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Highly Favorable Antiviral Activity and Resistance Profile of the Novel Thiocarboxanilide Pentenyloxy Ether Derivatives UC-781 and UC-82 as Inhibitors of Human Immunodeficiency Virus Type 1 Replication

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SUMMARY

The novel human immunodeficiency virus type 1-specific thiocarboxanilide derivatives that contain either a substituted furanyl (UC-781) or thienyl (UC-82) ring linked to the thiocarboxy group and a pentenyloxyether chain linked to the 4-chlorophenyl ring in meta position show highly favorable antiviral properties. Compounds UC-781 and UC-82 discovered by scientists at Uniroyal Chemical Ltd. proved to be ≥5–10-fold more inhibitory to wild-type human immunodeficiency virus type 1 strains (EC_{50} ~ 0.002 μg/ml) than the thiocarboxanilide oxime ether UC-10 and other non-nucleoside reverse transcriptase inhibitors such as nevirapine, bis(heteroaryl)piperazine, and tetrahydroimidazo[4,5,1-jk][1,4]-benzodiazepin-2(1*H*)-one. In addition, the compounds were able to knock out virus replication in cell culture at concentrations that were 20–50-fold lower than those of nevirapine or bis(heteroaryl)piperazine. They were also highly efficient (EC_{50} ≤ 0.02 μg/ml) in suppressing the replication of mutant virus strains that contained mutations in their reverse transcriptase that conferred resistance to other non-nucleoside reverse transcriptase inhibitors (i.e., Tyr181 to Cys, Lys103 to Asn, Val106 to Ala, and Leu100 to Ile). The compounds selected for virus mutants that were only marginally resistant to the thiocarboxanilides (<10–20-fold). The antiviral activity of the compounds was only slightly affected by the presence of high concentrations of human serum, and the compounds were shown to be highly stable in the presence of human serum for at least 24 hr at room temperature.

Several different classes of NNRTIs of HIV-1 have been described (for an overview, see Refs. 1–4). These compounds share a number of characteristics, including their high specificity toward HIV-1, their low toxicity in cell culture and patients, an action targeted at the HIV-1 RT, and the rapid emergence of resistant virus strains both *in vitro* and *in vivo* (for an overview, see Refs. 1–5).

Resistance development is usually due to the appearance of point mutations in the RT gene, leading to virus mutants with >10-fold reduced sensitivity to the particular compounds. The cross-resistance/sensitivity spectrum of such mutant virus strains to NNRTIs other than those against which they were selected varies markedly depending the nature of the amino acid change and the particular NNRTI. However, several amino acid mutations have been identified that tend to markedly affect the antiviral activity of most, if not all, NNRTIs that have been described. Two such mutations that affect the activity of the majority of NNRTIs (in particular, NNRTIs that are currently the subject of clinical trials, such as nevirapine, BHAP, and α-anilinophenylacetamide) are Tyr181Cys and Lys103Asn. We recently described a series of oxathiin(thio)carboxanilide esters and various structurally closely related derivatives that show a favorable resistance spectrum in that they were able to suppress the Tyr181Cys RT mutant virus at drug concentrations that

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ABBREVIATIONS: NNRTI, non-nucleoside reverse transcriptase inhibitor; RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; BHAP, bis(heteroaryl)piperazine; TSAO-m^3T, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide]-N^3-methylthymidine; TIBO, tetrahydroimidazo[4,5,1-jk][1,4]-benzodiazepin-2(1*H*)-one; HEPT, 1-(2-hydroxyethoxymethyl-6-(phenylthio)thymine; MKC-442, 1-(ethoxymethyl)-6-benzyl-5-isopropyluracil; hu-PBL, human peripheral blood lymphocyte; SCID, severe combined immunodeficiency; PE, phycoerythrin; PCR, polymerase chain reaction.

were significantly lower than those required for other compounds, such as nevirapine and BHAP (6–8).

However, these thiocarboxanilide ester derivatives proved to be poorly stable in plasma (due to aspecific esterase activity) and showed cross-resistance to the Lys103Asn RT mutant virus. These compounds also predominantly selected for Lys103Asn/Thr mutant virus strains when HIV-1-infected cell cultures were propagated in the presence of these test compounds (8). We describe several novel thiocarboxanilide derivatives that are highly stable in serum (due to an aliphatic ether function instead of the ester function) and that are able to markedly suppress both the Tyr181Cys and Lys103Asn mutant virus strains in cell culture. In addition, the compounds were found to select in cell culture for only marginally resistant mutant virus strains and to knock out the virus from the HIV-1-infected cell cultures at concentrations that were 10–50-fold lower than those required for other NNRTIs to knock out HIV-1 under the same experimental conditions.

