Ring Expanded Nucleoside Analogues Inhibit RNA Helicase and Intracellular Human Immunodeficiency Virus Type 1 Replication

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A series of ring expanded nucleoside (REN) analogues were synthesized and screened for inhibition of cellular RNA helicase activity and human immunodeficiency virus type 1 (HIV-1) replication. We identified two compounds, **1** and **2**, that inhibited the ATP dependent activity of human RNA helicase DDX3. Compounds **1** and **2** also suppressed HIV-1 replication in T cells and monocyte-derived macrophages. Neither compound at therapeutic doses was significantly toxic in ex vivo cell culture or in vivo in mice. Our findings provide proof-of-concept that a cellular factor, an RNA helicase, could be targeted for inhibiting HIV-1 replication.

Introduction

Human immune deficiency virus type 1 (HIV-1) is a retrovirus with an RNA genome of ~9 kilobases. HIV-1 is the etiological agent for the acquired immunodeficiency syndrome (AIDS). Since the first report of AIDS in 1981, HIV-1 has cumulatively caused 33 million deaths worldwide. Current HIV therapeutics target viral proteins such as reverse transcriptase, protease, integrase, and gp120. Because HIV-1 can mutate its genome rapidly, a consequence of this treatment approach has been the selection for, and the rapid emergence of, drug-resistant HIV-1's.[1] Thus, there is a compelling need to evolve therapeutic strategies that do not readily elicit resistant mutations.

One way to circumvent the mutability of the HIV-1 genome is to target cellular factors used by the virus for replication.[2] Several such cellular factors have been characterized,[3–8] including a cellular RNA-helicase reported as necessary for HIV-1 to replicate optimally in human cells.[9,10] Mammalian RNA-helicases include proteins that have DEAD-, DEAH-, DExH-, and DExD- motifs.[11,12] These helicases participate in many aspects of cellular RNA metabolism including transcription, RNA splicing and stability, mRNA- export and translation, and mitochondrial gene expression.[13–16] While all RNA helicases apparently conserve the same eight discrete protein motifs,[12] there is some evidence that individual RNA helicases can be sufficiently different that each could be targeted with some degree of specificity.[17–22] Thus, there are reports of compounds that inhibit with relative specificity the RNA-helicases encoded by viruses such as hepatitis C, West Nile, herpes simplex, and Japanese encephalitis.[18–22]

HIV-1 does not encode a viral helicase, but it does engage several cellular RNA-helicases for replication.[23] In this study, we asked if novel compounds could be discovered that target cellular RNA helicases and whether such compounds would inhibit HIV-1 replication in cultured cells. Toward these goals, we screened several ring-expanded nucleoside (REN[a]) analogues (Figure 1), some of which were previously described to be inhibitors of the NTPase/helicase of West Nile virus, hepatitis C virus, and Japanese encephalitis virus.[21,22] We identified two RENs, **1** and **2**, that inhibited the activity of human RNA helicase DDX3 in vitro and intracellular HIV-1 replication. Encouragingly, neither **1** nor **2** was toxic to cells or to mice at concentrations that were inhibitory for DDX3 and HIV-1. Our findings offer proof-of-principle for treating HIV-1 infection using small molecules that target a cellular protein.

Results

Compounds 1 and 2 Inhibit Productive HIV Replication in T Cells and Macrophages. We tested a panel of RENs (Figure 1) for inhibition of HIV-1 replication in MT4 cells. Among 12 compounds, two strongly inhibited peak HIV-1 replication (10 μM concentration) on day 4 of infection in cultured cells (Figure 2). We selected **1** and **2** (Figure 3A), the two that were most potent, for further characterization of their virus-inhibitory activities.

