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Antiretroviral Activity of Thiosemicarbazone Metal Complexes

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Thiosemicarbazones display a wide antimicrobial activity by targeting bacteria, fungi, and viruses. Here, we report our studies on the antiviral activity of two thiosemicarbazone metal complexes, [bis-(citronellalthiosemicarbazonato)nickel(II)] and [aqua(pyridoxalthiosemicarbazonato)copper(II)] chloride monohydrate, against the retroviruses HIV-1 and HTLV-1/-2. Both compounds exhibit antiviral properties against HIV but not against HTLVs . In particular, the copper complex shows the most potent anti-HIV activity by acting at the post-entry steps of the viral cycle.

Introduction

Thiosemicarbazones are compounds endowed with a large variety of biological properties. Currently, the principal concern of researchers is to enhance their antitumor and antiprotozoal activity.[1,2] The thiosemicarbazone antiviral properties were exploited in the 1960s, with 1-*N*-methylisatin β-thiosemicarbazone, which was used to treat smallpox,[3] but since then they have been neglected for a long time and only in the past 10 years has there been a rediscovery of these compounds as antivirals.[4−7] In the year 2000, Horton and Varela[8] reported antiviral activities of three 3-deoxy-D-erythro-hexos-2-ulose bis(thiosemicarbazone) complexes of copper(II), platinum(II), and palladium(II) ions against poliovirus type 1. Four years later, Genova et al.[9] discovered the antiviral properties against herpes simplex virus strain 1 and 2, on acyclovir resistant cells, of benzyl bis(thiosemicarbazone) and 3,5-diacyl-1,2,4-triazole bis(4-methylthiosemicarbazone) palladium-(II) complexes. The antiviral activity of these metal complexes are higher than that of the thiosemicarbazone ligands, and it is proposed that the biochemical mechanism of action of the complexes is probably due to a synergetic effect between the ligands and the palladium(II) center. A thiosemicarbazone, menthone thiosemicarbazone, was evaluated for the first time by Mishra et al.[10] against the human immunodeficiency virus (HIV) types 1 and 2, and the compound revealed a promising antiviral activity. In 2005, Bal et al.[11] have reported a marked anti-HIV-1 activity also for a series of isatin β-thiosemicarbazone derivatives designed on the basis of a 3D-pharmacophoric mapping of the existing non-nucleoside reverse transcriptase inhibitors (NNRTIs[a]) with the thiosemicarbazone moiety (=N-NH−CS-N<).

HIV-1 and human T-cell leukemia viruses type 1 and 2 (HTLV-1/-2) are retroviruses belonging to the *lentivirinae* and *oncovirinae* subfamilies, respectively. The HIV and HTLV have a similar replicative cycle, the same target cells (T lymphocytes), and common modes of transmission, however, the HIV-1 infection is characterized by a considerable virion production and lytic effects on infected cells, while HTLV propagation is predominantly due to clonal proliferation of infected cells (immortalization) with a low infectious virion release. HIV-1 is the causative agent of acquired immunodeficiency syndrome (AIDS), whereas HTLV-1 is associated with subsequent development of adult T-cell lymphoma or leukemia. An etiological role for HTLV-2 in human diseases has not been clearly demonstrated so far. Although HTLV-1 and HTLV-2 retroviruses share 70% of nucleotide sequence homology, they induce different effects on metabolic pathways and on the cell cycle of their target cells. Epidemiological surveys have indicated that a high percentage of injection drug users (IDUs) are dually infected with HIV-1 and HTLV-1 or HTLV-2.[12−14] In Europe, the IDUs are frequently coinfected (up to 10%) with HTLV-2.[15] Regarding the pathophysiological consequences occurring in the course of HIV-1/HTLV coinfection, HTLV-1 provokes an accelerated progression toward AIDS,[16] whereas HTLV-2 seems to negatively interfere with HIV-1 replication.[15] Although considerable progress has been made in identifying HIV-1 treatments with limited side effects and low capacity of inducing the emergence of drug resistant variants, little effort has been made to study the effects of HIV therapeutic strategy in the presence of concomitant infections. The use of drugs that interfere with concomitant infectious agents during HIV infection can dramatically alter not only the progression of HIV disease but also the development of diseases provoked by these agents. Therefore, the investigation of new anti-HIV drugs should also include the study of their activity against coinfecting microbes.

