Antiviral Research 162 (2019) 101–109

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Discovery of 4-oxoquinolines, a new chemical class of anti-HIV-1 compounds

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ARTICLE INFO

Keywords:
Anti-HIV
4-Oxoquinoline
Antiviral compound
Integrase strand-transfer inhibitor
Latently infected cell
Tat

ABSTRACT

Antiretroviral therapy (ART) against HIV-1 infection offers the promise of controlling disease progression and prolonging the survival of HIV-1-infected patients. However, even the most potent ART regimens available today cannot cure HIV-1. Because patients will be exposed to ART for many years, physicians and researchers must anticipate the emergence of drug-resistant HIV-1, potential adverse effects of the current drugs, and need for future drug development. In this study, we screened a small-molecule compound library using cell-based anti-HIV-1 assays and discovered a series of novel anti-HIV-1 compounds, 4-oxoquinolines. These compounds exhibited potent anti-HIV-1 activity (EC_{50} < 0.1 μM) with high selectivity indexes (CC_{50}/EC_{50} > 2500) and favorable pharmacokinetic profiles in mice. Surprisingly, our novel compounds have a chemical backbone similar to the clinically used integrase (IN) strand transfer inhibitor (INSTI) elvitegravir, although they lack the crucial 3-carboxylate moiety needed for the common INSTI diketo motif. Indeed, the new 4-oxoquinoline derivatives have no detectable INSTI activity. In addition, various drug-resistant HIV-1 strains did not display cross resistance to these compounds. Interestingly, time-of-addition experiments indicated that the 4-oxoquinoline derivative remains its anti-HIV-1 activity even after the viral integration stage. Furthermore, the compounds significantly suppressed p24 antigen production in HIV-1 latently infected cells exposed with tumor necrosis factor alpha. These findings suggest that our 4-oxoquinoline derivatives with no 3-carboxylate moiety may become novel lead compounds in the development of anti-HIV-1 drugs.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). Anti-HIV drugs have expanded immensely in the last three decades to more than 40 approved drugs belonging to five classes: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors, and integrase (IN) strand transfer inhibitors (INSTIs). The INSTIs, such as raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG), are currently used as a highly potent “key drug” with a favorable safety profile in the management of HIV-1 (Markowitz et al., 2007; Walmsley et al., 2013). INSTIs inhibit HIV-1 IN-catalyzed integration of proviral DNA into the host chromosome through a mechanism in which the ß-diketo acid moiety of the INSTI binds to two metals at the IN catalytic center. DTG is a second-generation INSTI that represents a potent genetic barrier to HIV-1 resistance. Several mutations are required in HIV-1 IN to confer high-level resistance to DTG [(Hachiya et al., 2017; Kobayashi et al., 2011), and reviewed in (Mesplede and Wainberg, 2015; Wainberg et al., 2012)], and a limited number of case reports have described no virological responses to a DTG-containing regimen in patients (Danion et al., 2015; Hardy et al., 2015). The second-generation INSTIs include cabotegravir, which is currently in clinical trials, and bictegravir, which was recently approved for use in the United States (Margolis et al., 2017; Sax et al., 2017). In the NRTI class, the tenofovir prodrug, tenofovir alafenamide (TAF), is being substituted for

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https://doi.org/10.1016/j.antiviral.2018.12.012
Received 8 July 2018; Received in revised form 5 December 2018; Accepted 18 December 2018
Available online 21 December 2018

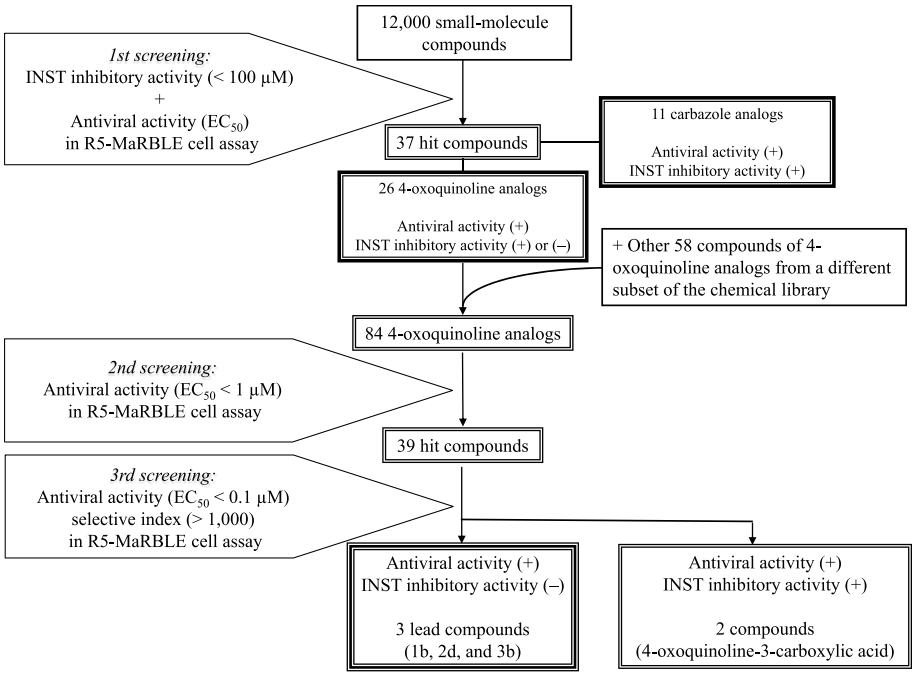

Fig. 1. The scheme of our screen and the number of hit compounds. A total of 12,000 small-molecule compounds were first screened through the *in vitro* INST assay and the R5-MaRBLE reporter cell-based assay. Twenty-six 4-oxoquinoline analog compounds from the 37 hit compounds, and an additional 58 4-oxoquinoline analogs from a different subset of the chemical library were screened through the 2nd assay. From these, we narrowed down to the compounds to those with an EC_{50} < 1 μM. The resultant 39 hits were further screened through the 3rd set of assays, which included a toxicity assay (selectivity index: the cytotoxic concentration (CC_{50})/the antiviral activity (EC_{50}) > 1000-fold). Five 4-oxoquinoline analog compounds (1b, 2c, 2d, 3a, and 3b) were obtained for their potent anti-HIV-1 activity and lack of INST activity. From the five, thee (1b, 2d, and 3b) were selected as our initial lead compounds. Of note, two 4-oxoquinolin-3-carbolilic acid analog compounds exhibited potent antiviral activity and an INST inhibitory effect *in vitro*. INST: integrase strand transfer; EC_{50}: the concentration resulting in 50% inhibition of viral replication; CC_{50}: the concentration resulting in 50% cytotoxicity.

original tenofovir disoproxil fumarate (TDF) in antiretroviral therapy (ART). TAF elicits decreased renal and bone toxicity compared to TDF because of a 90% reduction in the tenofovir concentration of plasma (Ruane et al., 2013; Sax et al., 2015). Thus, improvements in the efficacy and toxicity of ART offers the promise of controlling disease progression and prolonging the survival of HIV-1-infected patients.