RENs were previously found to inhibit virus encoded RNA helicases.[21,22] We asked if **1** or **2** could inhibit the activity of a cellular RNA helicase, DDX3. DDX3 was identified earlier to be important for HIV-1 replication in human cells.[9] As an initial test, **1** and **2** were queried for their inhibition of DDX3′s ability to unwind in vitro a partially double-stranded RNA. A partially double-stranded RNA substrate was generated (see Experimental
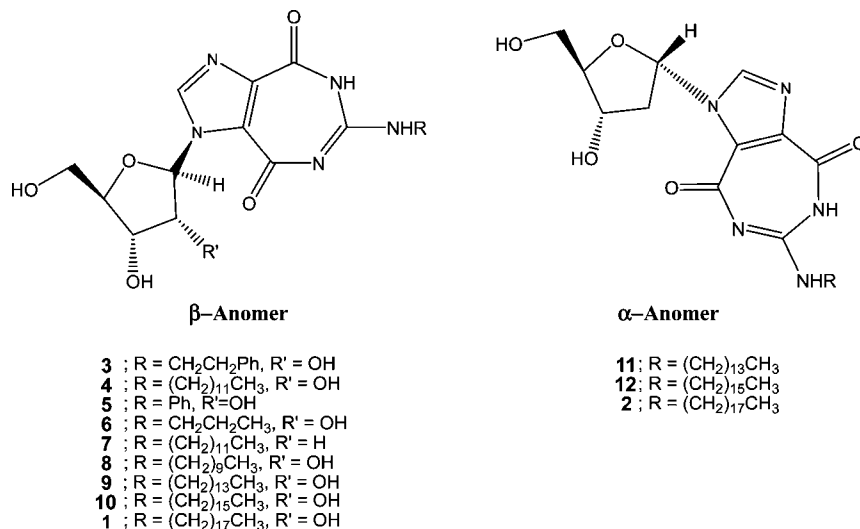
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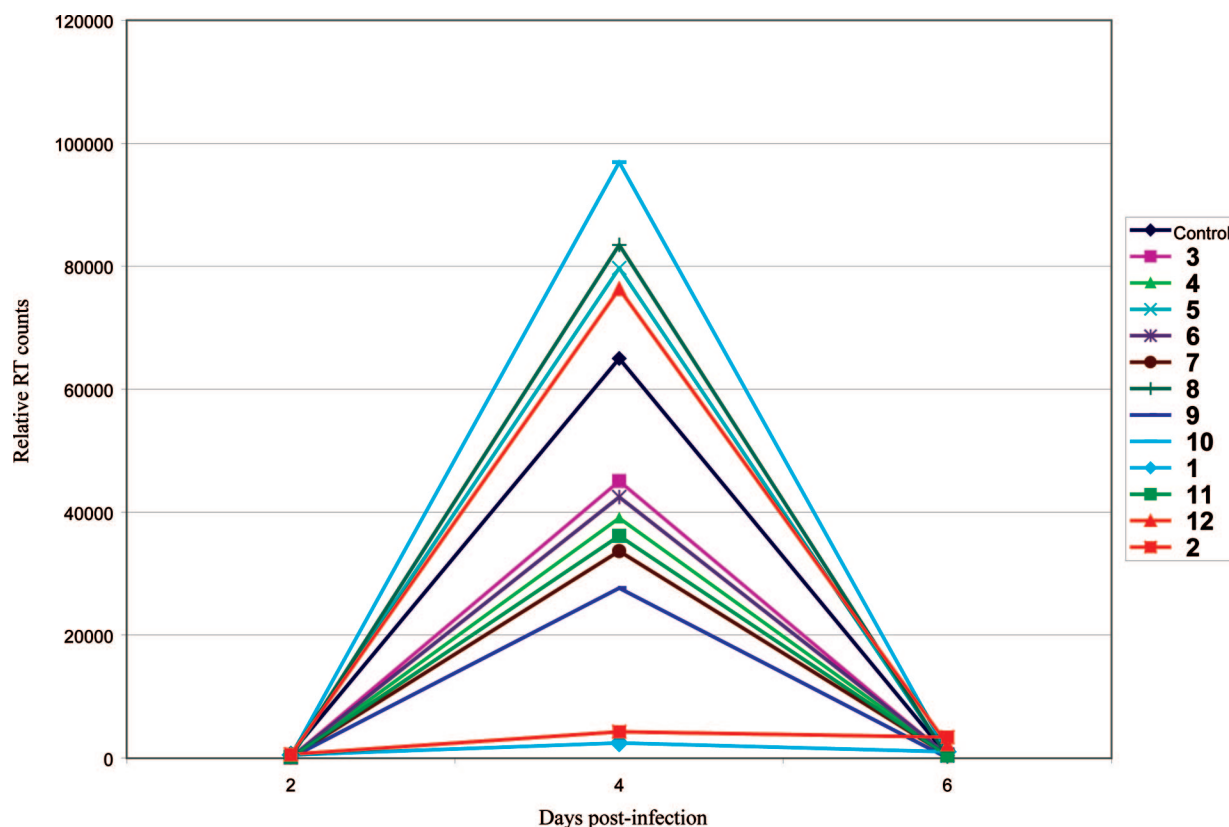
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[a] Abbreviations: REN, ring expanded nucleoside; MDM, monocyte derived macrophages; DMSO, dimethyl sulfoxide; RT, reverse transcriptase; CCR5, chemokine (C−C motif) receptor 5; CXCR4, chemokine (C−X−C motif) receptor 4; NTPase, nucleoside triphosphatase; TFA, trifluoroacetic acid.

β–Anomer

3 ; R = CH₂CH₂Ph, R' = OH
4 ; R = (CH₂)₁₁CH₃, R' = OH
5 ; R = Ph, R'=OH
6 ; R = CH₂CH₂CH₃, R' = OH
7 ; R = (CH₂)₁₁CH₃, R' = H
8 ; R = (CH₂)₉CH₃, R' = OH
9 ; R = (CH₂)₁₃CH₃, R' = OH
10 ; R = (CH₂)₁₅CH₃, R' = OH
1 ; R = (CH₂)₁₇CH₃, R' = OH

α–Anomer

11; R = (CH₂)₁₃CH₃
12; R = (CH₂)₁₅CH₃
2 ; R = (CH₂)₁₇CH₃

**Figure 1.** α- and β-anomers of ring-expanded nucleoside analogues.

Figure 2. Screening of RENs for inhibition of HIV-1 replication. Twelve REN compounds were synthesized and used at 10 μM concentration to analyze their effects on HIV-1 replication in T cell line (MT4 cells). MT4 cells were infected with 20000 RT counts of virus and treated with the indicated compounds. Cell supernatants were collected every 2 days and assayed for RT activity. Compounds **1** and **2** were the most potent for inhibiting HIV-1 replication. Note that, by day 6, because no new cells are being added to the tissue cultures, continuous HIV-1 replication in cells that are not inhibited by drugs results in cell killing and a reduction of RT production to baseline.

Section), and this was incubated with purified WT DDX3 in the presence of ATP. We observed that the double-stranded substrate could indeed be unwound by WT-DDX3 to release single-stranded ³²P labeled RNA (Figure 3B, lane 2). However, when either **1** or **2** was added into the reaction, DDX3's unwinding activity was inhibited (Figure 3B, lanes 3 and 4).

We next tested **1** and **2** for inhibition of HIV replication in cultured cells. We first assayed **1** and **2** on HIV-1 infection of a suspension T cell line, MT4. In this setting, over the time

course of 8 days, an essentially complete suppression of HIV-1 replication was seen when **1** or **2** was used at 25 μM (Figure 4A,B).