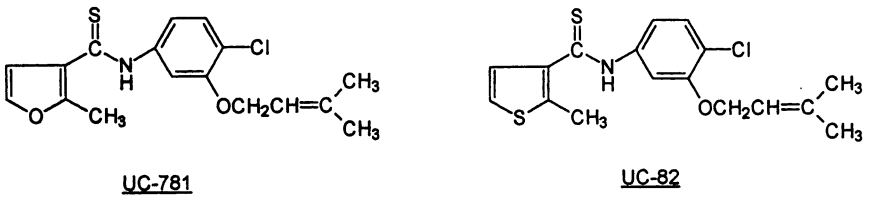
Materials and Methods

Test compounds. The compounds UC-781, UC-82, UC-10, UC-83, and UC-284 were supplied by Uniroyal Chemical Ltd. (Guelph, Ontario, Canada), and their synthesis will be published elsewhere. The structural formulae are depicted in Fig. 1. Nevirapine was kindly provided by Dr. P. Ganong (Boehringer Ingleheim, Ridgefield, CT); BHAP U90152 was provided by Dr. Kirsch (Hoechst AG, Frankfurt, Germany); TSAO-m^{3}T was provided by Dr. Camarasa [Instituto de Quimica Medica (Consejo Superior de Investigaciones Cientificas), Madrid, Spain]; TIBO R82913 was provided by Dr. Zhang-Hao (National Cancer Institute, National Institutes of Health, Bethesda, MD); MKC-442 was provided by Prof. M. Baba (Kagoshima, Japan); and dideoxyinosine was provided by Dr. D. G. Johns (National Cancer Institute, National Institutes of Health).

Cells. Human lymphocyte CEM cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% (v/v) inactivated fetal calf serum (GIBCO), 2 mM L-glutamine (Flow Laboratories, Irving, UK), and 0.075% (v/v) $NaHCO_3$ (Flow Laboratories). Cells were subcultured every 3–4 days. Human C8166 cells and Molt 4/clone 8 were obtained from Prof. P. La Colla (Cagliari, Sardinia) and Dr. N. Yamamoto (Nagoya, Japan), respectively, and cultured as described for the CEM cells. The procedure for purification of monocytes from the blood has been described in detail previously (9). Briefly, peripheral blood mononuclear cells obtained from the blood of HIV-seronegative healthy volunteers by centrifugation over Ficoll-Hypaque gradient were cultured for 5 days in 48-well plates (Costar, Cambridge, MA) in RPMI 1640 medium plus 2 mM L-glutamine and penicillin/streptomycin (GIBCO, Paisley, Scotland) supplemented with 20% mycoplasma- and endotoxin-free fetal calf serum (Hyclone Labs, Logan, UT). A total of 1.8×10^6 macrophages/cm^2 and /ml were used. Nonadherent cells were removed by repeated washings with warm medium 5 days after plating. Cells obtained at this point are >95% pure macrophages, as assessed by morphology and fluorescence-activated cell-sorting analysis.

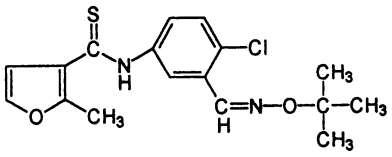
Viruses. HIV-1(III_B) was kindly provided by Prof. R. C. Gallo (National Cancer Institute, National Institutes of Health) (10). HIV-1/HE represents a clinical isolate obtained from a patient with acquired immune deficiency syndrome in Leuven, Belgium. HIV-1 (Ba-L) was obtained from the American Type Culture Collection.

Antiviral activity of test compounds. CEM cells were suspended at 250,000 cells/ml of culture medium and infected with 100 $CCID_{50}$ (1 $CCID_{50}$ is the 50% cell culture infective dose) of HIV-1(III_B) or mutant HIV-1 strains selected for resistance against TIBO R82150 (HIV-1/Ile100) (11), TIBO R82913 (HIV-1/Asn103) (11), TSAO-m^{3}T (HIV-1/Lys138) (12), nevirapine (HIV-1/Ala106) (13), or pyridinone L697,661 (HIV-1/Cys181) (11). Then, 100 μl of the infected cell suspensions was added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds (i.e., 100, 20, 4, 0.8, 0.16, 0.032, and 0.006 μg/ml). The inhibitory effect of the test compounds on HIV-1-induced syncytium formation in CEM cells was examined on day 4 after infection as described previously (11, 13). The EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%. In the monocyte/macrophage assays, 5-day adherent macrophages were treated with various concentrations of test compounds and immediately thereafter challenged with 300 minimum infectious doses of the monocytotropic strain of HIV/Ba-L. Virus excess was removed at 2 hr after virus challenge, and the macrophages were cultured in the presence of the same drug concentrations as before. Virus production was assessed 14 days after virus challenge by a commercially avail-

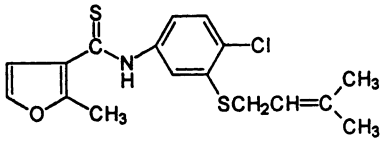


UC-781

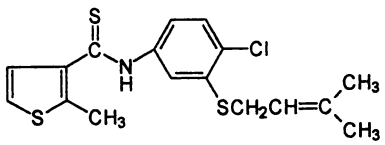
UC-82



UC-10



UC-83



UC-284

Fig. 1. Structural formulae of thiocarboxanilides.

able immunoenzymatic method (Abbott, Pomezia, Italy) that could be used to detect HIV-p24 antigen in the supernatants. All samples were run in quadruplicate.

