Highly Potent Oxathiin Carboxanilide Derivatives with Efficacy against Nonnucleoside Reverse Transcriptase Inhibitor-Resistant Human Immunodeficiency Virus Isolates†

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The structure-activity relationships of a series of compounds related to the nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) oxathiin carboxanilide have been described (R. W. Buckheit, Jr., T. L. Kinjerski, V. Fliakas-Boltz, J. D. Russell, T. L. Stup, L. A. Pallansch, W. G. Brouwer, D. C. Dao, W. A. Harrison, R. J. Schultz, J. P. Bader, and S. S. Yang, *Antimicrob. Agents Chemother.* 39:2718–2727, 1996). From these studies, the furanyl-containing analog UC10 was identified as the most potent inhibitor of human immunodeficiency virus type 1 (HIV-1) replication and a promising candidate for further development. Three new UC analogs (UC040, UC82, and UC781) have been determined to inhibit laboratory-derived and low-passage-number, primary virus isolates at low nanomolar concentrations in both established and fresh human cells. Each of the compounds synergistically interacted with the nucleoside analogs zidovudine, dideoxyinosine, dideoxycytosine, and lamivudine to inhibit HIV-1 replication. As a group, the UC compounds were found to be less active against viruses with the L100I, K103N, and Y181C amino acid changes in the RT and, upon *in vitro* selection, yielded resistant virus with the Y181C mutation in the RT. The most potent of the three new compounds, UC781, contains a furanyl side chain, similar to UC10, but differs in having an extended ether side chain instead of an oxime chain. The broad therapeutic index of UC781 (>62,000) resulted in effective inhibition of NNRTI-resistant virus isolates at high nanomolar concentrations. Furthermore, UC781 and the NNRTI costatolide were able to synergistically inhibit HIV-1 replication when used in combination, suggesting that UC781 may interact with the RT differently than the other UC analogs. The favorable anti-HIV properties of the UC compounds suggest they should be considered for further clinical development.

The nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) selectively inhibit RT through reversible interaction with the RT at the nonnucleoside binding pocket of the palm domain of the p66 subunit of the RT (11, 12, 15, 28). Although they are relatively nontoxic to host cells, the high specificities of the compounds result in the rapid selection of drug-resistant virus isolates *in vitro* (23). High-level resistance is conferred by a variety of single-amino-acid changes within the binding pocket, including A98G, L100I, K101E, K103N, V106A, V108I, E138K, T139I, Y181C, Y188C, G190A, and P236L (23). The use of NNRTIs in patients is now dependent on defining appropriate combinations of agents which will prevent or significantly retard the selection of drug-resistant virus populations or which will result in the selection of drug-resistant virus isolates in which mutation of critical amino acid residues renders the RT less fit to support virus reproduction.

Oxathiin carboxanilide (UC84) was first defined as an antiviral agent by the National Cancer Institute's anti-human immunodeficiency virus (anti-HIV) screening program (1) and was subsequently defined as a member of the NNRTI pharmacological class (6, 14). The anti-HIV activity of a second more potent member of the UC series (UC38) was recently

reported (22). Synthesis of a large number of UC analogs has resulted in the delineation of detailed structure-activity relationships and significant improvements in the antiviral activities of the compounds (2, 8, 22). Recent research with two of the initial members of this series (UC84 and UC38) demonstrated that each of the UC analogs bound to different mechanistic forms of the ribonucleoprotein complex required for reverse transcription in the cell (14) and defined the first synergistic interactions between two NNRTIs (8, 13, 14). Synergistic inhibition of HIV-1 resulted from the interaction of UC84 with the RT-primer-template complex, while UC38 interacted with the RT-primer-template-deoxynucleoside triphosphate ternary complex (14). Cross-resistance evaluations and the selection of drug-resistant virus isolates in cell culture have defined the site of interaction of the various UC compounds with the HIV type 1 (HIV-1) RT. Selection for amino acid changes at positions 100, 101, 103, 106, 138, 181, and 190 have been described for the UC compounds (2, 8, 19).

In this report, we present the anti-HIV activity profiles of three newly discovered UC analogs which exhibit more potent activity than previously described members of the UC series and which have cross-resistance profiles which indicate greater potential utility than the previously reported UC compounds. Although these compounds possess the characteristics commonly associated with the NNRTIs, the antiviral properties of the compounds described in this report suggest that they may be attractive candidates for further development. One of these compounds, UC781, has been extensively evaluated in phar-

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† This paper is dedicated to the memory of Melinda J. Snow (1965–1996).