Because the readout for HIV-1 replication in the MT4 cells was an in vitro assay of reverse transcriptase (RT) activity produced by the replicating virus and because **1** and **2** are nucleoside analogues that can potentially interfere with the RT assays, we were concerned that the observed reduction in RT activity could simply be an inhibition of the enzymatic assay
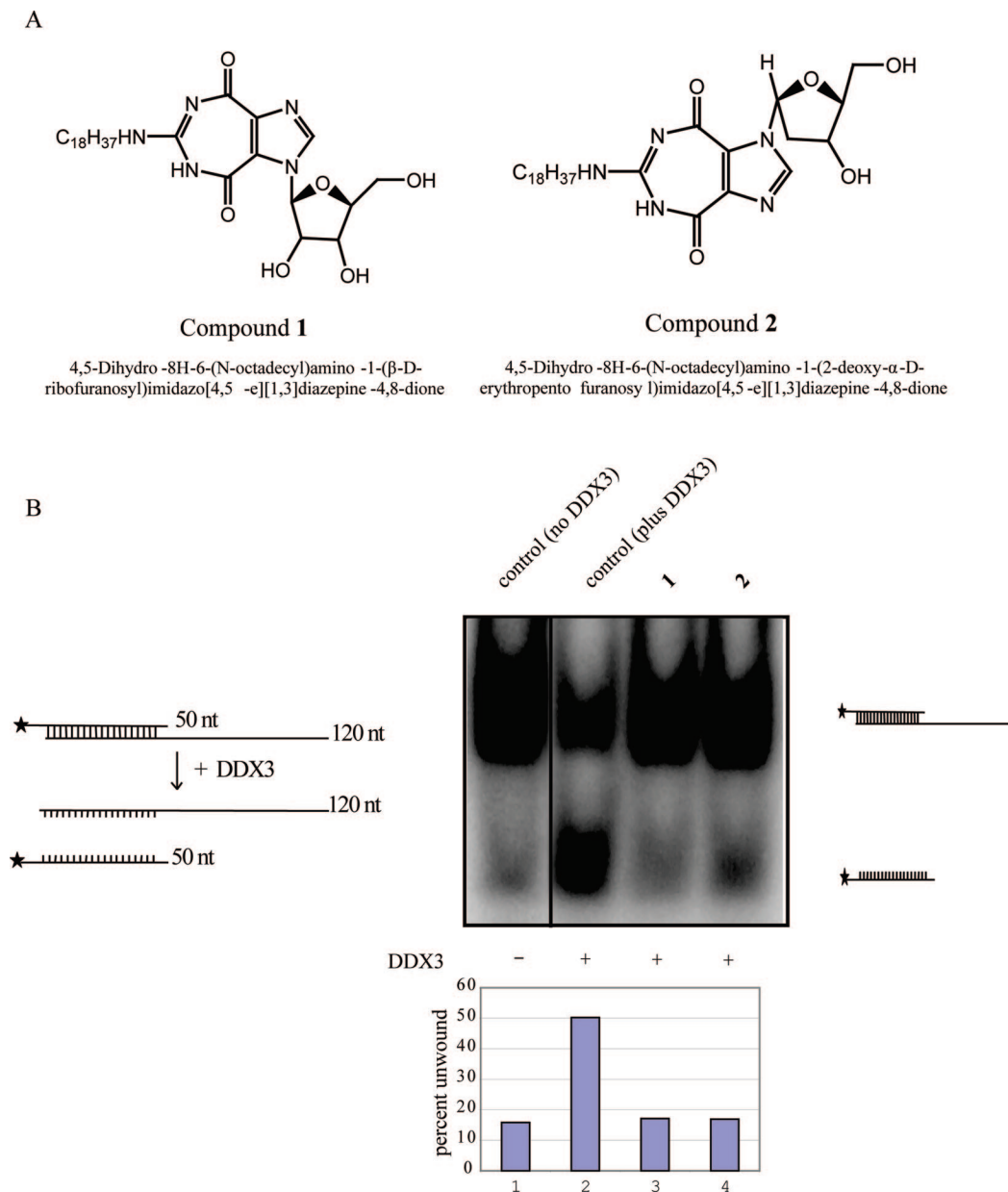
Figure 3. Inhibition of helicase activity in vitro. (A) Structures of **1** and **2**. (B) Inhibition of helicase activity of DDX3 by **1** and **2**. The pBluescript vector was digested to completion with *Kpn*I and transcribed with T3 polymerase to generate a 120 base long transcript. The vector was also digested with *Eco*RI and transcribed with T7 polymerase in the presence of (α32p) UTP. The two complementary transcripts (50 nt and 120 nt) were purified and hybridized at 95 °C for 20 min. Note that the 120 nt is unlabeled, while the 50 nt strand is ^{32}P-labeled. Autoradiography of the slower migrating band is due to duplex formation between the labeled and unlabeled strands (lane 1). Duplexed RNA was unwound by DDX3 in the presence of ATP, resulting in increased release of the labeled strand (50 nt; lane 2) and a reduction in intensity of the duplexed strands. Both **1** and **2** inhibited the ATP dependent RNA unwinding activity of DDX3 as measured by the reduced release of the 50 nt strand (lane 3 and 4). The bar diagram indicates percent duplex unwound by DDX3 in the presence and absence of **1** and **2**.

and not an actual suppression of virus replication in the infected MT4 cells. To exclude this possibility, HIV-1 replication in MT4 cells was quantified using an enzyme-linked immunosorbent assay (ELISA) measuring the viral p24 capsid protein. Indeed, similar to the RT results, the p24 ELISA findings (Figure 4C) confirmed a significant reduction of virus production (p24) by **1** (similar results were achieved with **2**, data not shown). We also asked whether **1** and **2** inhibited HIV-1 gene expression in transfected HeLa cells using a Western blotting assay (Figure 4D). On the basis of protein blotting using hyperimmune patient serum, we observed that both **1** and **2** greatly reduced the synthesis of viral proteins in the HIV-1 transfected HeLa cells

(Figure 4D), consistent with a mechanism affecting a postintegration step in viral replication.