PBLs from a normal HIV-negative human volunteer were obtained by the Ficoll/Hypaque technique, washed twice with phosphate-buffered saline, and cultured with phytohemagglutin (2 μg/ml) for 3 days at 37° in a CO_2-controlled incubator. Phytohemagglutin-stimulated PBL cells were washed twice with phosphate-buffered saline and then infected with a concentrated HIV-1 (HTLV-III_B) stock in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, L-glutamine (2 mM), gentamycin (50 μg/ml), recombinant interleukin-2 (10 units/ml), and Polybrene (2 μg/ml). After 60-min incubation at 37°, nonadsorbed virus was removed by successive washing steps with fresh culture medium, and the HIV-1-infected PBL cells were then suspended at 5×10^5 cells/ml in RPMI 1640 medium and cultured in the presence of varying concentrations (20, 4, 0.8, 0.16, 0.032, and 0.006 μM) of the test compounds in 96-well culture plates (200 μl/well). HIV-1 p24 core antigen was quantified in the cell culture supernatants at 7 days after infection by an antigen-capture assay using an enzyme-linked immunosorbent assay.

Determination of the amino acid sequence of the RT of drug-resistant virus strains. The procedure of CEM cell infection with mutant HIV-1 strains, preparation of the samples for PCR assays, amplification of the proviral DNA, and sequencing of the 727-bp fragment covering amino acid residues 50–270 have been reported previously (11–13). The first set of primers (5′-GTAGAATTCTGTTGACTCAGATTGG-3′ and 5′-TTCTGCCAGTTCTAGCTCTGCTTCT-3′) gave a 900-bp product of the proviral RT gene. The second set of primers (5′-CCTGAAAATCCATACAATACTCCAGTATTTG-3′ and 5′-AGTGCTTTGGTTCCTCTAAG GAGTTTAC-3′) gave the 727-bp RT fragment covering amino acids 50–270. The PCR products were purified from 0.1% low-melting agarose gel by Magic PCR Preps (Promega, Madison, WI) or on a microspin column and directly sequenced with a Taq Dye Deoxy Terminator sequencing kit (Applied Biosystems) or an AutoRead T-7 Sequencing kit (Pharmacia, Piscataway, NJ). Sequence analysis was then performed on a model 373A DNA sequencer (Applied Biosystems, Norwalk, CT) or an ALF DNA sequencer (Pharmacia).

RT assay. The RT assays using recombinant HIV-1 RT were performed as described previously (14).

Selection of HIV-1(III_B) mutant strains resistant to HIV-1-specific RT inhibitors administered as single drugs at fixed concentrations. HIV-1(III_B) was subjected to two or three passages in 5-ml CEM cell cultures (4×10^5 cells/ml) in the presence of several fixed concentrations of the test compounds in 25-cm^2 culture flasks (Falcon flasks; Becton Dickinson, San Jose, CA) to produce mutant HIV-1 strains. The multiplicity of the initial infection was 1000-fold the IC_{50} value. Passages were performed every 3–4 days by adding 0.5–1.0 ml of the infected culture supernatants to 4–4.5 ml of a suspension containing 4×10^5 uninfected CEM cells/ml. Syncytium formation was used as a parameter of virus breakthrough in the cell cultures.

Grafting mice with PBL, delivery of compounds, infection with HIV, and recovery of human cells from hu-PBL-SCID mice. CB.17 scid/scid (SCID) mice were bred at the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Leuven, Belgium) in a sterile environment. hu-PBL-SCID mice were generated by reconstituting adult SCID mice through intraperitoneal injection of 20×10^6 PBL, as described previously (15). For the isolation of PBL, buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven and kindly provided by Dr. O. Souw. All hu-PBL-SCID mice were housed in microisolator caging and maintained in a sterile environment. At 14 days after reconstitution, hu-PBL-SCID mice were implanted with osmotic Alzet 35 2002 micropumps (Alza, Palo Alto, CA) for the delivery of the compounds. The pumps have a reservoir volume of 200 μl and can pump for 14 days at a pumping rate of 0.5 μl/hr. They were implanted in the dorsolateral side of the mice under sterile conditions. The compounds UC-10 and BHAP U-90152 were diluted at 125 mg/ml (in a first, preliminary experiment) or 25 mg/ml (in a second preliminary experiment) in dimethylsulfoxide. Therefore, the pumps delivered 7.5 or 1.5 mg of compound/day/mouse. The mice were injected intraperitoneally 16 hr later with 1×10^4 50%-tissue culture infective dose HIV-1 in a Biosafety level 3 containment facility. The HIV-1 strains used were III_B (10) and SF-2 (16). At 13 days later, mice were bled by cardiac puncture, and the spleens were collected. Single cell suspensions of the spleen were obtained by gently teasing the tissue in sterile medium and passing the disrupted tissue through a sterile wire mesh.

Flow cytometric analysis and antibodies. Three-color flow cytometric analysis was performed on a FACScan 228 (Becton Dickinson). Data were acquired and analyzed with CellQuest 228 software (Becton Dickinson) on a Apple Macintosh Quadra computer. The following antibodies, specific for human cell surface markers, were purchased from Becton Dickinson: CD8 (fluoresceinisothiocyanate), CD4 (PE), and CD45 (peridine chlorophyl). Mouse isotype controls were also obtained from Becton Dickinson. Single cell suspensions from the spleen of each hu-PBL-SCID were stained with the antibodies CD4-PE, CD8-fluoresceinisothiocyanate, and CD45-peridine chlorophyl and analyzed, and the percentage of human $CD4^+$ T cells was calculated of the total number of human $CD45^+$ T cells.