Despite highly effective inhibition of HIV-1 replication, ART cannot cure individuals with HIV-1/AIDS. It is assumed that several mechanisms contribute to HIV persistence during ART, including HIV latency, immune dysfunction, and residual low-level viral replication (Barton et al., 2016; Martinez-Picado and Deeks, 2016). Consequently, long-term ART is still required, and we must anticipate drug-resistance and side effects caused by the currently used drugs. In addition, it is necessary to continue providing novel drug development pipelines for HIV-1/AIDS therapy.

We previously performed random screening of a diverse library of 12,000 small-molecule compounds using our *in vitro* HIV-1 integrase-mediated strand-transfer (INST) assay and our additional cell-based assay, and identified a novel chemical compound, calbazole, and its derivatives, which possess potent inhibitory effects on HIV-1 INST (Yan et al., 2005). In this study, we further screened the other hit compounds that differed from the calbazole derivatives and discovered a series of novel anti-HIV compounds, 4-oxoquinolines. The compounds exhibited superior selectivity indexes [ratios of the 50% cytotoxic concentration (CC_{50}) relative to the 50% effective concentration (EC_{50})] *in vitro* and good pharmacokinetic profiles in mice. In addition, PI-resistant, NRTI-resistant, NNRTI-resistant, and INSTI-resistant HIV-1 displayed no cross resistance to these compounds. Time-of-addition experiments suggested that the new compound expert its anti-HIV-1 activity even after the integration stage in viral replication. Furthermore, the 4-oxoquinoline derivatives suppressed tumor necrosis factor-alpha (TNF)-α-induced p24-antigen production in HIV-1 latently infected cells in a dose-dependent manner. Interestingly, the structure-activity relationships of these compounds are quite different from those of previously reported anti-HIV compounds. These findings suggest that the 4-oxoquinoline derivatives may become novel lead compounds in the development of anti-HIV-1 drugs.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293T (293T) and TZM-bl (Wei et al., 2002) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (HyClone), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Thermo Fisher Scientific). MT-2 and HIV-1 chronically infected OM-10.1 (Butera et al., 1994) cells, were maintained in Roswell Park Memorial Institute (RPMI) 1640 growth medium (Sigma-Aldrich) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). The TZM-bl and OM-10.1 cells were obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH. R5-MaRBLE cells (Chiba-Mizutani et al., 2007) were maintained in RPMI 1640 growth medium containing 250 μg/mL G418 (Roche Applied Science), 150 μg/mL hygromycin B (Thermo Fisher Scientific), and 0.1 μg/mL puromycin dihydrochloride (Sigma-Aldrich) and were cultured in RPMI 1640 growth medium without the selection antibiotics only when antiviral activity and cell cytotoxicity assays were performed. Of note, the R5-MaRBLE cells line is derived from the human T-cell line HPB-Ma, which was established by transfection of the CCR5 expression plasmid, the HIV-1 long terminal repeat-driven firefly luciferase plasmid and the cytomegalovirus promoter-driven Renilla luciferase plasmid as previously described (Chiba-Mizutani et al., 2007). Peripheral blood mononuclear cells (PBMCs) were isolated from three healthy donor blood samples using Ficoll-Paque density gradient centrifugation (GE healthcare), activated with 1 μg/mL of phytohemagglutinin (PHA) (Pharmacia) in RPMI 1640 growth medium for 72 h, and maintained in the growth medium supplemented with 20 U/mL interleukin-2 (IL-2) (Roche Applied Science) before infection.

2.2. Virus production

To prepare supernatants containing viruses, provirus DNA was transfected into 293T cells using FuGENE HD (Promega). At 12 h after transfection, the culture medium was gently changed with fresh DMEM growth medium. The supernatant was harvested at 48 h, clarified by centrifugation and filtration, and used for infection. The proviral DNA used in this study was as follows: HIV-1 subtypes C, D, F1, G, Group O,