Macrophage (M) tropic HIV-1 differs from T-cell tropic HIV-1 in that the former uses the CCR5 (not CXCR4) coreceptor and is the predominant viral genotype found in newly infected individuals. The above HIV-1/MT4 assays tested the effect of **1** and **2** on a T-tropic (CXCR4-utilizing) infection. Next, we queried whether **1** and **2** would also inhibit the replication of a M-tropic HIV-1 AD8 (a CCR5 using virus) in macrophages. We compared mock infected macrophages to macrophages infected with HIV-1 AD8 and found that virus replication in macrophages was indeed substantially suppressed
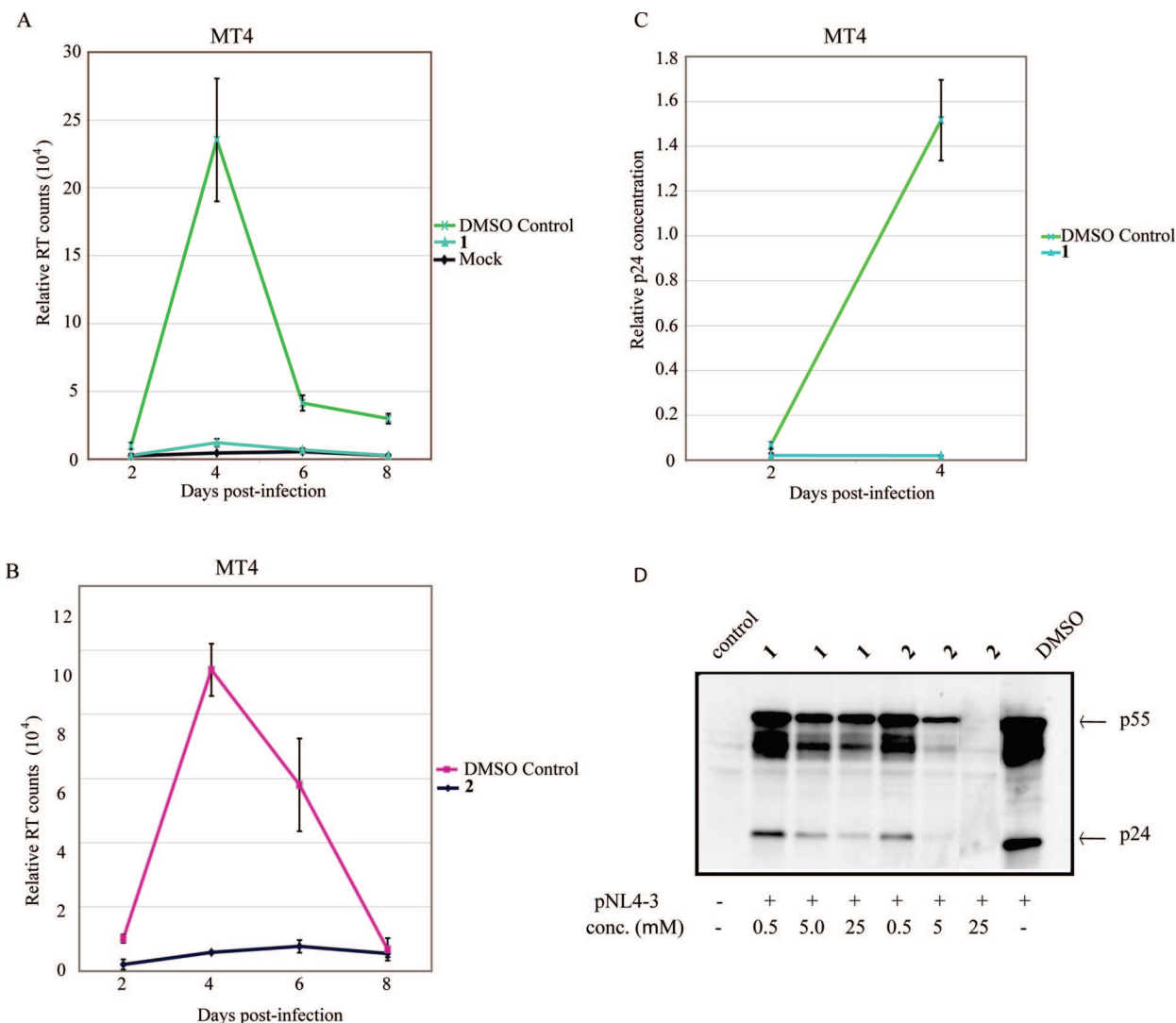
Figure 4. Measurement of **1** and **2** inhibition of HIV-1 replication using RT, p24, and Western blotting assays. MT4 cells were infected with 20000 RT counts of virus and treated with the indicated compounds. The experiments were repeated three times. Cell supernatants were collected every 2 days and assayed for both RT activity (A and B) and p24 ELISA (C). ELISA shows a corresponding decrease in the amount of p24 present in supernatants collected from infected MT4 cultures similar to the results from the RT assays. (D) **1** and **2** suppressed HIV-1 protein expression in HeLa cells transfected with HIV-1 molecular clone pNL4-3. HeLa cells were transfected with 2 μg pNL4-3, and 3 h post-transfection, the cells were treated with 25 μM concentration of **1** and **2**. Cell lysates were prepared 24 h later and analyzed by Western blotting using anti-HIV-1 patient IgG.

by both **1** and **2**, respectively, as assayed by measuring RT (Figure 5A) or p24 (Figure 5B) production.

Lack of Toxicity of 1 and 2 in Cultured Cells and in Mice. Potentially useful candidate antiviral compounds should have acceptable toxicity profiles. To check **1** and **2** for cytotoxicity, we employed a colorimetric assay that monitors cell viability after treatment with the compounds. MT4 cells were incubated individually with **1** or **2** at 1, 10 or 100 μM concentrations. Over a 6 day period, we compared **1** or **2** treated cells to mock treated control cells and found no significant differences in cell growth and viability (Figure 6A).

We next checked **1** and **2** for in vivo toxicity in mice. Mice, in groups of four, were injected intraperitoneally twice weekly with drug boluses calculated to achieve final **1** or **2** body distributions of 0.2, 2, 20, and 200 μM. The mice were injected and monitored for 5 weeks. Control mice were injected with either normal saline or DMSO. At the end of the study period, none of the mice injected with **1** or **2** displayed any symptoms or showed any visible signs of toxicity. Detailed necropsies and histologies were performed

on multiple organs from the **1**, **2**, and control mice. No difference was seen in blood smears, liver, spleen (Figure 6B), kidney, heart, and lung histologies (data not shown). These results are consistent with a lack of in vivo toxicity elicited by **1** and **2** over the studied period.