Results

Inhibitory effect of thiocarboxanilide pentenyloxy-(thio)ether derivatives against wild-type and mutant HIV-1 strains in CEM cell cultures. From a broad screening of several hundred oxathiincarboxanilide derivatives against HIV-1(III_B) in CEM cell cultures, a number of highly active and selective HIV-1-specific inhibitors emerged (6–8, 17–19). Structure-activity relationship studies revealed that ~30 compounds had EC_{50} values in the range of 1–10 ng/ml. The most promising inhibitors were thiocarboxanilide derivatives containing a 2-methyl-substituted thiophene (i.e., UC-82 and UC-284) or furanyl (i.e., UC-10, UC-781 and UC-83) ring linked to the thiocarboxy group and an aliphatic (branched pentenyl) ether function linked to the 4-chloroanilide moiety at the meta-position. The ethers could be either oxy-ether (i.e., UC-781 and UC-82) or thio-ether (i.e., UC-83 and UC-284) (Fig. 1). UC-10 represents a thiocarboxanilide oxime ether that has been identified (8) as a potent and specific inhibitor of HIV-1 replication (Fig. 1). It was included in our study as a reference compound together with nevirapine, BHAP U90152, TIBO R82913, the HEPT derivative MKC-442, and TSAO-m^3T.

The thiocarboxanilide (oxy)ethers UC-781 and UC-82 were exquisitely inhibitory to HIV-1 replication in CEM cells. Their EC_{50} was as low as 0.002–0.003 μg/ml (Table 1). The corresponding thioethers UC-83 and UC-284 were 2–3.5-fold less antivirally effective, whereas the oxime ether UC-10 was 5–10-fold less inhibitory to HIV-1. However, UC-10 inhibited HIV-1-induced cytopathicity at similar concentrations as nevirapine, BHAP, TIBO, and TSAO-m^3T (EC_{50} = 0.015–0.019 μg/ml) (Table 1). Unlike UC-10 and UC-82, the other thiocarboxanilide derivatives (UC-781, UC-83, and UC-284) were devoid of any cytostatic activity against CEM cells at a compound concentration as high as 100 μg/ml. The selectivity indexes of UC-781, UC-83, and UC-284 were ≥14,300 or even >50,000. In this respect, they exceeded the selectivity index of UC-10 by ≥1 or by >2 orders of magnitude. The superior antiviral activity of the novel thiocarboxanilides UC-781, UC-82, UC-83, and UC-284 over UC-10 and the other NNR-

TABLE 1

Cytostatic activity and inhibitory activity of thiocarboxanilide pentenyloxy(thio) ethers and other NNRTIs against different HIV-1 strains in different cell systems

Compound	EC_{50}[a]					CC_{50}[b] (CEM)	S.I. (CEM)
	III_B (CEM)	III_B (C8166)	HE (Molt)	BaL (M/M)	III_B (PBL)		
			μg/ml			μg/ml	ratio CC_{50}/EC_{50}
UC-781	0.002	0.002	0.008	0.080	0.001	>100	>50,000
UC-82	0.003	0.002	0.005	0.015	0.004	5.8	1,933
UC-83	0.007	0.010	0.08		0.002	>100	14,300
UC-284	0.007	0.006	0.065		0.001	>100	>14,300
UC-10	0.016	0.019	0.08	0.85	0.021	5.8	360
Nevirapine	0.019	0.032	0.05		0.006	>20	>1,000
BHAP U-90152	0.006	0.012	0.05			>20	>3,300
TIBO R82913	0.016	0.031	0.25	0.05	0.006[c]	>20	>1,250
MKC-442	0.001	0.003	0.009	0.02		>20	>20,000
TSAO-m^3T	0.015	0.026	0.06	0.08	0.046	>20	>1,300

[a] 50% Effective concentration or compound concentration required to inhibit HIV-1-induced cytopathicity in CEM and C8166 cells or p24 expression in M/M cell cultures.
[b] 50% cytostatic concentration or compound concentration required to inhibit CEM cell proliferation by 50%.
[c] Data obtained for TIBO R82150.

TIs was further confirmed against HIV-1(III_B) in C8166 cell cultures, clinical isolate HIV-1(HE) in CEM cell cultures, monocytotropic virus strain HIV-1(Ba-L) in freshly isolated monocyte/macrophage cell cultures, and HIV-1(III_B) in hu-PBL cell cultures (Table 1). None of the compounds were inhibitory to HIV-2(ROD) replication in CEM cells (data not shown). Thus, both UC-781 and UC-82 emerged as the most prominent anti-HIV-1 compounds among the thiocarboxanilides included in our study.