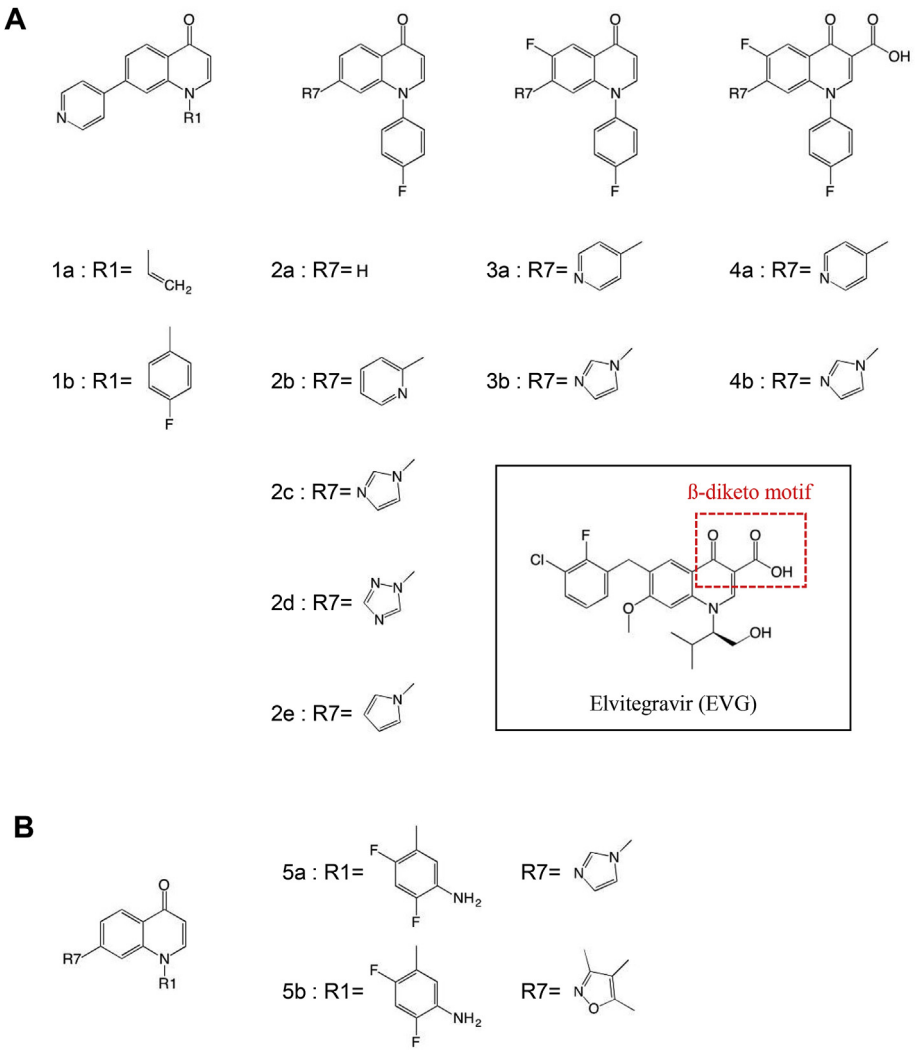

Fig. 2. Chemical structure of our hit compounds, the analogs and the INSTI, elvitegravir (EVG). (A) The hit compounds (1b, 2c, 2d, 3a, and 3b) have a common basic structure of 1-(4-fluorophenyl)-4-oxoquinoline and lack a ß-diketo motif (enclosed in red lines), whereas the inactive derivatives (4a and 4b) and the INSTI with a 4-oxoquinoline backbone, EVG, have a 3-carboxylate group for the ß-diketo motif. (B) Two additional analogs (5a and 5b) were synthesized on the basis of the hit compounds.

CRF06_cpx, and CRF02_AG (GenBank accession no. AB485645, AB485650, AB485658, AB485663, AB485667, AB485661, and AB485665, respectively) and the multidrug-resistant strains DR2510, DR5032, DR6175, and DR6190 (GenBank accession no. AB287371, AB253723, AB480694, and AB480300, respectively), which were from clinical specimens. The PI-resistant strains F719105-FY4, V42613_1 (GenBank accession no. KC109813 and KC109808, respectively), T448163_4 (the M46L/I54M/I84V/L90M/L10F/L33F/A71V/G73A mutant), and F390563_1 (the V32I/M46L/I54L/I84V/L90M/L10V/V11I/L33F/A71I/G73S/L89V mutant) were obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH. A PCR-based method for site-directed mutagenesis was used to introduce mutations into the proviral DNA plasmid, pNL4-3 (Adachi et al., 1986), and the plasmids for INSTI-resistant HIV-1 production (NL4-3 IN T66I, NL4-3 IN E92Q, NL4-3 IN S147G, and NL4-3 IN G140S/Q148H) were constructed. The primer sequences used for the mutagenesis are available upon request.

2.3. Preparation of test compounds

3′-Azido-3′ deoxythymidine (AZT), lamivudine (3 TC), efavirenz (EFV), RAL and darunavir (DRV) were purchased from Sigma-Aldrich. EVG was purchased from ChemScene. A diverse library of 12,000 small-molecule compounds and a subset of 4-oxoquinolines analogs were supplied by Toyama Chemical Co., Ltd. (Fujifilm Toyama, Japan). All test compounds were dissolved in dimethylsulfoxide and adjusted to a concentration of 2 mM.

2.4. Evaluation of in vitro antiviral effects

To determine the concentration resulting in an EC_{50}, antiviral activity was evaluated by using R5-MaRBLE cells according to a previously described procedure (Chiba-Mizutani et al., 2007) with slight modifications. Briefly, R5-MaRBLE cells (5×10^5 cells/well) were seeded in 96-well microtiter plates and infected with various test viruses. After incubation for 2 h at 37 °C, serial five-fold dilutions of compounds were added into the 96-well plates. Seven days post-infection, cells were lysed in 75 µL of luciferase assay reagent according to the manufacturer's protocol (Promega). Firefly and Renilla luciferase activities were sequentially quantified using a Dual-Glo luciferase reporter assay system (Promega) and an ARVO MX1420 Multilabel Counter (Perkin Elmer). Data were plotted as the percentage of luciferase activity versus the compound concentrations (log_{10}). The EC_{50} was determined by plotting the curve defined by the four-parametric sigmoidal equation f(x) = A + ([B – A])/(1 + [C/x]D) using Xlfit5 (IDBS).

Table 1
Profiles of antiviral activity (EC_{50}), cytotoxicity (CC_{50}), and pharmacokinetics of 4-oxoquinoline analogs.

Compound	Antiviral activity EC_{50}[a] (μM)	Cytotoxicity CC_{50} (μM)	Selectivity Index CC_{50}/EC_{50}	Concentration in mouse serum[b] 1hr/4hr (μg/mL)
1a	> 1.0	97.6 ± 0.2	< 97.6	NT[b]
1b	**0.028 ± 0.005**	**71.7 ± 2.3**	**2560.7**	**2.01/1.32**
2a	> 1.0	36.7 ± 0.5	< 36.7	NT[c]
2b	> 1.0	> 100	NC[d]	NT[c]
2c	0.042 ± 0.003	209.1 ± 1.4	4978.6	4.79/1.08
2d	**0.041 ± 0.008**	**120.3 ± 3.4**	**2934.1**	**2.17/0.30**
2e	> 1.0	37.5 ± 1.1	< 37.5	NT[c]
3a	0.085 ± 0.006	> 500	> 5882.4	1.48/0.14
3b	**0.054 ± 0.012**	**205.4 ± 1.4**	**3803.7**	**6.06/4.86**
4a	> 1.0	NT[c]	NC[d]	NT[c]
4b	> 1.0	NT[c]	NC[d]	NT[c]
5a	0.016 ± 0.042	17.6 ± 2.1	1100.1	5.34/1.64
5b	**0.001 ± 0.0001**	**26.2 ± 0.7**	**26200.0**	**3.92/1.23**
AZT	0.003	NT[c]	NC[d]	NT[c]

[a] EC_{50} was determined using HIV-$1_{JR\text{-}CSF}$.
[b] Single oral administration (25 mg/kg) to 6-weeks old male ICR mouse (n = 3).
[c] NT, not tested.
[d] NC, not calculated.