FIG. 1. Structure of oxathiin carboxanilide derivatives UC10, UC781, UC82, and UC040. The positions of the R-3 and R-4 substituents are indicated on the structure of UC10.

macokinetic and in vivo animal model assays to define its clinical potential and to provide a rationale for its use in patients (4).

MATERIALS AND METHODS

Chemistry. The structures of the compounds evaluated are presented in Fig. 1. These compounds were provided to the National Cancer Institute by Uniroyal Chemical Ltd. Research Laboratories (Guelph, Ontario, Canada) and were synthesized as described previously (16).

Cells and viruses. The established human cells, laboratory-derived virus isolates (including drug-resistant virus isolates), and low-passage-number clinical virus isolates used in these evaluations have previously been described in detail (8, 9). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Fresh human cells were obtained from the American Red Cross (Baltimore, Md.).

Materials. All experimental antiviral agents were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis and Centers, National Cancer Institute. Crystalline stock materials were stored at -70°C and were solubilized in 100% dimethyl sulfoxide. All stocks were diluted

at least 400-fold prior to performing drug susceptibility assays. Enzyme-linked immunosorbent assay plates were obtained from Coulter Cytometry (Hialeah, Fla.). Materials required for RT inhibition assays, anti-HIV assays, and the growth and maintenance of established and fresh human cells have been described previously (3, 8).

Antiviral and cross-resistance assays. The HIV inhibitory activities of the compounds were evaluated as described previously (8) in a microtiter anti-HIV assay with CEM-SS cells. The assay quantifies the ability of a compound to inhibit HIV-induced cell killing or HIV replication. Quantitation was performed by using the tetrazolium dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), which is metabolized to a colored, formazan product by viable cells. Antiviral and toxicity data are reported as the quantity of drug required to inhibit 50% of virus-induced cell killing or virus production (50% effective concentration [EC₅₀]) and the quantity of drug required to reduce cell viability by 50% (50% inhibitory concentration [IC₅₀]). The results provided in Tables 1 to 6 are representative of those results from one of at least two or three replicate assays. In our studies, the standard deviations from the mean antiviral and toxicity indices were determined to be less than 10% of the respective means.

Combination anti-HIV assays. Analysis of the efficacy of drug combinations was performed in CEM-SS cells acutely infected with the IIIB strain of HIV-1 as described previously (8) by using the anti-HIV assay methodology described above. In each assay, five serial twofold dilutions of drug A were evaluated in combination with each of eight twofold dilutions of drug B. The high test concentration used for each drug was two times the EC₅₀ of each drug, as determined in the microtiter anti-HIV assay. The high test concentrations of each drug used in the combination anti-HIV assays are noted in Table 2 and in each case were determined to not yield any measurable toxicity to the target cells. Statistical evaluations of the data obtained were performed by using MacSynergy II (26). The results of the combination assays are presented three dimensionally at each combination concentration, yielding a surface of activity extending above (synergy) or below (antagonism) the plane of additivity. The volume of the surface is calculated and expressed as a synergy volume (square micromolar percent) calculated at the 95% confidence interval (26). On the basis of our experience with these assays, synergy is defined in these studies as drug combinations yielding synergy volumes greater than 50 µM²%. Slightly and highly synergistic anti-HIV activities have been defined as yielding synergy volumes of 50 to 100 and >100 µM²%, respectively. Synergy volumes of between 0 and 50 µM²% are considered additive, and synergy volumes less than 0 µM²% are considered antagonistic.

RT inhibition assays. Analysis of the drug sensitivity of RT containing defined amino acid substitutions was performed as described previously (3).

Selection of drug-resistant strains. Resistant virus isolates were selected in cell culture by serial passage of the IIIB strain of HIV-1 in CEM-SS cells in the presence of increasing concentrations of antiviral compound. The initial selection was performed with a drug concentration of two times the EC₅₀ of the compound as determined in the microtiter anti-HIV assay. With successive passages the drug concentration was increased twofold to enhance the selective pressure on the virus. Upon selection of a drug-resistant virus isolate, cross-resistance testing was performed by the methods described above for the performance of antiviral assays. Resistance has been defined in this study as a greater than fivefold increase in the EC₅₀ compared to the activity of the compound against the wild-type (IIIB) isolate.