In this paper, we report the evaluation of anti-HIV activity of thiosemicarbazone derivatives which have also been tested

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[a] Abbreviations: CC_{50}, the 50% cytotoxic concentration; CCR5, C−C chemokine receptor type 5; CXCR4, C-X-C chemokine receptor type 4; FBS, fetal bovine serum; HTLV-1/-2, human T cell leukemia viruses type 1/type 2; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; rIL-2, recombinant interleukin-2; SD, standard deviation; $TCID_{50}$, tissue culture infectious dose 50%.

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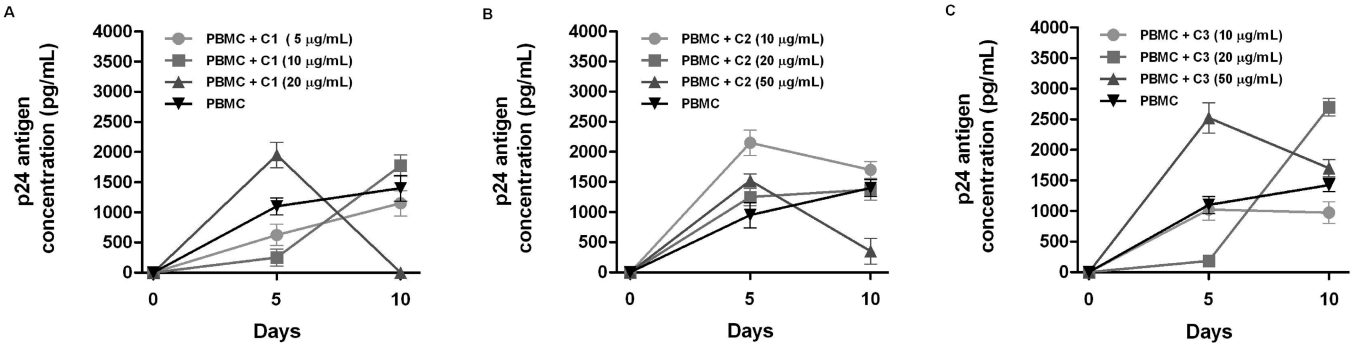


Figure 1. Antiviral activity of thiosemicarbazone derivatives on endogenous replication of HIV-1 R5 strain. The treatments were administered at day 0 of culture. The supernatants were collected at days 5 and 10 for the monitoring of HIV-1 production as p24 antigen determination. Each data point represents the mean ± SD value of two independent experiments with different HIV-1-infected patients. Each experiment was performed in triplicate. C1 = compound **1**; C2 = compound **2**; C3 = compound **3**.

against HTLV. We have set up a series of experiments using two coordination compounds and the thiosemicarbazone of menthone (**1**), a compound of known activity[10] for comparative purposes. These two complexes, apart from the common thiosemicarbazide fragment, present markedly different physicochemical properties, particularly regarding shape and polarity. The nickel derivative, [bis(citronellalthiosemicarbazonato)-nickel(II)] (**2**),[17,18] contains the long hydrophobic citronellal chain that, as demonstrated by its octanol−water partition ratio coefficient (log P_{ow}), is fairly hydrophobic, while the copper derivative, [aqua(pyridoxalthiosemicarbazonato) copper(II)] chloride monohydrate (**3**)[19] is a polar compound which presents a very low partition ratio value.

To check the antiviral activity of these compounds, we planned endogenous virus replication assays. Our findings showed that compounds **2** and **3** were able to counteract HIV-1 propagation but proved inactive against HTLV replication. In addition, we have also tried to clarify the way they work and, more specifically, to understand whether their activity is due to their ability to prevent the virus entering into the cell or if their antiviral activity is inside the infected cell. To do this, endogenous replication assays were conducted. The results indicate that the copper complex displays its antiviral potency during the post-entry stages of infection, probably interfering with the host cell factor functions utilized by HIV-1 to favor itself.