Inhibitory effect of thiocarboxanilide pentenyloxy-(thio)ether derivatives against mutant HIV-1 strains in CEM cell cultures. The thiocarboxanilides were evaluated for their inhibitory activity against a series of mutant HIV-1 strains that had emerged in the presence of a variety of NNRTIs as described previously (11–13). The most relevant mutations shown to occur in HIV-1-infected individuals treated with NNRTIs (5) were included in this study. The NNRTIs nevirapine, BHAP, TIBO, and TSAO-m^3T markedly lost inhibitory potency against several of the mutant virus strains by 1, 2, and even 3 orders of magnitude (depending on the nature of the NNRTI as well as the nature of the amino acid substitution in the RT of the mutant virus strains) (Table 2). In marked contrast, the thiocarboxanilides retained high potency against the mutant viruses evaluated. In fact, UC-781 and UC-82 inhibited the Leu100Ile, Val106Ala, Glu138Lys, and Val179Asp RT mutant viruses within the 0.004–0.010 μg/ml range (i.e., at only 2–3-fold higher concentrations than those required to inhibit wild-type virus). Even more importantly, the Tyr181Cys RT mutant and Lys103Asn RT mutant virus strains proved to be highly susceptible to the inhibitory effects of UC-781 and UC-82. They were inhibited by these compounds at concentrations that ranged between 0.014 and 0.023 μg/ml (i.e., at drug concentrations that were only 5–10-fold higher than the concentrations required to inhibit wild-type virus and similar to the concentrations required for nevirapine, BHAP, TIBO, and TSAO-m^3T to inhibit wild-type virus; Table 2).

The thiocarboxanilide oxime ether UC-10 and thioethers UC-83 and UC-284 showed a relative greater loss in inhibitory potency against the Leu100Ile, Val106Ala, Glu138Lys, and Val179Asp RT mutant virus strains than UC-781 and UC-82. However, like UC-781 and UC-82, the thioethers UC-83 and UC-284 retained marked activity against the Lys103Asn and Tyr181Cys RT mutant viruses (EC_{50} = 0.035–0.080 μg/ml).

Effect of human serum on the anti-HIV-1 activity of thiocarboxanilide pentenyloxy ether derivatives. Because drug binding to serum proteins may affect the antiviral activity of the test compounds *in vivo*, we performed an experiment in which the anti-HIV-1 activities of UC-10, UC-781, and UC-82 were examined in the presence of 10% fetal

TABLE 2

Antiviral activity of thiocarboxanilide pentenyloxy(thio) ethers and other NNRTIs against mutant HIV-1 strains

Compound	EC_{50}[a]						
	WT[b]	Leu100Ile	Lys103Asn	Val106Ala	Glu138Lys	Val179Asp	Tyr181Cys
				μg/ml			
UC-781	0.002	0.008	0.023	0.005	0.004	0.006	0.015
UC-82	0.003	0.006	0.020	0.005	0.006	0.012	0.014
UC-83	0.007	0.035	0.080	0.060	0.030		0.080
UC-284	0.007	0.035	0.035	0.080	0.035		0.070
UC-10	0.016	0.13	0.20	0.05	0.05	0.17	0.030
Nevirapine	0.019	0.065	1.5	0.80	0.12	0.25	6.8
BHAP U-90152	0.006	0.46	0.27	0.16	0.016	0.13	0.25
TIBO R82913	0.016	1.75	≥5	0.50	0.30		2.0
TSAO-m^3T	0.015	0.041	0.11	0.23	>20	0.097	0.33

[a] 50% effective concentration or compound concentration required to inhibit HIV-1-induced cytopathicity in CEM cell cultures by 50%.
[b] Wild-type HIV-1(III_B).

calf serum and increasing concentrations of human serum (i.e., 0%, 5%, 12.5%, and 50%) (Table 3). Although the test compounds progressively showed decreased antiviral potency at increasing serum concentrations, the decrease was only 3.5–5-fold in the presence of the highest human serum concentration tested (i.e., 50%). The EC_{50} values under our experimental conditions were as low as 0.014 μg/ml for UC-781, 0.025 μg/ml for UC-82, and 0.075 μg/ml for UC-10. A similar minimal effect of human serum on the anti-HIV-1 potency of the HEPT derivative MKC-442 was observed (Table 3), with the latter compound chosen as the clinical candidate compound among the HEPT derivatives based on its relatively low serum binding properties.

Breakthrough of mutant HIV-1 strains in the presence of thiocarboxanilide pentenyloxy ether derivatives. The thiocarboxanilides UC-781, UC-82, and UC-10 were administered to HIV-1-infected CEM cells at fixed concentrations of the test compounds (i.e., 0.001, 0.025, 0.1, 0.25, and 1.0 μg/ml). Cell cultures were passaged twice a week in the presence of the test compounds and further subcultured in the absence of the test compounds after the 10th passage. Although UC-10 fully suppressed virus breakthrough in the CEM cell cultures at 1 μg/ml but not at lower drug concentrations, UC-781 and UC-82 did so at concentrations of 1, 0.25, and 0.1 μg/ml (Table 4). At a concentration as low as 0.01 μg/ml (i.e., at a 3–5-fold higher concentration than the EC_{50} value), UC-781 and UC-82 were able to markedly delay virus breakthrough (27–30 days versus 3 days in control cultures for the occurrence of 50% cytopathicity). The HIV-1-infected CEM cell cultures that were treated with UC-10 at 1.0 μg/ml or with UC-781 or UC-82 at 0.1, 0.25, or 1.0 μg/ml were shown to be fully cleared from the virus as demonstrated by the lack of cytopathicity, absence of proviral DNA, and inability to detect viral p24 antigen after the drugs were removed and the cell cultures were further incubated for an additional five passages.

