Novel In Vivo Model for the Study of Human Immunodeficiency Virus Type 1 Transcription Inhibitors: Evaluation of New 6-Desfluoroquinolone Derivatives

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Two novel 6-desfluoroquinolone derivatives, HM-12 and HM-13, were evaluated for anti-human immunodeficiency virus (anti-HIV) activity in acutely, chronically, and latently HIV type 1 (HIV-1)-infected cell cultures and were found to behave as potent HIV-1 transcription inhibitors. In order to extend this result in vivo, we developed an artificial hu-SCID mouse model for HIV-1 latency based on SCID mice engrafted with latently HIV-1-infected promyelocytic OM-10.1 cells in which HIV-1 can be reactivated in vivo by the administration of human tumor necrosis factor alpha (hTNF-α). Treating these SCID mice with HM-12 or HM-13 prior to hTNF-α stimulation resulted in a pronounced suppressive effect on viral reactivation. Since both quinolone derivatives were able to inhibit the reactivation of HIV-1 from this artificial viral reservoir in vivo, we provide encouraging evidence for the use of quinolones in the control of HIV-1 infections.

AIDS, caused by the human immunodeficiency virus (HIV), remains a health threat of global significance. The use of combination therapy consisting of HIV type 1 (HIV-1) reverse transcriptase and/or protease inhibitors results in a dramatic reduction of plasma viral loads to undetectable levels and leads to a significant decline in the onset of AIDS and AIDS-related morbidity and mortality. Despite the notable success of highly active antiretroviral therapy (HAART), infectious HIV-1 continues to replicate and to reside latently in resting CD4+ T cells (14, 16, 47). Postintegration latency appears to result from the reversion of productively infected CD4+ T cells to a resting memory state in which viral transcription is minimal and is limited to the production of short, abortive HIV-1 transcripts (20, 23). This population of cells with a memory phenotype has a relatively long half-life and serves as a potential source of reactivation of viral replication, thereby creating a major obstacle for the eradication of the virus from HIV-infected patients. Since HAART fails to cure the infection, and considering the fact that the viral transactivator Tat protein has been shown to upregulate HIV-1 gene expression in peripheral blood mononuclear cells from patients on HAART (24), a new anti-HIV treatment strategy may arise based upon HIV-1 transcription inhibitors (for a review, see reference 42).

A unique class of drugs that may contribute to the control of the latent HIV-1 reservoir includes the quinolone derivatives. Quinolones were first reported as an important class of broad-spectrum antibacterials based on the inhibition of prokaryotic type II topoisomerases, namely, DNA gyrase and, in a few cases, topoisomerase IV (39). In addition to their antibacterial properties, the quinolones have been shown to inhibit HIV-1 replication in vitro in both acutely and chronically HIV-infected cell lines by interfering with Tat-mediated transcription (4, 5, 11, 34, 38, 40, 41, 44). Richter and coworkers found that the mechanism of anti-HIV action could be ascribed to the interaction of the quinolone with the bulge of the HIV-1 TAR RNA element, resulting in the inhibition of Tat-TAR complex formation (38). This antiviral approach has also been described for many other classes of anti-HIV compounds, including peptoids (such as CGP64222 or TR87) (19, 21), Tat peptide mimetics (13), polyamide oligomers (27), arginine-aminoglycoside conjugates (25), intercalators (32), chemically modified aptamers (15, 22), and TAR RNA decoys (8, 29, 43). In addition, quinolones have been shown not only to inhibit HIV replication but also to be inhibitory to human cytomegalovirus, varicella-zoster virus, and herpes simplex virus types 1 and 2 (41, 46). The mechanism of inhibition of these human herpesviruses will be discovered.