We further verified **1** for good in vivo systemic distribution in the injected mice. Absorption of the drug into the bloodstream and its distribution throughout the body are desirable properties for drug candidates. To measure if peritoneal injection of **1** distributed well in the host, we collected sera from compound **1** injected mice and analyzed the samples by HPLC. The sera from mice injected with 200 μM of **1** (Figure 7C) were extracted with 3 volumes of ethyl acetate and passed through a C4 Vydac 214TP1010 column (10 mm × 250 mm), and then eluted with a gradient of 25% (H_2O-0.1%TFA) and 75% (CH_3CN-0.1% TFA). This analysis revealed an absorption peak that corresponded to a **1**-specific signal in the positive control (Figure 7A), and in **1**-treated mice "spiked" with additional **1** compound (Figure 7D).
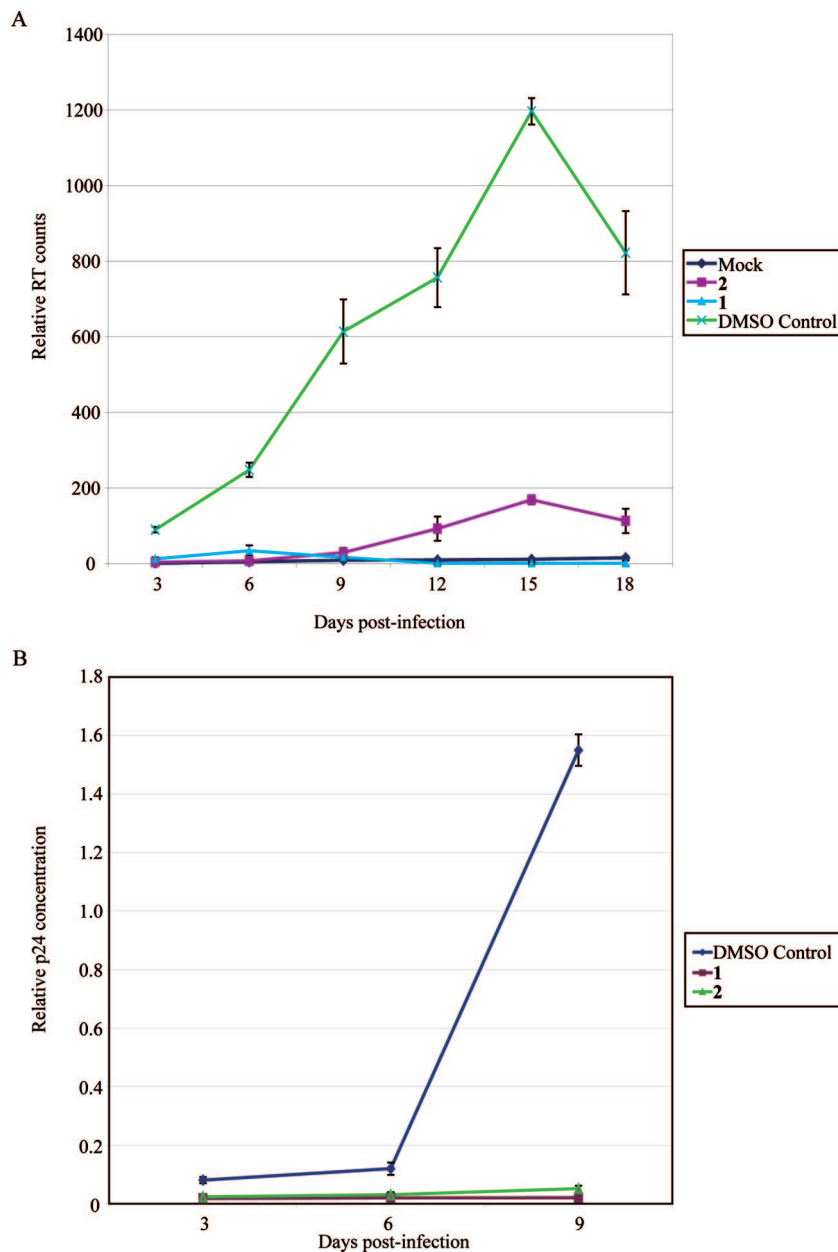
Figure 5. Compounds **1** and **2** suppressed HIV-1 replication in monocyte-derived-macrophages (MDMs). MDMs were infected with M-tropic HIV-1 AD8 with 100000 RT counts of virus on day 5 in culture and were treated with **1** or **2**. Supernatants were collected every 3 days and assayed for virus replication by (A) RT activity and (B) p24 ELISA. RT assays and p24 ELISA showed that both **1** and **2** suppressed HIV-1 replication. The experiments were repeated three times.

Conversely, the "compound **1** peak" was absent from the sera from DMSO-treated mice (Figure 7B).

Discussion

The increased prevalence of HIV-1 resistant to current antiviral drugs highlights an urgent need to develop new treatment approaches. Existing therapies are targeted to virus-encoded proteins (e.g., reverse transcriptase, protease, integrase, and gp120). Because of the relaxed fidelity of HIV-1's reverse transcriptase enzyme, virus-encoded proteins can rapidly mutate under drug selection.[1,24] Here, we have tested the concept of targeting HIV-1 replication using small molecules that inhibit the activity of a cellular RNA helicase, previously identified as important for virus replication in human cells.

We chose to target a cellular RNA helicase for two reasons. First, we and others[9,25−28] have begun to broach the possibility that cellular proteins which do not mutate rapidly under drug selection could be bona fide targets for inhibiting virus replication. Second, targeting helicase activity is attractive because there is a body of literature that virus-encoded RNA helicases from hepatitis C virus, West Nile virus, herpes simplex virus, and Japanese encephalitis virus can be inhibited with small molecule drugs.[18−22] While inhibiting a cellular protein risks cytotoxicity, we reasoned that there could be a therapeutic window of drug concentration which affects differentially the virus versus the host cell. We note that inhibition of cell-encoded enzymes in medical therapy is not an unprecedented strategy. Several clinical examples exist for the use of drugs to inhibit cellular enzymes.[27,29−33]