Results and Discussion

Chemical Data. The compounds used in this work are menthone thiosemicarbazone (**1**), [bis(citronellalthiosemicarbazonato)nickel(II)] (**2**), and [aqua(pyridoxalthiosemicarbazonato)copper(II)] chloride monohydrate (**3**). We also studied the corresponding ligands, citronellalthiosemicarbazone (**4**) and pyridoxalthiosemicarbazone (**5**). All of these compounds have already been carefully chemically characterized and studied.[10,17,18] The complexes have been preliminarily tested for their hydrophobicity as a standard procedure in view of a potential uptake in cells by diffusion. Generally, compounds with log P_{ow} values less than 1.5 tend to exhibit minimal distribution into lipid membranes while compounds with log P_{ow} ranging between 2 and 4 tend to exhibit excellent partitioning in membranes.[20,21] In our study, the two coordination compounds we have chosen possess partition ratios with values as low as 0.35 for the copper complex (**3**) and 2.98 for the nickel citronellal complex (**2**). The latter has a value that is strikingly more hydrophobic than the corresponding ligand (log P_{ow} 1.66). This behavior can be explained by the fact that in the metal complex the polar moiety of the ligand is hidden upon coordination, and thus the apolar role of the long aliphatic chains is enhanced. The log P_{ow} of 0.35 of the copper complex on the contrary is very low because of the ionic form of the complex. The partition ratio of menthone thiosemicarbazone (**1**) results to be 2.65. The partition coefficients of compounds **4** and **5** were calculated and resulted to be 3.28 ± 0.56 and 0.09 ± 0.66, respectively.[22]

Inhibition of Endogenous HIV-1 Replication by Complexes. We preliminarily determined the CC_{50} of compounds **2** and **3** on cultures of unstimulated peripheral blood mononuclear cells (PBMCs), obtained from healthy donors. For this purpose, we tested each compound at 10, 20, and 50 μg/mL, and we observed that both **2** and **3** did not exhibit any cytotoxic effect at all the evaluated concentrations (viability >80%; data not shown). Then we assessed the anti-HIV activity of compounds by two independent endogenous replication assays culturing PBMCs purified from HIV-1-infected patients. The chosen patients are drug-naïve and characterized by a clinical latency condition, which is sustained predominately by R5 strains. These isolates use the coreceptor CCR5 to infect target cells, preferentially monocytes and macrophages, although they are also easily propagated in primary T-cells from peripheral blood samples. Immediately after being seeded, PBMCs were cultured in the presence of each compound alone at 10, 20, and 50 μg/mL for a 10-day course. The kinetic of HIV-1 progeny was determined as HIV-1 p24 gag production and shown in Figure 1. The antiviral activity of thiosemicarbazone derivatives was compared to that obtained for compound **1** (Figure 1A), used as a reference positive control,[9] and to the untreated control. After checking the noncytotoxic effect of compound **1** on unactivated primary cells (data not shown), this compound was used in the inhibitory assays at 5, 10, and 20 μg/mL. Compound **2** showed a dose dependent reduction of HIV-1 growth (Figure 1B). At the highest concentration (50 μg/mL), it exhibited the highest antiviral activity (HIV-1 reduction by 75%), while at lower concentrations, it did not influence the viral production. Interestingly, the antiviral effect of compound **2** was only observed from day 5 until day 10 of culture, suggesting an ability of the compound to inhibit viral replication at later time points. By contrast, the inhibitory effects of compound **3** resulted independent of the dose and were already appreciable in the early stages of the infection. In fact, compound **3** was able to suppress virus replication during the first 5 days of culture (reduction by 80%), afterward a burst in HIV-1 replication was observed, probably as

a consequence of the complete metabolization of the compound by the cells (Figure 1C). The kinetics related to the treatment with compound **3** was similar to that induced by compound **1**, even if at a concentration that is 2-fold that of the reference compound, suggesting that both of them act in a similar way. This appears to be different from the mechanism displayed by compound **2** that requires, to be effective, a higher concentration than other two compounds.

