC_{50} of dUY11 on HSV-1 infectivity to different cell lines

Cell type	IC_{50} ± SD (μM)
Vero	0.048 ± 0.012
HFF	0.042 ± 0.021
HEK293	0.037 ± 0.010
HeLa	0.044 ± 0.011

Table S3. IC_{50} of dUY11 on the infectivity of enveloped and nonenveloped viruses

Virus	IC_{50} ± SD (μM)
HSV-1 KOS	0.048 ± 0.012
HSV-2 186	0.049 ± 0.009
HSV-2 333	0.055 ± 0.005
VSV	0.005 ± 0.001
SIN	0.011 ± 0.005
HCV	0.183 ± 0.001
PV	>200*
AdV	>20*

*No concentration tested inhibited the infectivity of poliovirus (PV) or adenovirus (AdV) by 50%.

Table S4. IC_{50} of dUY11 on HSV-1 and Sindbis virion infectivity and on the infectivity of progeny virions produced by cells treated for 1 or 23 h after infection

Infectivity of	IC_{50}± SD (μM)
Exposed HSV-1 virions	0.048 ± 0.012
Extracellular progeny HSV-1 virions (1-h cell treatment)	6.25 ± 4.474
Intracellular progeny HSV-1 virions (1-h cell treatment)	7.1 ± 0.925
Extracellular progeny HSV-1 virions (23-h cell treatment)	0.63 ± 0.338
Intracellular progeny HSV-1 virions (23-h cell treatment)	0.42 ± 0.261
Exposed SIN virion	0.011 ± 0.005
Extracellular progeny SIN virions (1-h cell treatment)	1.52 ± 0.017
Extracellular progeny SIN virions (23-h cell treatment)	0.011 ± 0.005