Table 2
Antiviral and cytotoxic effects of the 4-oxoquinoline analogs in PBMC.

Compound	Antiviral activity[b]	Cytotoxicity	Selectivity Index
	EC_{50} (μM)	CC_{50} (μM)	CC_{50}/EC_{50}
5b	0.007 ± 0.005	> 1.0	> 143
3 TC[a]	0.026 ± 0.017	> 1.0	> 38

Data are the mean ± SD (n = 3).
[a] 3 TC, lamivudine.
[b] The EC_{50} was determined based on the p24 amounts in supernatants at day 11 postinfection.

The antiviral effects of the compounds were also evaluated in a virus replication assay system using MT-2 cells and PBMCs. Briefly, MT-2 cells (2×10^5 cells/well, 2 mL) and PBMCs (2×10^5 cells/well, 2 mL) were infected with HIV-$1_{NL4\text{-}3}$ and HIV-$1_{JR\text{-}CSF}$ (Koyanagi et al., 1987), respectively. After incubating for 4 h at 37 °C, the cells were washed twice with fresh culture medium and maintained with culture medium changes every two days. For the virus replication kinetics assay in MT-2 cells, test compounds were added into the wells of plates at 90% viral replication inhibitory concentration (EC_{90}). The supernatants were sampled at the indicated day points after the initial incubation, and the amount of p24 in the supernatants was measured with an HIV-1 p24 antigen ELISA kit (ZeptoMetrix).

2.5. Cell cytotoxicity assay

R5-MaRBLE cells and PBMCs (1×10^5 cells/well, 100 μL) were seeded in 96-well microtiter plates. Serial 2.5-fold dilutions of compounds were added into the 96-well plates and incubated at 37 °C for 4 days (R5-MaRBLE) or 11 days (PBMCs). Cell viability was determined using the ATP-based luminescence assay, Cell Titer-Glo (Promega). Data were plotted as the percentage of luciferase activity versus the compound concentrations (log_{10}). The CC_{50} was determined by the curve defined by the four-parametric sigmoidal equation f (x) = A + ([B – A])/(1 + [C/x]D) using the Xlfit5 software.

2.6. Pharmacokinetic experiments and HPLC analysis

Male ICR mice (6 weeks old) were purchased from the Charles River Laboratories International, Inc. The selected compounds were orally administered to mice at 25 mg/kg once. One or four hours after the compounds were administered, blood samples were collected from CO_2-euthanized mice, with three mice used for each time point. To determine the compound concentrations in the blood samples, serum was obtained from each whole blood sample using a serum separation kit (Eiken Chemical Co.), which was stored at −80 °C until high-performance liquid chromatography (HPLC) analysis.

Compound concentrations in all samples were measured using a Shimadzu Prominence HPLC system (Shimadzu Co.). The HPLC analysis was carried out using a Symmetry C18 column (5 μm 4.6 × 150 mm) and a mobile phase composed of 25–35% acetonitrile, with a flow rate of 1.0 mL/min and UV detection at 254 nm.

2.7. Time-of-addition experiments

TZM-bl cells (3.2×10^5 cells/well) were plated in 12-well plates at 16 h prior to infection and then infected with HIV-$1_{NL4\text{-}3}$ (30 ng of p24 antigen/well) for 0.5 h. Then, the cells were washed twice with fresh DMEM growth medium, followed by addition of the test compounds (5 μM) at various time points (1, 2, 4, 6, 8, 10, 12, 14, and 16 h). 24 h after infection, the cells were placed on ice and the suparnatants were removed. Subsequently, the viral infection was determined by measuring luciferase activity using Bright-Glo reagents (Promega).

2.8. Analysis of antiviral effect in latently infected cells

Antiviral effects of the test compounds on chronic HIV-1 infection were analyzed on the basis on the inhibition of p24 antigen production in OM-10.1 cells after TNF-α stimulation (Wako Chemicals). Briefly, OM-10.1 cells (5×10^5 cells/well) were incubated with various concentrations of test compounds (0, 0.064, 0.32, 1.6, 8, 40, and 200 nM) in the presence of 0.5 ng/mL TNF-α for 72 h at 37 °C. The suparnatants were collected and assayed for the p24 antigen levels using the p24 ELISA kit. The percentage (%) of relative p24 production was calculated

Table 3
Antiviral activity (EC_{50}) of the 4-oxoquinoline analogs against non-B subtype HIV-1 strains.

Compound	Antiviral activity EC_{50} (μM)						
	subtype C	subtype D	subtype F1	subtype G	group O	CRF02_AG	CRF06_cpx
1b	0.132 ± 0.017	0.057 ± 0.006	0.045 ± 0.002	0.055 ± 0.004	0.044 ± 0.012	0.143 ± 0.002	0.458 ± 0.031
2d	0.268 ± 0.084	0.161 ± 0.020	0.090 ± 0.009	0.083 ± 0.010	0.073 ± 0.030	0.262 ± 0.041	0.981 ± 0.029
3b	0.212 ± 0.020	0.132 ± 0.014	0.090 ± 0.029	0.119 ± 0.014	0.092 ± 0.018	0.291 ± 0.006	0.872 ± 0.032
5b	0.003 ± 0.000	0.003 ± 0.000	0.001 ± 0.000	0.002 ± 0.000	0.001 ± 0.000	0.003 ± 0.000	0.013 ± 0.000
EFV[a]	0.001	0.0006	0.0007	0.001	0.027	0.002	0.007
DRV[b]	0.039	0.003	> 1	0.002	0.027	0.002	0.2
RAL[c]	0.001	0.0006	0.001	0.001	0.001	0.0007	0.008

[a] EFV, efavirenz.
[b] DRV, darunavir.
[c] RAL, raltegravir.