Animal models have played an important role in HIV pathogenesis studies and in preclinical evaluations of therapeutic strategies. Two well-established xenochimeric models have been developed by transplanting immunodeficient mice with either human peripheral blood leukocytes (hu-PBL-SCID mice) (30, 31) or pieces of human fetal tissues containing hematopoietic cells (SCID-hu Thy/Liv mice) (28, 33). Furthermore, thymopoiesis in the SCID-hu Thy/Liv mouse model was also used to generate latently infected cells, thus further expanding its utility (3, 9). More recently, multiple researchers...
have been able to develop a functional human immune system in central and peripheral lymphoid organs of newborn Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice by injection of human CD34<sup>+</sup> hematopoietic cells (6, 7, 48). This mouse model of HIV infection shows great promise for future pathogenesis studies as well as for the evaluation of new drug treatments. Although these in vivo models closely resemble HIV infection in humans, we developed a rather simple and artificial SCID mouse model of HIV-1 latency to evaluate the potential of HIV reactivation inhibitors in a faster, more cost-effective way. This xenobiotic model is based on the engraftment of latently HIV-1-infected promyelocytic OM-10.1 cells into SCID mice in which HIV-1 can be reactivated in vivo by the administration of human tumor necrosis factor alpha (hTNF-α). This study represents the first proof of concept that quinolone-based drugs are inhibitory to HIV-1 replication in vivo and that they may prevent virus reactivation from the artificial viral reservoir.

**MATERIALS AND METHODS**

**Compounds and plasmid constructs.** The structures of the 6-desfluoroquinolone (6-DFQ) derivatives HM-12 and HM-13 are shown in Fig. 1. Their syntheses are reported elsewhere (45). The construct pHIV-RT-Q was made as follows. After a reverse transcription reaction on total RNA isolated from OM-10.1 cells to create cDNA, a PCR was performed with the forward primer HIV-RT-F (CTTCCCTTTGATGATTATGGA) and the reverse primer HIV-RT-R (TGTCATTGACAGTCCAGCTGTCT) in order to amplify a 93-bp fragment from the HIV-1 RT gene for cloning into the TOPO cloning vector (Invitrogen, Merelbeke, Belgium). The construct was verified by sequencing and used as a standard for quantitative real-time PCR. pGAPDH-Q was used as an internal control in the quantitative real-time PCR and was constructed by cloning the complete glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the cDNA mix, using the forward primer GAPDH-F (GACAGTCAGCCGCACCACTTAT) and the reverse primer GAPDH-R (CTTCCTCTTGCTCTGTCGTT), into the TOPO cloning vector. This construct was also verified by sequencing.

**Cells and viruses.** The latently HIV-1-infected promyelocytic OM-10.1 cell line (10) and latently HIV-1-infected promonocytic U1 cells (17) were maintained in RPMI 1640 medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Life Technologies), and 0.1% NaHCO<sub>3</sub> (Life Technologies) and incubated at 37°C in a humidified CO<sub>2</sub>-controlled atmosphere. Human primary monocytes/macrophages (M/M) were prepared and purified as described previously (1, 2). A monocytotropic HIV-1 strain was used for studies involving primary M/M. The characteristics and genomic sequence of this strain have been described previously (12, 18, 36, 37). The virus was incubated with M/M, and supernatants were collected, filtered, and stored at −80°C before use (36). The characteristics of viral stocks used for this study were 2.1 × 10<sup>8</sup> HIV RNA genomes/ml (corresponding to 35 ng/ml of p24 antigen) and 5 × 10<sup>3</sup> 50% tissue culture infective doses per ml, as assessed by virus titration in other primary M/M cultures.

**Assessment of antiviral drug activity in acutely infected M/M.** One day after separation (i.e., 6 days after being plated), M/M were treated with various concentrations of drugs (HM-12 and HM-13) and then exposed to 2,000 pg/ml of HIV-1 (BaL). Two hours after virus challenge, M/M were washed to remove the virus inoculum, and complete medium containing the appropriate drugs was replaced. M/M were then cultured for the duration of the experiments by replenishing them with fresh complete medium and drugs every 7 days. Supernatants were collected at different time points (7 and 14 days) for assessment of virus production by analysis of HIV-1 p24 production with a commercially available kit (Bio-Rad). The p24 antigen evaluation was repeated at later time points in selected experiments. The geometric mean of p24 Gag antigen production for replicates in each experiment was used to determine the effective drug concentration where 50% or 90% of viral replication was inhibited (EC<sub>50</sub> and EC<sub>90</sub>, respectively) by linear regression of the log of the percent HIV-1 p24 production (compared to that in untreated controls) versus the log of the drug concentration.