When evaluated under similar experimental conditions, nevirapine and TIBO R82913 prevented virus breakthrough only at compound concentrations that were 25–50-fold higher than those required for UC-781 and UC-82 (data not shown).

Characterization of mutant virus strains emerging in the presence of the thiocarboxanilide pentenyloxy ether derivatives. The virus strains that emerged in the presence of UC-10 (0.1 and 0.25 μg/ml), UC-781 (0.01 and 0.025 μg/ml), and UC-82 (0.01 and 0.025 μg/ml) were characterized in terms of amino acid mutations in their RT and their sensitivity/resistance spectrum against a series of NNRTIs (Tables 5 and 6). The virus strains that emerged at the lowest concentration of UC-10 (0.1 μg/ml) and UC-781 (0.01 μg/ml) did not contain any detectable mutations in their RT within the 50–270-amino acid region that was sequenced. However, the virus strains that emerged in the presence of UC-10 at 0.25 μg/ml, UC-781 at 0.025 μg/ml, or UC-82 at 0.01 and 0.025 μg/ml contained single amino acid mutations at positions Val179, Leu100, Glu138, and Val106. The mutations at amino acid positions 179 and 100 were transversion mutations resulting in the amino acid changes of Val179Asp and Leu100Ile. The mutations at amino acid positions 138 and 106 were transition mutations resulting in the amino acid changes of Glu138Lys and Val106Ala (Table 5). All of these four mutations have been reported to emerge in the presence of NNRTIs such as pyridinone and PETT (Val179Asp mutation) (20, 21); TIBO, BHAP, and thiocarboxanilide esters (Leu100Ile mutation) (8, 11, 13, 20, 22); TSAO and oxathiincarboxanilide ester derivatives (Glu138Lys mutation) (6, 12); and nevirapine (Val106Ala mutation) (13, 23, 24).

When the mutant HIV-1 strains that emerged under treatment with the thiocarboxanilide pentenyloxy ether derivatives were evaluated for their sensitivity to UC-10, UC-781, UC-82, and a series of other NNRTIs and nucleoside analogues such as ddI and AZT, only low level resistance was observed for the thiocarboxanilide pentenyloxy ether derivatives (Table 6). Indeed, the activity of UC-10 against the mutant HIV-1 strains was decreased by no more than 10–20-

TABLE 3

Effect of human serum concentrations on the anti-HIV-1 activity of thiocarboxanilides

Compound	EC_{50}				
	Concentration of human serum				
	0%	5%	12.5%	25%	50%
	μg/ml[a,b]				
UC-781	0.003	0.007	0.010	0.017	0.014
UC-82	0.005	0.009	0.020	0.03	0.025
UC-10	0.02	0.04	0.045	0.07	0.075
MKC-442	0.003	0.007	0.008	0.015	0.014

[a] 50% effective concentration or compound concentration required to reduce the HIV-1-induced cytopathicity by 50%.
[b] Data are the mean of two independent experiments.

TABLE 4

HIV-1 breakthrough in CEM cell cultures in the presence of thiocarboxanilide derivatives at fixed concentrations

From day 36 (passage 10), cell cultures were maintained in the absence of the test compounds.

Compound	Concentration	Initial breakthrough of virus	50% Cytopathicity
		μg/ml	
UC-781	0.01	11	30
	0.025	32	42
	0.1	>50	>>50
	0.25	>50	>>50
	1	>50	>>50
UC-82	0.01	15	28
	0.025	36	38
	0.1	>>50	>>50
	0.25	>>50	>>50
	1	>>50	>>50
UC-10	0.1	11	27
	0.25	32	42
	1.0	>>50	>>50
Control	0	2	3

TABLE 5

Amino acid mutations in the RT of the virus strains that emerged in HIV-1-infected cell cultures in the presence of thiocarboxanilides

Compound	Concentration	Position of the amino acid mutations in the RT	Amino acid change in the RT	Codon mutation in the RT
	μg/ml			
UC-781	0.01	None		
	0.025	100	Leu → Ile	TTA → ATA
UC-82	0.01	138	Glu → Lys	GAG → AAG
	0.025	106	Val → Ala	GTA → GCA
UC-10	0.1	None		
	0.25	179	Val → Asp	GTT → GAT

TABLE 6
Antiviral activity of thiocarboxanilides against mutant HIV-1-infected CEM cells

Compound	EC_{50}[a]				
	HIV-$1_{(IIIB)}$	HIV-1/Ile 100	HIV-1/Ala 106	HIV-1/Lys 138	HIV-1/Asp 179
			μg/ml		
UC-10	0.011 ± 0.008	0.24 ± 0.05	0.16 ± 0.06	0.1 ± 0.0	0.17 ± 0.09
UC-781	0.0018 ± 0.0004	0.035 ± 0.009	0.023 ± 0.004	0.0065 ± 0.0021	0.006 ± 0.04
UC-82	0.0018 ± 0.0004	0.50 ± 0.14	0.025 ± 0.0	0.010 ± 0.007	0.012 ± 0.008
TSAO-m^3T	0.11 ± 0.08	0.070 ± 0.017	1.15 ± 0.49	>20	0.097 ± 0.055
BHAP U-90152	0.015 ± 0.007	1.33 ± 0.58	0.15 ± 0.07	0.033 ± 0.011	0.13 ± 0.06
Nevirapine	0.033 ± 0.011	0.50 ± 0.14	≥5	0.065 ± 0.007	0.25 ± 0.07
Dideoxyinosine[a]	5.0 ± 1.4	4.83 ± 2.02	13.5 ± 9.2	12.0 ± 11.3	6.90 ± 6.18
AZT[a]	0.0016 ± 0.0	0.0025 ± 0.0007	0.008 ± 0.0	0.003 ± 0.0	0.017 ± 0.020