Table 4
Antiviral effects (EC_{50}) of the 4-oxoquinoline analogs on PI-resistant HIV-1.

Compound	Antiviral activity EC_{50} (μM) (fold change)[a]				
	NL4-3	F719105-FY4	T448163_4	V42613_1	F390563_1
1b	0.062 ± 0.022 (1.0)	0.057 ± 0.004 (0.9)	0.045 ± 0.005 (0.7)	0.100 ± 0.025 (1.6)	0.063 ± 0.006 (1.0)
2d	0.077 ± 0.044 (1.0)	0.116 ± 0.010 (1.5)	0.070 ± 0.014 (1.5)	0.195 ± 0.030 (0.9)	0.118 ± 0.011 (2.5)
3b	0.133 ± 0.066 (1.0)	0.120 ± 0.005 (0.9)	0.084 ± 0.009 (0.6)	0.165 ± 0.010 (1.2)	0.123 ± 0.020 (0.9)
5b	0.004 ± 0.001 (1.0)	0.003 ± 0.000 (0.8)	0.002 ± 0.000 (0.5)	0.007 ± 0.001 (1.8)	0.003 ± 0.000 (0.8)
DRV[b]	0.006 (1.0)	0.562 (93.7)	0.133 (22.2)	0.049 (8.2)	0.194 (32.3)

[a] The fold change was calculated as the ratio of drug-resistant HIV-1 EC_{50} to the reference, HIV-1_{NL4-3}, EC_{50}.
[b] DRV, darunavir.

Table 5
The EC_{50} of the 4-oxoquinoline analogs for INSTI-resistant HIV-1.

Compound	Antiviral activity EC_{50} (**nM**) (fold change)[a]				
	WT	IN T66I	IN E92Q	IN S147G	IN G140S/Q148H
5b	1.62 ± 0.39 (1.0)	0.89 ± 0.25 (0.5)	1.15 ± 0.19 (0.7)	1.55 ± 0.03 (1.0)	1.38 ± 0.33 (0.9)
EVG[b]	0.32 ± 0.01 (1.0)	1.32 ± 0.33 (**4.1**)	2.65 ± 0.75 (**8.3**)	1.79 ± 0.15 (**5.6**)	> 400 (> **1250**)
RAL[c]	0.66 ± 0.24 **(1.0)**	0.61 ± 0.39 (0.9)	1.73 ± 0.71 (2.6)	0.88 ± 0.18 (1.3)	294 ± 15.5 (**445**)

[a] The fold change was calculated as the ratio of mutant EC_{50} to the HIV-1_{NL4-3} WT EC_{50}.
[b] EVG, elvitegravir.
[c] RAL, raltegravir.

Table 6
The EC_{50} of the 4-oxoquinoline analogs for NRTI- and NNRTI-resistant HIV-1.

Compound	Antiviral activity EC_{50} (μM) (fold change)[a]				
	WT	DR2510	DR5032	DR6175	DR6190
NRTI Resistance Mutations:	–	M41L, D67N L210W, T215Y	D67N, T69D M184V, L210W T215F	M41L, D67N, M184V, L210W T215Y	M41L, D67N L210W, T215Y
NNRTI Resistance Mutations	–	K101E, E138K	A98G, K101N V108I, Y181C G190A	–	–
1b	0.028 ± 0.005 (1.0)	0.020 ± 0.006 (0.7)	0.102 ± 0.022 (3.6)	0.038 ± 0.001 (1.4)	0.042 ± 0.003 (1.5)
2d	0.041 ± 0.008 (1.0)	0.028 ± 0.013 (0.7)	0.205 ± 0.027 (5.0)	0.101 ± 0.011 (2.5)	0.093 ± 0.004 (2.3)
3b	0.054 ± 0.012 (1.0)	0.035 ± 0.007 (0.6)	0.198 ± 0.035 (3.7)	0.075 ± 0.009 (1.4)	0.090 ± 0.002 (1.7)
5b	0.001 ± 0.000 (1.0)	0.001 ± 0.000 (1.0)	0.003 ± 0.000 (3.0)	0.002 ± 0.000 (2.0)	0.002 ± 0.000 (2.0)
EFV[b]	0.0008 (1.0)	0.0007 (0.9)	> 1 (> 1250)	0.0005 (0.6)	0.0008 (1.0)

[a] The fold change was calculated as the ratio of mutant EC_{50} to the wild-type EC_{50}.
[b] EFV, efavirenz.

relative to that of control (0 μM of drug), which was set at 100%.

3. Results

3.1. Antiviral 4-oxoquinoline analogs screened from the chemical library

A diverse library of 12,000 small-molecule compounds was initially screened using an *in vitro* HIV-1 INST assay as previously reported (Yan et al., 2005). As shown in Fig. 1, two types of compounds, carbazoles (Yan et al., 2005) and 4-oxoquinolines, were identified as potential inhibitors of INST. Because of the high cytotoxicity of carbazoles, we targeted the 4-oxoquinolines and further tested the antiviral activity (EC_{50}) of eighty-four compounds from our 4-oxoquinoline library in a cell-based assay using R5-MaRBLE cells. The cells were infected with HIV-1_{JR-CSF}, after which serial five-fold dilutions of compounds were added to the infected cells. Viral replication was monitored via firefly luciferase activity after 7 days post-infection. The corresponding EC_{50} values were calculated based on the dose-response curves. Thirty-nine compounds showed an anti-HIV-1 effect with EC_{50} values less than 1 μM. Next, we assessed the inhibitory effects of the hit compounds on