All RNA helicases share generally conserved motifs; however, recent findings have shown that small molecule inhibitors can work against some viral helicases but not others, suggesting that target discrimination between different
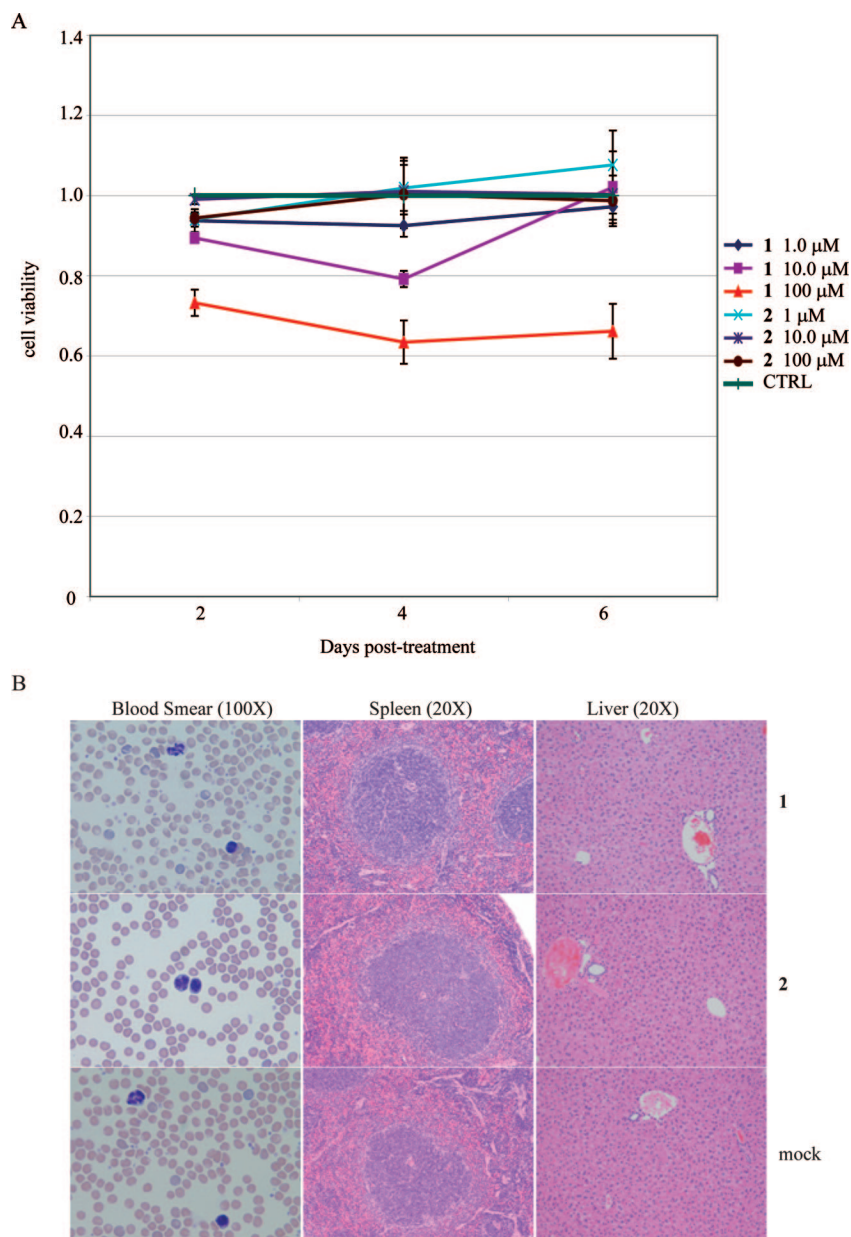
Figure 6. Lack of toxicity of **1** and **2** in cells and in mice. (A) 4×10^6 MT4 cells in 2 mL were incubated in culture media containing 1, 10, and 100 μM concentrations of **1** or **2**. Then 100 μL of each cell suspension were incubated with 10 μL of WST8 (water-soluble tetrazolium) for 2 h and then subjected to colorimetric measurements. Production of color is a measurement of the metabolism of viably dividing cells. (B) In vivo toxicities of **1** and **2** were evaluated in 8−10 week old BALB/C mice. Mice, in groups of four, were injected intraperitoneally twice weekly with drug boluses calculated to achieve final body **1** or **2** distributions of 0.2, 2, 20, and 200 μM respectively for 4 weeks. None of the mice demonstrated any signs of toxicity from intraperitoneal injections of **1** or **2**. At the end of 5 weeks of drug injection and observation, all mice were sacrificed and histological examinations of tissues were performed. Examples of tissues from **1** and **2** injected mice compared to tissues from control mice are shown.

helicases may be possible.[18−22] For example, human herpes viruses encoded helicases have been shown in recent studies to be targeted by two new classes of drugs, aminothiazolyphenyl-molecules[18] and thiazole amide derivatives,[19] with little apparent toxicity on cellular helicases. Our current results extend these earlier antiviral studies by testing the idea that a small molecule (e.g., **1**) can target a cellular helicase and inhibit viral (HIV-1) replication. Indeed, our results suggest a therapeutic level for **1**, which appears to be well-distributed systemically in vivo, that can inhibit HIV-1 replication without eliciting significant host toxicity. We note, however, that **1** and **2** could have additional complex intracellular effects; further studies are needed before one

can conclusively determine that their anti-HIV-1 activities arise from direct inhibition of RNA helicases.