Inhibition of Exogenous HIV-1 Replication by Complexes. The inhibitory activity of compound **2** and **3** was further evaluated by infecting in vitro activated healthy PBMCs with HIV-1. Initially, we chose to check if compounds **2** and **3** at the concentrations of 50 and 20 μg/mL (the most effective in reducing viral infection as previously demonstrated), respectively, were toxic for activated cells. Both compounds were found to be toxic at the above concentrations, so we used lower doses. We determined that compound **2** was toxic at all tested concentrations (1, 10, 25, 50 μg/mL), while compound **3** showed a $CC_{50} > 10$ μg/mL (data not shown). For these reasons, only compound **3** was used in the following assays. To investigate which events can be considered the potential target of the compounds in the HIV replicative cycle, we designed different experiments to discriminate whether the inhibitory effect occurred during viral entry or after the internalization of the virus into the host cells. In the first assay, the cells were pretreated with compound **3** at 10 μg/mL and then infected with either R5 and X4 strains of HIV-1.[23] In this case, we did not observe any significant variation in the amount of HIV-1 progeny when treated and untreated infected cells were compared (Figure 2A,B). When we carried out the second assay where the compound was added after the adsorption step and maintained throughout the experiment, we found a significant reduction of HIV-1 expression on treated cells in comparison to the untreated ones (Figure 2C,D). No significant difference was observed between peaks and troughs of HIV-1 production determined at days 8 and 12, respectively, in the first and the second assay. Furthermore, there was no difference in the results obtained in both assays by using either isolates of HIV-1. Taken together, these results indicated that compound **3** showed the same inhibitory effect against both isolates, suggesting that its antiviral activity is not related to HIV coreceptor tropism and that the blockage did not occur during virus−cell interaction but at sometime following viral entry. The antiviral activity of the compounds against HIV was further investigated in a T-cell line, the HTLV-1 transformed C8166 cell line, by infecting exponentially growing cells under two different experimental conditions (Figure 3). Because only T-tropic viruses are able to infect T-cell lines, the C8166 cells (which express the coreceptor CXCR4) were inoculated with the X4 strain. As a first step, we determined the cytotoxic activity of compounds on uninfected cells and obtained that compound **2** was extremely toxic for C8166 cells at 50, 25, 10, and 1 μg/mL, while compound **3** resulted in being not toxic at 10 μg/mL with a measured percentage of viable cells similar to that of the control (90% versus 98%; data not shown), and therefore we decided to test only compound **3**. In the first assay, the infected cells were treated with compound **3** at 10 μg/mL only at time 0 of culture, while in the second experiment, a supplemental fresh dose of the compound was added daily from day 3 onward. In both assays, the supernatants were collected after every 24 h for the assessment of extracellular level of p24 antigen. The results showed a progressive decrease in virus yield in comparison to the control samples, with a remarkable reduction from the sixth day of culture, corresponding to the start of productive infection. Moreover, when we compared the amounts of p24 antigen detected every 24 h in the two experiments, we revealed a significant variation of HIV-1 replication only at day 7 postinfection, after which a fresh dose of compound failed to improve the inhibitory effects, likely due to the short period of treatment and the dosage tested that resulted ineffective in condition of superinfection.

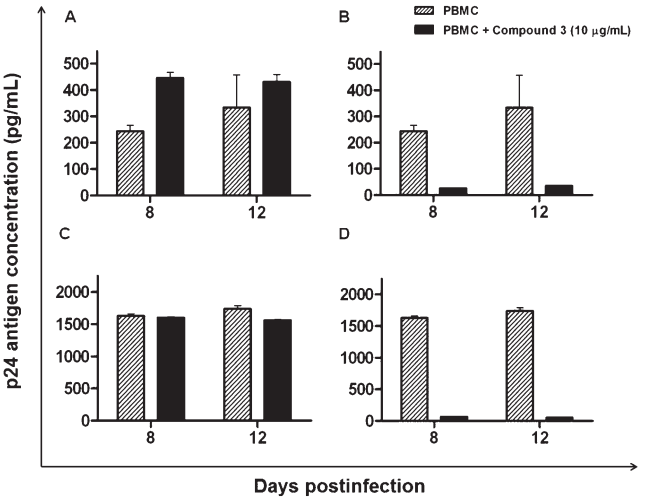


Figure 2. Antiviral activity of thiosemicarbazone derivatives on exogenous replication of HIV-1 R5 and X4 strains. In a first assay, the cells were treated with compound **3** (10 μg/mL) and then infected with the R5 (A) and X4 (C) strains. In a second assay, the cells were infected with the R5 (B) and X4 (D) viruses and subsequently treated with compound **3** (10 μg/mL). Infection was monitored as the p24 antigen level in the supernatants. Values are the mean of three independent experiments. Error bars represent ± SD.

Low Effort of the Complexes in Counteracting HTLV-2 Propagation. To evaluate the antiviral activity of compound **2** and **3** against HTLV, we used cell lines C10 and BJABGu, which were transformed by HTLV-1 and HTLV-2, respectively. First, we determined that the CC_{50} of each compound was > 10 μg/mL on both cell lines. Then we exposed the cells to the compounds at 1, 5, and 10 μg/mL for 4 days of culture. The amount of HTLV progeny in the supernatants was measured by HTLV p19 antigen detection. No antiviral activity of both compounds was noted against HTLV-1 (data not shown). A relative low drop (not significant) of HTLV-2 replication was observed after treatment with both compounds. This variation was dose-dependent and more relevant when compound **3** was tested (Figure 4). These observations led to the conclusion that the compounds have a limited effect against HTLV, maybe as a consequence of the predominant oligoclonal expansion of the virus[24,25] which, in most infected cells, remain confined in the nucleus of infected cells and not in the cytoplasm where the thiosemicarbazones are predominantly localized.[26]

Cytotoxic Activities of the Ligands. Prior to testing the anti-HIV activity of compounds **4** and **5**, we determined their cytotoxic potentials. To this aim, we planned to expose primary cells isolated from healthy donors to each of the compounds. The cells were seeded at the concentration of 1×10^6 cells/well and cultivated for 1 day before adding each compound at the concentrations of 10, 20, and 50 μg/mL. To monitor the percentage of viable cells, we performed a MTT-based assay in accordance with the manufacturer's instructions.