**Assessment of antiviral drug activity in chronically infected M/M.** M/M were defined as being chronically infected when no new rounds of infection occurred in cultures in vitro and the p24 production remained stable. Our previous experience demonstrated that such a chronic infection status starts on day 10 after virus challenge. For this purpose, M/M were challenged with 2,000 pg/ml of HIV-1 BaL (in the absence of any drug) on day 0. At the time that chronic infection was established, M/M were carefully washed at least twice to remove any virus present in the supernatants, replenished with fresh complete medium containing HM-12 or HM-13 at the same dose used for the acute treatment, and cultured under the same conditions as described before. Each drug concentration was run in triplicate or quadruplicate, while positive controls were run in sextuplicate. The supernatants of HIV-1-infected M/M were collected on days 13, 17, and 20, and the drugs were re-added at these time points at the appropriate concentrations. The supernatants of the cell cultures taken at 17 and 20 days were assessed to determine virus production in the presence or absence of drugs by measuring HIV p24 production.

**Assessment of antiviral drug activity in latently infected M/M.** The activity values for the quinolone derivatives against latent HIV-1 infection were based on the inhibition of p24 antigen production in OM-10.1 and U1 cells after stimulation with hTNF-α (Roche Diagnostics Belgium) and phorbol myristate acetate (PMA; Sigma Chemical Co., Bornem, Belgium). Briefly, OM-10.1 and U1 cells (500,000 cells/ml) were incubated in the presence or absence of the compounds for 2 h in 48-well plates. After this short incubation period, the cell cultures were stimulated with 1 ng/ml of hTNF-α or 0.02 μM of PMA, followed by two transfers of 200 μl to a 96-well plate for cytopotoxicity evaluation. After a 2-day incubation period at 37°C, the cell culture supernatants were collected from the 48-well plates and examined for their p24 antigen levels with an HIV-1 p24 enzyme-linked immunosorbent assay kit (NEP, Brussels, Belgium). The cytopotencies of the compounds for both latently HIV-1-infected OM-10.1 and U1 cells in the 96-well plates were based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability staining, as previously described (35).

**Quantitative real-time PCR.** Quantitative determinations of full-length viral RNA in the latently HIV-1-infected cell lines OM-10.1 and U1 were done by real-time detection based on TaqMan technology, using the plasmid pHIV-RT-Q as a standard and pGAPDH-Q as an internal control. Cells were seeded into 24-well plates at a density of 750,000 cells per well and exposed to different concentrations of the compounds for 1 h. Next, 1 ng/ml of hTNF-α or 0.02 μM of PMA was added to the cell cultures, and they were incubated further over...
night at 37°C. The next day, cells were collected, and RNAs were extracted using the TRizol method (Invitrogen, Merelbeke, Belgium). PCRs were performed in 96-well optical reaction plates, with a final volume of 25 μl per well. Each PCR mix contained 5 μl RNA sample added to a mixture of 12.5 μl of TaqMan one-step reverse transcription-PCR master mix, 1.25 μl endogenous GAPDH control mixture, a 600 nM concentration (each) of primers HIV-RT-F and HIV-RT-R, and 250 nM of the TaqMan HIV RT probe (ATAAATGGAGACGACACGCTAGCTTATAGCTGCGCAAG, with the reporter dye 6-carboxyfluorescein at the 5’ end and the quencher dye 6-carboxytetramethylrhodamine at the 3’ end). Real-time PCR was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) under the following conditions: 30 min at 48°C for reverse transcription, 10 min at 95°C for enzyme activation, and 45 cycles of amplification (15 s at 95°C and 1 min at 60°C), with measurement of fluorescence at the end of each elongation step. All assays included two negative controls (water) and a dilution series of the plasmid containing the human GAPDH gene. The detection of GAPDH RNA was conducted using a TaqMan predeveloped assay reagent containing a VIC/6-carboxytetramethylrhodamine-labeled TaqMan probe (Applied Biosystems, Foster City, CA). A standard curve of the cycle threshold values was constructed for each PCR assay in order to automatically calculate the sample quantities, using software for data analysis (26). All samples were performed in duplicate.

**Animal experiments.** Male SCID mice of reproductive age (4 to 6 weeks old) were bred at the Rega Institute under specific-pathogen-free conditions and were used throughout the experiments. SCID mice were inoculated intraperitoneally with 40 μl of HIV-1 inoculum obtained from Pieter Rottiers of the Department for Molecular Biomedical Research (VIB/UGent). The 6-DFQ derivatives HM-12 and HM-13 inhibit HIV-1 replication in acutely and chronically HIV-1-infected M/M. The uninoculated controls (water) and a dilution series of the plasmid containing the human GAPDH gene. The detection of GAPDH RNA was conducted using a TaqMan predeveloped assay reagent containing a VIC/6-carboxytetramethylrhodamine-labeled TaqMan probe (Applied Biosystems, Foster City, CA). A standard curve of the cycle threshold values was constructed for each PCR assay in order to automatically calculate the sample quantities, using software for data analysis (26). All samples were performed in duplicate.