The benefit of targeting a cellular protein versus a viral protein is that cellular genes do not mutate rapidly like viral genes. Hence in cells treated with **1**, HIV-1 cannot develop drug resistance through mutation of its viral genome. Consistent with this proposed concept, a synthetic immunomodulator, Murabutide, was shown recently to suppress HIV-1 replication in macrophages and T cells.[34] Intriguingly, Murabutide was found to inhibit the activity of a cellular RNA helicase, RH116, which works at the level of HIV-1 gene transcription rather than at post-transcriptional RNA processing like DDX3.[23] Collectively, the Murabutide results
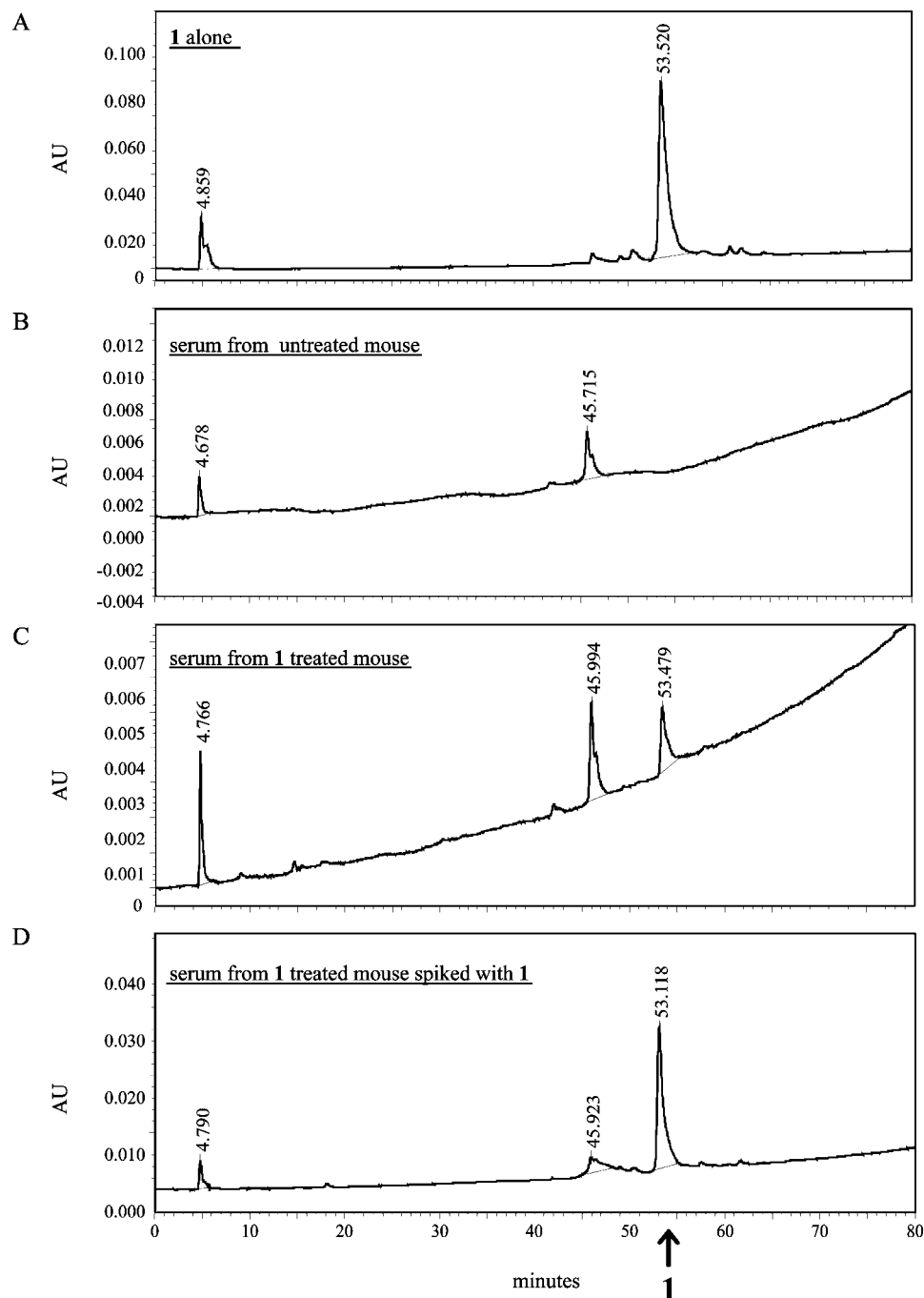
Figure 7. Systemic distribution of **1** in vivo. Sera collected from mice injected intraperitoneally with **1** were analyzed by HPLC for the presence of **1**. In (A) and (B), HPLC was performed using **1** dissolved in MeOH:AcCN (1:1) and serum from an untreated mouse respectively to establish control profiles. The sera from mice injected with **1** (C) showed an absorption peak corresponding to the control **1** peak in (A). A serum sample from a mouse injected with **1** was deliberately spiked with more **1** (D), and this produced an increase in the absorbance peak corresponding to the **1** peak in serum from a mouse injected with **1** (panel C) and the **1**-alone control (A).

and our present work encourage further explorations of helicase inhibitors as anti-HIV-1 agents.

Experimental Section

Compounds. The chemical synthesis of ring-expanded nucleosides (RENs) with a β-configuration, including but not limited to **1**, have been previously reported.[21,22] The procedure for the synthesis of RENs with an α-configuration is described below.

A General Procedure for the Synthesis of α-Deoxy Ring-Expanded Nucleoside (REN) Analogues. An appropriate alkylamine (6 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (1.244 g, 6 mmol, 97%) was refluxed in methanol (20 mL) for 2 h. The solvent was evaporated under vacuum, dried

in a desiccator over P_2O_5 under vacuum for two nights. Without purification, it was dissolved in anhydrous methanol (6.0 mL), cooled to 0 °C, and a solution of methanolic sodium methoxide (25 wt % in methanol, 3.15 mL) was added dropwise. The reaction mixture was kept in ice bath for 0.5 h. Solution from the reaction mixture was directly filtered into butyl 1-(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl-α-D-erythropentofuranosyl)-4,5-imidazoledicarboxylate[21] (1.38−1.81 mmol) in anhydrous methanol (30 mL). The ring closure reaction went complete after stirring the reaction mixture overnight. The product was purified by silica gel chromatography, eluting successively with a mixture of chloroform:methanol (30:1), chloroform:methanol (10:1), and methanol. During solvent evaporation of the collected fractions, solid

started to precipitate from solution. The precipitate was filtered and dried over P_2O_5 under vacuum.

4,5-Dihydro-8*H*-6-(*N*-tetradecyl)amino-1-(2′-deoxy-α-D-erythro-pentofurano- syl)imidazo[4,5-*e*]diazepine-4,8-dione (compound 11). Yield: 92.6%, mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 10.37 (brs, 1H, NH, exchangeable with D_2O), 8.18 (s, 1H, imidazole), 7.10 (brs, 1H, NH, exchangeable with D_2O), 6.62 (d, $J = 5.9$ Hz, 1H, 1′-H), 5.10 (m, 1H, 3′-OH, exchangeable with D_2O), 4.87 (m, 1H, 5′-OH, exchangeable with D_2O), 4.24 (m, 2H, 3′,4′-H), 3.42 (m, 2H, 5′-$H_{1,2}$), 3.20 (m, 2H, CH_2), 2.62 (m, 1H, 2′-H_1), 2.01 (d, $J = 14.3$ Hz, 1H, 2′-H_2), 1.46 (m, 2H, CH_2), 1.22 (m, 22H, $(CH_2)_{11}$), 0.83 (t, $J = 5.9$ Hz, 3H, CH_3); Anal. Calcd for $C_{25}H_{41}N_5O_5 \cdot H_2O$ (509.64): C, 58.92; H, 8.50; N, 13.74. Found: C, 58.67; H, 8.29; N, 13.58.