[a] EC_{50} expressed in μM.

fold, as was the antiviral potency of UC-781. The EC_{50} values of UC-781 against the four mutant virus strains did not exceed 0.03 μg/ml. A similar behavior was noted for UC-82, except for the Leu100Ile mutant virus strain that showed a more pronounced decrease of sensitivity to UC-82 (but not to UC-781). In contrast with UC-781 and UC-82, the NNRTIs TSAO-m^3T, BHAP, and nevirapine showed a 100–200-fold reduction in their inhibitory activity against HIV-1/Lys138, HIV-1/Ile100, and HIV-1/Ala106. All thiocarboxanilide derivatives showed a moderate (5–10-fold) reduction in activity against HIV-1/Asp179, which is of the same order as that found for TSAO-m^3T, BHAP, and nevirapine against this virus strain. As expected, both ddI and AZT virtually maintained full potency against all of the mutant virus strains (Table 6), except for HIV-1/Asp179, which showed a 10-fold reduced sensitivity to AZT.

Activity of thiocarboxanilide pentenyloxy ether derivatives against HIV-1 RT. UC-10, UC-781, and UC-82 were evaluated for their inhibitory effect on recombinant HIV-1 RT. Poly(C)/oligo(dG) was used as the (preferred) template/primer, and [^{3}H]dGTP (2 μM) was used as the radiolabeled substrate. Although UC-10 inhibited the enzyme activity by 50% at 0.1 μg/ml, UC-781 and UC-82 did so at 5-fold-lower compound concentrations (IC_{50} = 0.02 μg/ml). These drug concentrations were 5–20-fold lower than those required for nevirapine and BHAP to inhibit HIV-1 RT activity (IC_{50} = 0.4 and 0.1 μg/ml, respectively). When the kinetic properties of UC-781 and UC-82 against HIV-1 RT were examined, noncompetitive or uncompetitive inhibition was noted for the thiocarboxanilide pentenyloxy ether derivatives with respect to dGTP and the template/primer (data not shown). These observations are in agreement with earlier observations for other NNRTIs (1–4) and suggest that the thiocarboxanilides interact with HIV-1 RT at a site that is different from the substrate and template/primer binding site.

Antiviral activity of UC-10 and BHAP U-90152 in HIV-1-infected hu-PBL-SCID mice. In two preliminary experiments, the HIV-$1/III_B$ and HIV-1/SF-2 strains were used at the same $TCID_{50}$, as defined by culture with mitogen-activated PBL, to infect hu-PBL-SCID mice 2 weeks after reconstitution with PBL. Five animals per group were examined to determine the number of human $CD4^+$ and $CD8^+$ T cells at 2 weeks after infection (Table 7). After HIV-$1/III_B$ infection, 16 ± 2% of the $CD45^+$ human cells were $CD4^+$ T cells, whereas the percentage for the control group, which was not injected with HIV-1, was 32 ± 4%. Percentages for the two groups treated with BHAP or UC-10 were 33 ± 4% and 40 ± 14% $CD4^+$ T cells, respectively. The other experiments with the HIV-1 SF-2 strain gave comparable results, although in addition to a different HIV-1 strain, a different donor was used. The percentage of $CD4^+$ T cells in the human $CD45^+$ cell population of these mice was 24 ± 3% in the uninfected group, whereas it decreased to 13 ± 2% in the SF-2-infected group. Again, the two compounds delivered at a 5-fold-lower concentration (i.e., 75 mg/kg/day) completely protected the $CD4^+$ T cells from destruction by HIV. Under our experimental conditions, no signs of toxicity of these compounds were observed in the hu-PBL-SCID mice.

TABLE 7
Effect of BHAP U-90152 and UC-10 on the percentage of human $CD4^+$ $CD45^+$ T cells in HIV-1-infected hu-PBL SCID mice

Groups of five mice were treated with the drugs through implanted osmotic minipumps. In Experiments 1 and 2, the concentration of the drugs in the pumps was 125 and 25 mg/ml, respectively. In Experiment 1, the HIV-1 (III_B) strain was used, and in Experiment 2, the HIV-1/SF-2 strain was used. The percentages of human $CD4^+$ $CD45^+$ T cells in the spleens were determined by flow cytometric analysis at 14 days after intraperitoneal injection of HIV-1. Values are mean ± standard error.