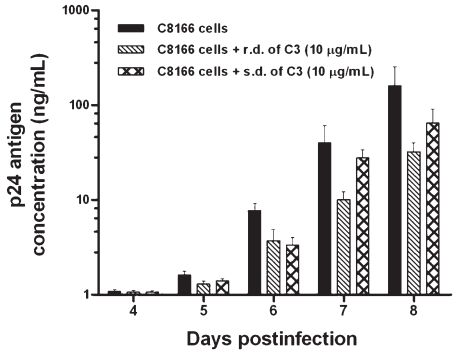


Figure 3. Antiviral activity of compound **3** against X4 replication in C8166 cells. The C8166 T-cell line, which expresses CXCR4, was infected by the HIV-1 IIIB strain. After 2 h of adsorption, the cells were washed and then cultured for 10 days in the presence of a single dose (sd) of compound **3** (C3), added at the concentration of 10 μg/mL at the day 0 of culture. Otherwise, a fresh repeated dose (rd) of C3 was further added daily from day 3 onward. Concentration of HIV p24 antigen was measured on supernatants collected every 24 h, from the fourth day of postinfection. Values are the mean of three independent experiments. Error bars represent ± SD.

Each cytotoxicity assay was performed in triplicate. The results showed that both compounds, at all used concentrations, were toxic to cells already after 24 h of treatment. A significant toxic effect induced by both compounds at concentrations of 1 and 5 μg/mL was also observed when the assays were repeated (data not shown). The compounds were also tested to calculate CC_{50} on C10 and BJABGu. The cells were cultured at 1×10^5cells/well for 1 day. After 2 days of cultivation, the cells were treated with each compound added at 10−20−50 μg/mL. Starting from 24 h of exposure, we observed an increasing amount of dead cells. Cell viability was determined by MTT-based assay. Results evidenced a percentage of viable cells of about 80% in the treated sample. A strong cytotoxic effect also persisted when the compounds were used at lower concentrations (1−5 μg/mL) (data not shown).

The ligands were not tested for their antiretroviral potency because of their high cytotoxicity. This behavior is not unexpected because it is commonly observed for thiosemicarbazones that the metal complexation almost systematically increases their activity and contributes to mitigating the side effects of the ligands.[2]

Conclusions

We have used two thiosemicarbazone metal complexes to evaluate their potential activity as antiviral agents. The compounds were first tested for their ability to enter cells by diffusion. From the log P_{ow} parameter, the nickel derivative (**2**) appears to be more prone to diffuse through the cell membrane, while the copper derivative, being charged, is in principle less prone to passive diffusion. Nevertheless, it is the copper complex (**3**) that presents the better results. In fact, it inhibits HIV-1 replication in primary T cells and in cell line culture, displaying its antiviral activity during the early post-entry step of HIV-1 replication. Furthermore, it has been proven to act specifically against HIV-1 and not against other retroviruses such as HTLV.

In this study for the first time the discovery of new potential anti-HIV drugs has taken into account the screening of the compounds against other retroviruses which frequently coinfect HIV-1 positive patients and interfere with AIDS progression. These results warrant further research and development aimed

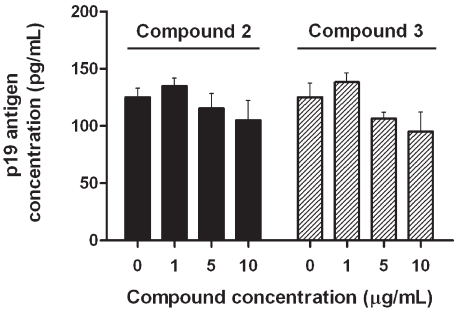
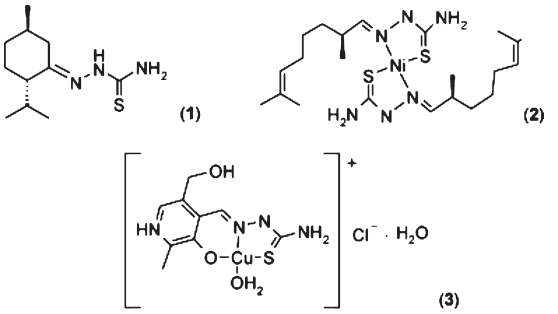