4,5-Dihydro-8*H*-6-(*N*-hexadecyl)amino-1-(2′-deoxy-α-D-erythro-pentofuranosyl)imidazo[4,5-*e*]diazepine-4,8-dione (compound 12). Yield: 99.0%, mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 10.56 (brs, 1H, NH, exchangeable with D_2O), 8.19 (s, 1H, imidazole), 7.11 (brs, 1H, NH, exchangeable with D_2O), 6.63 (d, $J = 6.6$ Hz, 1H, 1′-H), 5.11 (m, 1H, 3′-OH, exchangeable with D_2O), 4.87 (m, 1H, 5′-OH, exchangeable with D_2O), 4.24 (m, 2H, 3′,4′-H), 3.42 (m, 2H, 5′-$H_{1,2}$), 3.20 (m, 2H, CH_2), 2.64 (m, 1H, 2′-H_1), 2.01 (d, $J = 13.9$ Hz, 1H, 2′-H_2), 1.47 (m, 2H, CH_2), 1.22 (m, 26H, $-(CH_2)_{13}$), 0.83 (t, $J = 6.0$ Hz, 3H, CH_3). Anal. Calcd for $C_{27}H_{45}N_5O_5 \cdot 1.5H_2O$ (546.70): C, 59.32; H, 8.85; N, 12.81. Found: C, 59.71; H, 8.60; N, 13.05.

4,5-Dihydro-8*H*-6-(*N*-octadecyl)amino-1-(2′-deoxy-α-D-erythro-pentofuranosyl)imidazo[4,5-*e*]diazepine-4,8-dione (compound 2). Yield: 86.7%, mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 10.30 (brs, 1H, NH, exchangeable with D_2O), 8.19 (s, 1H, imidazole), 7.06 (brs, 1H, NH, exchangeable with D_2O), 6.62 (d, $J = 6.6$ Hz, 1H, 1′-H), 5.10 (d, $J = 2.2$ H, 1H, 3′-OH, exchangeable with D_2O), 4.87 (t, $J = 5.1$ Hz, 1H, 5′-OH, exchangeable with D_2O), 4.24 (m, 2H, 3′,4′-H), 3.43 (m, 2H, 5′-$H_{1,2}$), 3.21 (m, 2H, CH_2), 2.64 (m, 1H, 2′-H_1), 2.01 (d, $J = 14.3$ Hz, 1H, 2′-H_2), 1.46 (m, 2H, CH_2), 1.22 (m, 30H, $(CH_2)_{15}$), 0.84 (t, $J = 6.4$ Hz, 3H, CH_3). Anal. Calcd for $C_{29}H_{49}N_5O_5 \cdot H_2O$ (565.75): C, 61.57; H, 9.09; N, 12.38. Found: C, 61.88; H, 9.19; N, 12.18.

Antiviral Assays. HIV-1 T-cell-tropic molecular clone (NL4-3) and macrophage-tropic molecular clone (AD8) were used. HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum. Infectious virus stocks were generated by transfecting HeLa cells with either pNL4-3 or pAD8 plasmids. Culture supernatant was collected 36 h post transfection, and the virus stock was quantified by RT assay.[35] MT4 cells were maintained in RPMI supplemented with 10% fetal bovine serum. Usually 4×10^6 cells were infected with 20000 normalized RT counts of NL4-3, and compounds were added 4 h post infection. Afterward, 50% of the media was replaced every 2 days, with fresh media containing the compounds at the initial dosing level. Human monocyte derived macrophages (MDMs) were generated by culturing elutriated blood monocytes in RPMI-1640 containing 10% pooled human AB serum and M-CSF. Cells were cultured for 5−7 days before infecting with 10^5 RT counts of HIV-1 AD8.

RNA Unwinding Assay. RNA unwinding assay was performed as described previously.[9] Briefly, pBluescript plasmid was used to generate partially double-stranded RNA for the RNA unwinding assay. The pBluescript vector was digested to completion with *Kpn*I and transcribed with T3 polymerase to generate a 120 base long transcript. The vector was also digested with *Eco*RI and transcribed with T7 polymerase in the presence of (α32p) UTP. The two complementary transcripts were purified and hybridized at 95 °C for 20 min. Double-stranded RNA was then incubated with purified DDX3 (with or without 4 mM **1** and **2**) in ATPase/helicase (20 mM Tris-HCl, pH 8.0, 70 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 15 units RNasin, and 2 mM ATP). After incubation for 30 min at 37 °C, the reactions were stopped by the addition of a solution containing 10 mM EDTA, 40% glycerol, bromphenol blue, xylene cyanol. The reaction was resolved in a 10% polyacrylamide gel for 2 h. The gel was dried and exposed to a phosphorimaging plate.

Toxicity Assays. Four $\times 10^6$ suspension T cells (MT4) were incubated with or without **1** or **2**. 50% of the media was removed every 2 days and replaced with an equivalent amount of complete media containing the inhibitors. At time of each harvest, 100 μL of the cell suspension were measured for viablilty and compared to a similar amount of control (untreated) cells using the Cell Counting kit-8 (Dojindo) according to manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader. For in vivo studies, 8−10 weeks old BALB/c mice were injected with various concentrations of **1**, **2**, DMSO, or normal saline twice a week intraperitoneally for 4 weeks and sacrificed for necropsy after week 5. Blood from the mice was collected once per week via the tail vein.

HPLC Analysis of Mouse Serum. Serum samples for HPLC were extracted with 3 volumes of ethyl acetate and lyophilized. Each mouse serum sample was dissolved in a mixture of 100 μL of water and 20 μL of acetonitrile. The samples were analyzed by reverse-phase HPLC using a C4 Vydac 214TP1010 column (10 mm × 250 mm). The injection for each run was 20 μL with monitoring at λ_{max} 214 and 254 nm. The column was equilibrated with 75% developer A (water, 0.1%TFA) and 25% developer B (CH_3CN, 0.1% TFA), and a gradient was employed to give a final composition of 25% developer A and 75% B over a period of 80 min at 3 mL/min. For the HPLC analysis of **1** alone without the mouse serum, the compound was dissolved in a mixture of MeOH: AcCN (1:1).

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