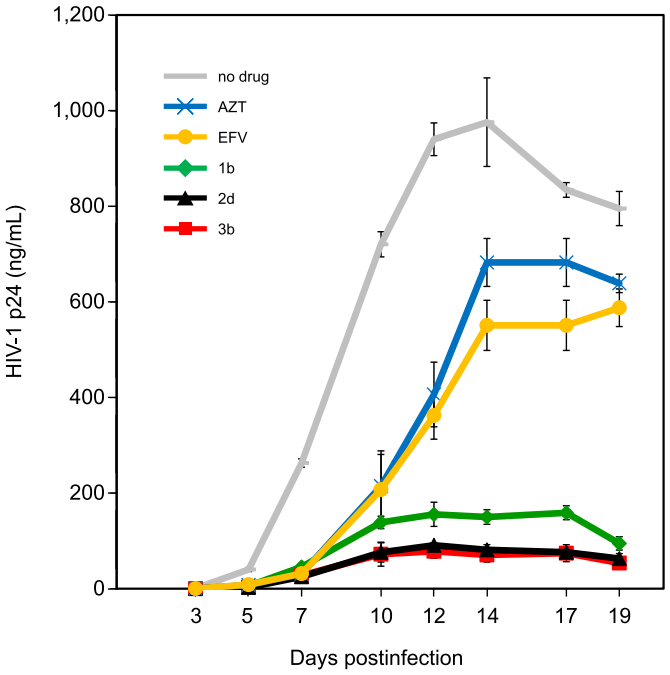

Fig. 3. Effect of the 4-oxoquinoline analogs on HIV-1 replication kinetics in the presence or absence of drugs. MT-2 cells were infected with HIV-1_{NL4-3} in the presence of each test compound and maintained for 17 days. The p24 antigen amounts in supernatants were quantified by the ELISA. Gray line, no drug; blue crosses, AZT (15.3 nM); orange circles, EFV (1.3 nM); green diamonds, black triangles and red squares represent the compounds 1b, 2d, and 3b, respectively.

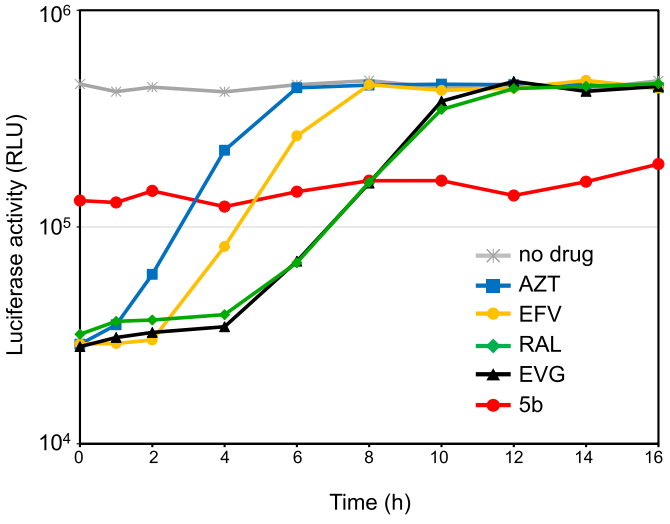

Fig. 4. Time-of-addition experiments. TZM-bl cells were infected with HIV-1_{NL4-3}, and then the compounds were added at different time points after infection. Viral infection, measured as luciferase activity (RLU, relative luciferase unit), was determined at 24 h postinfection. Stars, no drug control; squares, AZT (5 μM); yellow circles, EFV (5 μM); diamonds, RAL (5 μM); triangles, EVG (5 μM); red circles, 5b (5 μM). Experiment was performed in triplicate and the representative graph is shown.

the INST reaction and found two types of 4-oxoquinoline derivatives that differ in INST inhibitory activity. One group of compounds displayed INST inhibitory activity and had a carboxylate at position 3 of the oxoquinoline ring, whereas the other compounds lacked INST inhibitory activity had no 3-carboxylate moiety. The latter compounds were selected for further analyses to investigate anti-HIV-1 compounds with novel mechanisms. Cytotoxicity (CC_{50}) levels were determined

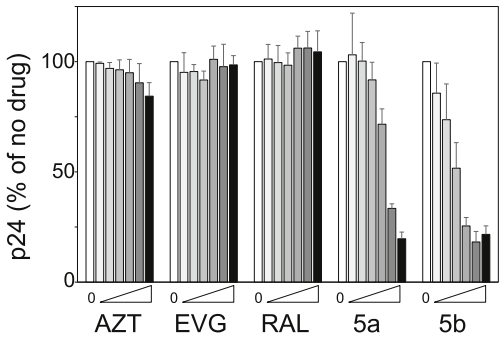

Fig. 5. Effect of the 4-oxoquinolines on TNF-α-induced production of HIV-1 in OM-10.1 cells. The cells were incubated with increasing concentrations of the indicated compounds, plus TNF-α (0.5 ng/mL) for 72 h. The amounts of p24 proteins in supernatants were quantified using the ELISA kit. The averages of triplicate assays are shown as the percent p24 levels relative to each control "0" for the compound. The grayscale bars represent productions at the compound concentration, 0.064, 0.32, 1.6, 8, 40, and 200 nM, respectively.

using the R5-MaRBLE cells, and used to calculate selectivity indexes (Cytotoxicity CC_{50}/Antiviral activity EC_{50}) (Fig. 2 and Table 1). Based on the EC_{50} (< 0.1 μM) and selectivity index (> 1000) criteria, 5 compounds (1b, 2c, 2d, 3a, and 3b) with potent antiviral activity and a high selectivity index (> 2500; range 2561–5882) were identified (Table 1). Comparing the chemical structures of the hits and their analogs, the five hit compounds all possess a 4-fluorophenyl moiety at the R1 position and a nitrogen-containing heterocyclic group at the R7 position (Fig. 2A). The EC_{50} value of the 4-fluorophenyl derivative 1b was 0.028 μM, whereas that of the 1-alyl derivative 1a showed no antiviral activity. In addition, the 7-heterocyclic derivatives 2b and 2e showed no significant inhibitory effect on HIV-1 replication (EC_{50} > 1 μM), suggesting that certain nitrogen-containing heterocyclic groups at the R7 position are required for high anti-HIV-1 activity. Notably, we found that the 3-carboxylic acid derivatives, 4a and 4b, have no significant inhibitory effects on HIV-1 replication (EC_{50} > 1 μM). These results validated our finding that our new 4-oxoquinolines that even lack a ß-diketo acid moiety exhibit potent anti-HIV-1 activity, but no detectable INST inhibitory effect. Detailed comparisons of the five hit compounds indicated that compounds 1b and 3a and compounds 2c and 3b have only a slight difference at the R6 position, with it either being fluorinated or not. Compound 2d, regardless of a different moiety at the R7, displays the EC_{50} level similar to that of 2c and 3b. Further analyses of pharmacokinetic profiles of the five compounds in mice demonstrated that compounds 1b and 3b are superior to 3a (Table 1).