**RESULTS**

The 6-DFQ derivatives HM-12 and HM-13 inhibit HIV-1 transcription in latently infected M/M. In order to investigate the effects of the quinolones on HIV-1 transcription, we quantified the full-length viral transcripts produced upon stimulation of latently HIV-1-infected cells with hTNF-α or PMA. In this context, we made a construct that contains a 93-bp fragment from the RT gene of HIV-1, as well as the endogenous control plasmid pGAPDH-Q, containing the complete human GAPDH gene. The detection of GAPDH RNA was conducted using a TaqMan predeveloped assay reagent containing a VIC/6-carboxytetramethylrhodamine-labeled TaqMan probe (Applied Biosystems, Foster City, CA). A standard curve of the cycle threshold values was constructed for each PCR assay in order to automatically calculate the sample quantities, using software for data analysis (26). All samples were performed in duplicate.

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closely correlated with their inhibitory effect on viral p24 production from the latently HIV-1-infected cells upon stimulation with hTNF-α and PMA.

Effects of the 6-DFQ derivatives HM-12 and HM-13 on viral reactivation in an artificial in vivo model of HIV-1 latency. We developed an artificial xenochimeric model of HIV-1 latency in order to determine whether the quinolone derivatives can also control the latent HIV-1 reservoir in vivo. Male SCID mice of reproductive age (4 to 6 weeks old) were inoculated intraperitoneally with 10^7 latently HIV-1-infected OM-10.1 cells and found to develop malignant tumors around the pancreas and the small and large intestinal canal. Four weeks after inoculation, the cancer became fatal to the mice. If mice were treated with 40 μg hTNF-α 3 weeks after inoculation with OM-10.1 cells, plasma viral loads were increased 10- to 100-fold compared to those in untreated mice without any visible signs of agony or fatality (Fig. 4). As shown in Fig. 4, the plasma viral load was monitored by HIV-1 p24 antigen (panel A) and HIV-1 RNA (panel B) measurements. It was found that both parameters (p24 concentration and number of HIV-1 RNA copies) correlated well with one another. Therefore, both parameters were used to evaluate the effects of the quinolone derivatives on hTNF-α-induced virus production in vivo. Initially, we investigated HM-13 by intraperitoneal administration at a drug dose of 50 mg/kg once daily for a period of 3 days prior to hTNF-α administration. Approximately 18 h after hTNF-α stimulation, the viral load was analyzed and found to contain as much HIV-1 as that found in the absence of stimulation. This result was repeated in an independent experiment and confirmed the complete suppressive effect of HM-13 on viral reactivation in this artificial in vivo model (Fig. 4A and B). Additionally, the quinolone derivative HM-12 at a dose of 50 mg/kg/day administered for a period of 3 days was also found to be endowed with a pronounced inhibitory effect on viral reactivation in vivo. In order to obtain insights on the hTNF-α plasma levels present 18 h after intraperitoneal administration to the mice, we quantified, in parallel with the viral p24 levels, the hTNF-α levels in the plasma and observed values ranging between 900 and 1,900 pg/ml under all tested conditions, in the absence or presence of HM-12 or HM-13 (Fig. 4C). Finally, pathological examination of the mice revealed poorly differentiated promyelocytic tumors involving the whole peritoneal cavity. No extra-abdominal localizations were found, however, either in thoracic organs or in the brain. Within the abdomen, tumor tissue was found along lymphatics in the peritoneum, the abdominal lymph nodes, and the liver (Fig. 5). Remarkably, the pancreas was heavily inflicted. At 4 weeks, almost no
pancreatic parenchyma was left, with major tumor growth resulting in death.