Figure 4. Effects of compounds on HTLV-2 propagation. The BJABGu cells, infected by the Gu strain of HTLV-2, were treated with each compound at several concentrations at day 0 of culture. The supernatants were harvested at day 4 of treatment and assessed by measuring p19 HTLV-2 antigen production. Values are the mean of two independent experiments. Error bars represent ± SD.

Scheme 1. General formulas for *S,S*-menthone thiosemicarbazone (**1**), [bis(*N,S*-citronellalthiosemicarbazonato)nickel(II)] (**2**), and [aqua(*O,N,S*-pyridoxalthiosemicarbazonato)copper(II)] chloride monohydrate (**3**)



to investigate the mechanism and the candidate factors by which these antiretrovirals act.

Experimental Section

Syntheses and Characterizations. The synthesis of compound **1**, **2**, and **3** (Scheme 1) and their characterizations are reported elsewhere.[9,17−19]

Endogenous HIV-1 Replication Assay. PBMCs from HIV-1 infected patients, showing a high RNA titer in plasma and a value of CD4+ about 300 cells/μL, were purified as described above. The cells were plated in a 96-well microplate at a density of 1×10^6 cells/mL in RPMI 1640 medium supplemented with 10% FBS in the presence of 25 U/mL rIL-2. At 24 h postseeding, the cells were treated with various concentrations (10, 20, and 50 μg/mL) of compounds **2** and **3**, which were maintained for a 10-day course. The culture medium was supplemented with a fresh dose of rIL-2 every 4 days. Each test was performed in duplicate. At days 5 and 10 of culture, the supernatants were collected and tested for HIV-1 production using the Ultrasensitive HIV-1 p24 Antigen Assay (Perkin-Elmer).

Exogenous HIV-1 Replication Assay. Healthy PBMCs were inoculated with both R5 and X4 strains, while C8166 cells were infected only by X4 virus. Before being infected, PBMCs were stimulated as previously described in order to favor in vitro HIV-1 replication. To test whether the compounds interfere with virus−cell contact or post-entry viral replication events, we planned two experimental conditions. In the former assay, an aliquot of cell suspension at the concentration of 1×10^6 cells/mL was pretreated with compound **3** (10 μg/mL) for 2 h. At the end of the treatment, the cells were washed with PBS and incubated alternately with 0.1 multiplicity of infection (MOI) of each viral stock for 2 h at 37 °C. After the viral adsorption, the cells were

repeatedly washed with PBS and then cultured at 37 °C for 12 days, during which rIL-2 was added every 4 days. In the latter experiment, the activated PBMCs were infected with either R5 or X4 strain (MOI = 0.1) and adsorbed for 2 h at 37 °C; subsequently, the cells were rinsed with PBS and cultured at 37 °C in the presence of compound **3** (10 μg/mL). During the culture, rIL-2 was readded as described above. In both assays, the supernatants were harvested at days 8 and 12 postinfection. Analogous procedures were carried out to determine the antiviral activity of compound **3** against X4 replication in C8166 cells. After being seeded at 4×10^5 cells/mL, the cells were immediately inoculated with 100 $TCID_{50}$ of viral stock. After 2 h of adsorption at 37 °C, the cells were washed five times with PBS and cultured for 10 days in the presence of 10 μg/mL of the compound. Besides, the compound was daily added from the fourth until the seventh day of postinfection. In this case, the supernatants were collected at 24 h intervals post-treatment. Untreated and infected cells (as positive controls) and untreated and uninfected cells (as negative controls) were also run for each experiment. All assays were performed in triplicate. Supernatants were harvested and assessed for HIV-1 infection analysis using the HIV-1 p24 Antigen Assay (VIDAS HIV p24, Biomerieux).

HTLV Antigen Detection. Supernatants from cell cultures were checked for the presence of HTLV p19 antigen by ELISA (RETROTEK HTLV p19 Ag ELISA; ZeptoMetrix). The concentration of antigen was determined by a linear regression analysis using different p19 standard amounts according to the manufacturer's instruction.

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Supporting Information Available. Chemical characterization of compounds; virus description; cell and viability assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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