Next, to further improve the antiviral activity of our 4-oxoquinoline derivatives, we synthesized two additional compounds, 5a and 5b, based on the compound 2c with the best selectivity index, and determined its profiles of antiviral activity, cytotoxicity, and pharmacokinetics *in vivo*. Compound 5a has a 5-amino-2,4-difluorobenzyl moiety at the R1 position, instead of a 4-fluorophenyl moiety (Fig. 2B). As shown in Table 1, the compound 5a has lower values of both EC_{50} and CC_{50} than 2c and 3b, although the pharmacokinetics in mice are comparable. Interestingly, a new compound 5b, that has a 3,5-dimethylisoxazole moiety at the R7, instead of a 1-imidazolyl moiety, exhibits significantly improved EC_{50} (~0.001 μM) with no significant increase in cytotoxicity, which led to a high selectivity index of 26,200. In addition, the compound 5b displays similar levels of antiviral activity and cytotoxicity in PBMCs (Table 2). Therefore, we selected four different types of the compounds 1b, 2d, 3b, and 5b for further assessment as our potential lead compounds.

3.2. Evaluation of compound efficacy against non-B subtype HIV-1 strains

The compounds 1b, 2d, 3b, and 5b were also tested in parallel with

EFV, DRV, and RAL for antiviral effects against various HIV-1 subtypes. The activities of the different subtypes were determined using the R5-MaRBLE reporter cell-based assay. The results are summarized in Table 3. RAL showed potent replication inhibition of all subtypes (EC_{50}: 0.6–8.0 nM), while EFV exhibited high antiviral activity against all tested strains (EC_{50}: 0.6–2.0 nM) except group O (EC_{50}: 0.027 μM). DRV had various inhibitory effects for different subtypes. Virus replication of the subtype D, subtype G, and CRF02_AG strains were inhibited at low concentrations of DRV (EC_{50}: 2.0–3.0 nM). However, a ten-fold higher concentration of DRV was required to inhibit the replication of subtype C and group O viruses. Of note, DRV had no effect on subtype F1 at a concentration of 1 μM. In contrast, all of the tested strains were sensitive to our compounds, with observed EC_{50} values of < 1 μM, although some variations in the EC_{50} values were observed among the different subtypes. Compound 5b showed the most potent antiviral activity among our lead compounds against all HIV-1 subtypes.

3.3. Antiviral activity of 4-oxoquinoline analogs against drug-resistant strains

The antiviral activities of compounds 1b, 2d, 3b, and 5b against PI-resistant strains were evaluated using R5-MaRBLE cells. The EC_{50} values of our compounds against three high-resistance strains were comparable to those observed against the NL4-3 strain, whereas the DRV EC_{50} values drastically increased (93.7-, 22.2-, and 32.3-fold against F719105-FY4, T448163_4 and F390563_1, respectively) (Table 4). In addition, the EC_{50} values of the compounds against the intermediate-resistance strain (V42613_1) only marginally increased, although the DRV EC_{50} against the intermediate strain showed an 8.2-fold increase (Table 4). These results demonstrate that compounds 1b, 2d, 3b, and 5b have potent antiviral effects against PI-resistant viruses, similar to those observed for the wild-type (WT) HIV-1.

Next, we tested the antiviral effects of the four compounds on INSTI-resistant HIV-1 replication using HIV-1_{NL4-3} containing either of INSTI resistance-associated mutations (T66I, E92Q, S147G, or G140S/Q148H). The HIV-1 G140S/Q148H showed remarkably reduced susceptibility to EVG and RAL with EC_{50} values of > 400 nM (> 1250 fold) and 294 nM (445 fold), respectively (Table 5). As expected, HIV-1 carrying T66I or S147G which are known as an EVG-specific resistance mutation exhibited a moderate reduction in EVG susceptibility, but not in RAL. In contrast, our compound 5b showed similar antiviral effects for WT as well as any of these INSTI-resistant HIV-1 assayed in this study (Table 5). These results indicate that our compounds have antiviral effects on INSTI-resistant HIV-1. To further evaluate the antiviral effect of 4-oxoquinolines on the other drug-resistant strains, we determined the antiviral activity against NRTI or NNRTI-resistant HIV-1. Although the antiviral activity of our compounds for DR2510 was slightly attenuated, the other tested strains were sensitive to our compound (EC_{50} < 0.1 μM) similarly to WT (Table 6).

3.4. Effect of 4-oxoquinoline on the replication kinetics of HIV-1

We confirmed the antiviral effects of compounds 1b, 2d, and 3b using an *in vitro* assay system for HIV-1 replication kinetics. In the absence of the compounds, the p24 antigen level in the culture supernatant increased greatly after 5 days and reached the maximum on day 14. In the presence of AZT or EFV, the virus was undetectable until 7 days post-infection, although the p24 amounts drastically increased, likely due to the emergence of revertants (Fig. 3). In contrast, the 4-oxoquinoline compounds significantly reduced the p24 antigen levels to minimal levels for 19 days, suggesting their persistent antiviral activity as lead compounds.

3.5. Time-of-addition experiments

To investigate at which stage of virus replication the 4-oxoquinolines exert its antiviral activity, we carried out time-of-addition experiments using TZM-bl cells synchronously infected with HIV-1_{NL4-3} (Fig. 4). Reference compounds (AZT, EFV, RAL, or EVG) with their action modes known, or compound 5b were added into the cells at various time points postinfection, and then maintained until the luciferase activity driven by Tat expression was measured at 24 h post-infection. As expected, the reference compounds lost their antiviral activity ranging between 6 and 12 h postinfection: AZT, at 6 h; EFV, at 8 h; RAL and EVG, at ~12 h. In contrast, compound 5b remained active even until 16 h postinfection, although the magnitude of antiviral effect at 0 h is significantly weaker than the reference compounds. These data demonstrate that the inhibitory action modes in viral infection clearly differ between EVG and compound 5b. In addition, the results suggest that compound 5b executes antiviral activity after virus integration, and mostly likely at viral gene expression.