	Percentage of human $CD4^+$ $CD45^+$ T cells	
	Experiment 1	Experiment 2
	%	
Mock-infected animals	32 ± 4	24 ± 3
HIV-1-infected animals	16 ± 2	13 ± 2
HIV-1-infected, BHAP-treated animals	33 ± 4	29 ± 7
HIV-1 infected, UC10-treated animals	40 ± 14	27 ± 6

Discussion

We previously reported that thiocarboxanilide derivatives qualify as potent and selective inhibitors of HIV-1 replication in cell culture. The vast majority of the compounds were ester derivatives that turned out to be unstable *in vivo*, presumably due to a nonspecific cleavage of the ester bond by cellular esterases. The cleaved compounds completely lost their potency against HIV-1 in cell cultures and their direct inhibitory effect on recombinant RT (8). We also reported on the antiviral properties of the enzymatically more stable thiocarboxanilide *t*-butyl oxime ether derivative UC-10. One of the major favorable properties of UC-10 is its pronounced activity against the Tyr181Cys RT mutant virus strain in cell culture, its marked stability in human plasma, and its high oral bioavailability in rats and dogs (≤80–90%) (8). However,

UC-10, as well as other closely related thiocarboxanilides, showed a relatively poor inhibitory effect on the Lys103Asn RT mutant virus. These thiocarboxanilides also selected for the Asn103 RT mutant virus strains.

The thiocarboxanilide pentenyloxy ether derivatives described here represent novel aliphatic ether derivatives that show a highly favorable activity spectrum against wild-type and mutant virus strains. These compounds (i.e., UC-781 and UC-82) proved to be exquisitely inhibitory to wild-type HIV-1 strains as well as to the mutant HIV-1 strains containing single amino acid mutations in their RT that emerge either in cell culture or in patients treated with one of the NNRTIs that are currently the subject of clinical trials (i.e., nevirapine, BHAP, TIBO, α-anilinophenylacetamide). Importantly, UC-781 and UC-82 not only inhibit wild-type HIV-1 at a concentration that is 5–10-fold lower than that required by nevirapine, BHAP, or TIBO but also are inhibitory to nevirapine-, BHAP-, and TIBO-resistant mutant virus strains at drug concentrations at which nevirapine and BHAP are active against wild-type virus.

It has been shown by Ho *et al.* (25) and Wei *et al.* (26) that nevirapine has a remarkable inhibitory potency against wild-type HIV-1 in drug-treated HIV-1-infected patients during the first week of treatment, resulting in a decrease of plasma virus load by ≥2–3 orders of magnitude. After this initial treatment period, a marked rebound of (drug-resistant) mutant virus has been seen that contains single mutations such as Cys181 and Asn103 in its RT. Because these mutant viruses can be efficiently suppressed in cell culture by UC-781 and UC-82, it is likely that these mutations may not have the chance to arise under the condition of thiocarboxanilide pentenyloxyether drug treatment. In fact, our knock-out experiments with UC-781 and UC-82 revealed that the drugs were able to efficiently suppress the emergence of any virus, regardless of whether mutated, at drug concentrations that were markedly lower than those for UC-10 or other NNRTIs such as nevirapine, TIBO, and BHAP. Furthermore, the virus mutants that arose in the presence of relatively low drug concentrations (i.e., with UC-781 or UC-82 at 0.025 or 0.01 μg/ml) contained the NNRTI-specific mutations (i.e., Ile100, Ala106, Lys138, or Asp179) in their RT and displayed minimal resistance to UC-781 and UC-82.

Thus, from our cell culture data, the thiocarboxanilide pentenyloxyether derivatives seem to possess markedly superior antiviral properties compared with NNRTIs that are the subjects of clinical trials (i.e., nevirapine, BHAP). In addition to their high antiviral potency, their activity against NNRTI-resistant virus strains, and their ability to knock out the virus in cell culture at low drug concentrations, the novel thiocarboxanilides UC-781 and UC-82 show a number of other favorable properties. First, their antiviral activity in the presence of high concentrations (≤50%) of human serum is only slightly affected. Second, although the oral bioavailability of the novel test compounds has not been determined, the oral absorption may well be in the same range as that found for the closely related UC-10 compound (70–90% in rats and dogs) (8). Third, in contrast with the thiocarboxanilide ester derivatives, the novel pentenyloxy ether derivatives UC-781 and UC-82 proved to be fully stable in human serum for 24 hr, a favorable property that they share with the oxime ether derivative UC-10. Fourth, the chemical synthesis of UC-781 and UC-82 is not very complicated and can be scaled-up to kilogram quantities.

Our preliminary results on UC-10 in hu-PBL-SCID mice demonstrate that despite a partial depletion (~50%) of the human $CD4^+$ T cells in the HIV-1-infected animals and even some variation in this depletion, UC-10 is active in inhibiting the HIV-1-induced destruction of $CD4^+$ T cells. Our data also show that although the hu-PBL-SCID mice do not provide a functional human immune system in an experimental animal (27), anti-HIV compounds can be evaluated in this animal model regarding their potential to protect human $CD4^+$ T cells from destruction by HIV-1.

In conclusion, a novel generation of thiocarboxanilide derivatives is presented that has proved to be superior to the existing NNRTIs in terms of their potency against both wild-type and mutant HIV-1 strains (resistant to other NNRTIs), their ability to knock out the virus in cell culture at low concentrations, and the poor effect of the presence of human serum proteins on their antiviral activity. These favorable properties make the thiocarboxanilide pentenyloxy ether derivatives excellent candidates to be further pursued for the chemotherapy of HIV-1 infection.

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