**DISCUSSION**

Previously, it was shown that a quinolone derivative bearing a methyl substituent at the N-1 position, an amino group at the C-6 position, and a 4-(2-pyridyl)-1-piperazine moiety at the C-7 position (WM5) can inhibit the Tat-mediated transcription process (34). In particular, WM5 binds with high affinity to the bulge of the TAR element, which results in inhibition of Tat-TAR complex formation (38). Subsequent structural investigations on a number of analogues of the lead compound WM5 allowed us to obtain insights into their structure-activity relationships (44). We successfully eliminated the amino group from the C-6 position and positioned a suitable 4-arylpiperazine at the C-7 position, resulting in the development of a new series of potent 6-DFQs (45). Indeed, we found that substitution at the N-4 piperazine core of m-trifluoromethylphenyl (HM-12) or benzothiazole (HM-13) resulted in pronounced anti-HIV properties, particularly in M/M cell lineages, with EC_{50} values in the ng/ml range. We observed, not only in the acute infection model but also in the chronic and latent infection models, pronounced antiviral activities for both quinolones HM-12 and HM-13 at drug concentrations as low as 40 ng/ml. In order to confirm that the inhibitory effects of both quinolones were located at the level of HIV-1 transcription, as previously reported for the parent WM5 compound, we quantified the viral transcripts in latently HIV-1-infected cells upon reactivation of the provirus with hTNF-α or PMA. Since we found that HM-12 and HM-13 inhibited the production of viral mRNAs at concentrations in the ng/ml range, we proved that both quinolone compounds act at a postintegrational step in the HIV-1 replication cycle by inhibiting HIV-1 transcription.

Since these results and all previous data concerning the quinolones were obtained in vitro, we aimed to establish the anti-HIV potency of these HIV-1 transcription inhibitors in vivo. For this purpose, we developed an artificial but novel xenochimeric model of HIV-1 latency in which HIV-1 could be recovered from the bloodstream within 18 h of exposure of the animals by using hTNF-α. Virus production was quantified by the p24 level as well as the viral RNA load in plasma. It was found that both parameters closely correlated with each other. Using this animal model, we provide the first evidence of a potent anti-HIV activity of quinolone-based drugs in vivo. Initially, we evaluated HM-13 for its effect on the reactivation of HIV-1 from the artificial viral reservoir and found that the compound had a pronounced suppressive effect on viral reac-

FIG. 3. Inhibitory effects of HM-12 and HM-13 on HIV-1 RNA transcription in latently HIV-1-infected OM-10.1 and U1 cell lines after stimulation with 1 ng/ml hTNF-α (black bars) or 0.02 μM PMA (white bars). Total RNA was isolated from the cells by the TRIzol RNA extraction method. Quantification of full-length viral RNA was assessed by real-time PCR, amplifying a 93-bp fragment of the HIV-1 RT gene by using TaqMan technology. In order to obtain HIV-1 RNA amounts, correction was included, using GAPDH as an endogenous control. All experiments were carried out in quadruplicate, and results are presented as mean values with standard deviations (1 μg/ml HM-12 equals 2.32 μM, and 1 μg/ml HM-13 equals 2.38 μM).
Activation in vivo under experimental conditions where no visible signs of drug toxicity could be detected before and after the SCID mice were sacrificed. The quinolone analogue HM-12 was also endowed with a marked anti-HIV activity in this artificial SCID mouse model of HIV-1 latency. Since both quinolones represent lead compounds, we plan to search and test for a larger number of compounds with optimal antiviral/cytotoxic properties in order to find clinically effective agents.

The quinolone derivatives are known as an important class of broad-spectrum antibacterials. The molecular mechanism of antibacterial action (i.e., prokaryotic DNA gyrase [topoisomerase II] and topoisomerase IV) is clearly different from the mechanism of anti-HIV action. Therefore, it is obvious that optimization of quinolone derivatives must show pronounced antiviral activity in the absence of appreciable antibacterial activity. This is important in view of the risk of potential resistance development in quinolone-exposed bacteria upon frequent or continuous drug exposure. Since the quinolones act at a postintegration stage in the replication cycle of HIV, they markedly slow down virus replication and should be interesting candidate drugs to be combined with entry, integrase, or reverse transcriptase inhibitors. It would also be interesting to find out whether the quinolone derivatives act synergistically when combined with HIV protease inhibitors that act at a late stage in the virus infection cycle.

In conclusion, we have shown that quinolone-based drugs are inhibitory to viral reactivation from an artificial latent HIV-1 reservoir both in vitro and in vivo. Since this study is the first to demonstrate anti-HIV activity of quinolones in vivo, it certainly prompts further investigations on the potential of the quinolones in the treatment of HIV-1 infection.

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