3.6. Inhibitory effect of 4-oxoquinolines on the virus production in HIV-1 latently infected cells

Finally, we examined whether 4-oxoquinolines actually inhibit the gene expression using HIV-1 chronically infected cells, OM-10.1. The cells stimulated with TNF-α were maintained in the presence of various concentrations of AZT, EVG, RAL, or test compounds 5a or 5b for 72 h. The amounts of p24 antigen produced in supernatants were measured by the ELISA. Of note, two compounds 5a and 5b that have relatively higher antiviral activity than others were used in this assay. As shown in Fig. 5, control drugs, EVG, and RAL, had no significant inhibitory effect on the p24 production, whereas AZT only marginally affects (a black bar, at 200 nM). In contrast, compounds 5a and 5b drastically reduced p24 antigen production in a dose-dependent manner. At a concentration of 200 nM, the production was suppressed to 18%, compared with those in the absence of the compounds. These results suggest that our 4-oxoquinolines exert potent inhibitory activity at HIV-1 gene expression in HIV-1 latently infected cells.

4. Discussion

In this study, we identified novel anti-HIV compounds with high selectivity indexes (CC_{50}/EC_{50} > 2500) *in vitro* and good pharmacokinetic profiles in mice. The compounds displayed inhibitory effects against various subtypes, and no cross resistance to these compounds was shown by PI-resistant, INSTI-resistant, NRTI-resistant, or NNRTI-resistant HIV-1. We found that these compounds all have a 4-oxoquinoline moiety as a common moiety. A fluorophenyl (or its modified) group at the R1 position and a nitrogen-containing heterocyclic moiety at the R7 position were required for their anti-HIV-1 activity (Fig. 2). Note that an addition of carboxyl acid at the C3 position of the 4-oxoquinolines abolishes their anti-HIV-1 activity, suggesting that our compounds differs from the 4-oxoquinoline-3-carboxylic acid derivatives, such as EVG, in the structure-activity relationships. Since the 1960s, some of the 4-oxoquinoline analogs, the 4-oxoquinoline-3-carboxylic acid derivatives, have been studied in the field of medicinal chemistry, because of their potent antibacterial activities and attractive pharmacokinetic profiles (Ahmed and Daneshtalab, 2012). The antibacterial activities of these derivatives are primarily caused by the inactivation of bacterial DNA gyrases. The molecular mechanism of gyrase inactivation occurs through the chelation of Mg^{2+} ions at the catalytic centers of bacterial gyrases. This mechanistic idea of enzymatic inactivation by chelating a divalent cation has been applied to the development of HIV-1 INSTIs (Nagasawa et al., 2011; Pasquini et al., 2008; Sato et al., 2006). EVG is an example of a successful modification of quinolone-3-carboxylic acids (Kobayashi et al., 2011; Sato et al., 2009). In these cases, a planar heteroatom chelating motif, the 4-ketone and 3-carbonyl moiety, is essential to chelate a pair of Mg^{2+} ions included in the IN. However, novel 4-oxoquinoline derivatives discovered in this study exhibited potent anti-HIV activity (EC_{50} < 0.1 μM)

despite the lack of a 3-carboxylate moiety in their chemical structures. As expected, these compounds displayed no detectable INSTI activity (EC_{50} > 100 μM). In addition, the 4-oxoquinolines exhibited potent antiviral activity against HIV-1 resistant to EVG.

Until now, only limited reports have described 4-oxoquinoline derivatives that display anti-HIV activity, except for INSTIs. The fluoroquinolone derivatives K-12 and K-37 (Baba et al., 1997, 1998) and 6-desfluoroquinolone (Tabarrini et al., 2008) inhibit HIV Tat-mediated transactivation and show potent inhibition of HIV replication in both acutely and chronically infected cells. Therefore, the 4-oxoquinoline derivatives might inhibit the same target(s) involved in TNF-α-induced viral gene expression and/or Tat-mediated transactivation, such as CDK9/cyclin T1. However, all the inhibitors that were previously reported fundamentally require the 3-carboxylic moiety for their biological activity. Therefore, our 4-oxoquinoline derivatives, lacking the 3-carboxylic moiety, may a new series of chemicals that differ from the fluoroquinolones or 6-desfluoroquinolone. Furthermore, oxoquinoline acyclonucleoside phosphonate analogs were reported as a new class of RT inhibitor (Faro et al., 2012; Santos et al., 2009). These compounds were designed as nucleobase analogs based on the 4-oxoquinoline moiety and were modified by the addition of 1-diisopropylphosphonate as a ribose of the acyclic chain to increase the anti-HIV activity. In the context of RT inhibitors, the chemical scaffold of our novel compounds is quite distinct from the nucleotide/nucleoside analogs. To date, we have not successfully identified a potential molecular target(s) that is involved in the anti-HIV-1 mechanism(s) of the novel 4-oxoquinoline derivatives. Notably, these compounds do not possess INST inhibitory activity in our *in vitro* enzymatic assay, and various drug-resistant HIV-1 strains do not display significant cross resistance to these compounds. These results suggest that our novel 4-oxoquinoline derivatives are likely a new class of compounds that interact with an unidentified target of cellular and/or viral molecules.

In summary, we identified a series of novel and potent anti-HIV compounds, 4-oxoquinolines, (EC_{50} < 0.1 μM) by screening a small-compound library using cell-based anti-HIV-1 assays. The 4-oxoquinolines exhibited high selectivity indexes (CC_{50}/EC_{50} > 2500) *in vitro* and favorable pharmacokinetic profiles in mice. In addition, various drug-resistant HIV-1 strains display no cross resistance to the compounds. Although no molecular target(s) for the compounds has been determined yet, these 4-oxoquinolines significantly reduce the p24 antigen production in HIV-1 latently infected cells, suggesting the inhibitory effect on viral gene expression. Interestingly, these compounds possess a chemical backbone that is similar to a current clinically used INSTI, EVG, although they lack a 3-carboxylate group that is a critical moiety for the diketo motif common in INSTIs. These findings may be important for the further development of a novel chemical class of anti-HIV-1 drugs.

Author disclosure statement

No competing financial interests exist.

Acknowledgments

The TZM-bl and OM-10.1 cells were provided by Drs. J. C. Kappes et al. and S. T. Butera, respectively, through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, NIH Reagent Program. This project was funded in part by a grant from the Human Sciences Foundation and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) and a grant for HIV/AIDS research from the Ministry of Health, Labor, and Welfare of Japan.

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