Analysis of RT mutations. Direct sequencing of PCR-amplified DNA by using dideoxynucleotide chain termination was performed with the Sequenase PCR Product Sequencing kit (United States Biochemicals-Amersham, Cleveland, Ohio) according to the manufacturer's protocol. Briefly, PCR-amplified DNA was derived from the first 750 bp of the HIV-1 RT gene by using the BLAA-NE1 primer set; five additional sequencing primers were used to provide DNA sequences spanning the entire amplified RT region. Following completion of the DNA sequencing reactions, the dideoxynucleotide chain-terminated reaction products were resolved on a 6% acrylamide-bisacrylamide denaturing gel containing urea and glycerol-tolerant buffer (United States Biochemicals-Amersham). Upon completion of electrophoresis, the DNA sequencing gels were dried (Bio-Rad, Richmond, Calif.) and were subjected to autoradiography by using BioMax MR2 film (Kodak, Rochester, N.Y.). The resulting autoradiographs were digitized (Hitachi, San Bruno, Calif.), and the reported DNA sequences were compiled and analyzed by using the Wisconsin Sequence Analysis Package, version 8.0 (Genetics Computer Group, Inc.).

RESULTS

Antiviral activities of selected UC compounds. We have previously described the structure-activity relationships of 27 compounds, representative of four subgroups of UC analogs, related to oxathiin carboxanilide (8). UC84, the original UC compound, and its expanded-spectrum analog UC38 have been described in detail (1, 22). In this report, we describe the antiviral properties of three new UC compounds (UC040, UC781, and UC82) with superior anti-HIV properties and

TABLE 1. Range of anti-HIV activity of UC compounds

Virus (isolate)	Cell ^a	EC ₅₀ (μM)				
		UC10	UC040	UC781	UC82	Nevirapine
HIV-1 (IIIB)	CEM-SS	0.4	0.5	0.01	0.03	0.1
HIV-1 (RF)	CEM-SS	0.1	0.1	0.02	0.004	0.2
HIV-1 (SK1)	CEM-SS	0.1	0.08	0.006	0.003	0.2
HIV-2 (ROD)	CEM-SS	>20	>20	>20	>20	>38
SIV (DeltaB670)	174×CEM	>20	>20	>20	>20	>38
HIV-1 (WEJO)	PBMC	0.2	0.5	<0.007	0.007	0.06
HIV-1 (BaL)	MAC	0.2	0.2	0.03	0.008	0.4
HIV-1 (IIIB)	174×CEM	0.6	0.1	0.01	0.008	0.1
HIV-1 (IIIB)	MT2	0.2	0.07	0.03	0.005	0.1
HIV-1 (IIIB)	U937	0.03	0.006	0.005	0.005	0.3
HIV-1 (IIIB)	AA5	1.9	0.2	0.02	0.009	0.7

^a Cellular phenotypes: CEM-SS, T cell; 174×CEM, T cell-B cell fusion; PBMC, fresh peripheral blood mononuclear cells; MAC, fresh peripheral blood monocytes; U937, macrophage; AA5, Epstein-Barr virus-infected B cell. The IC₅₀s of each compound in CEM-SS cells were 20 μM for UC10, 16 μM for UC82, >500 μM for UC781, 10 μM for UC040, and >100 μM for nevirapine.

compare their activities to that of UC10, the previously characterized lead compound (8). UC10 is a highly potent UC compound within a group of highly potent analogs possessing a furanyl side chain at R-3 (Fig. 1). Among several hundred analogs synthesized by the Uniroyal Chemical Ltd. Research Laboratories, UC781, UC82, and UC040 have been selected for further preclinical evaluation on the basis of their high levels of activity against HIV-1 and their extremely broad therapeutic indices (TIs). Similar to UC10, UC781 possesses a furanyl side chain but differs from UC10 by having an extended ether side chain instead of the oxime chain at the R-4 position. Both UC040 and UC82 have a thiophene substitution at R-3. While UC82 has the same extended ether side chain at R-4 as UC781, UC040 has an extended thioether side chain. The parent compound, UC84, exhibits anti-HIV activity (EC₅₀ = 0.6 μM, IC₅₀ = 180 μM), with a TI of 300. Although UC10, UC040, and UC82 are all potent anti-HIV agents (EC₅₀s = 0.008, 0.016, and 0.008 μM, respectively), they exhibit relatively greater toxicity to lymphocytes than UC781 (IC₅₀s = 20, 10, and 16 μM, respectively) and narrower TIs (50% TIs [TI₅₀], = 2,500, 625, and 2,000, respectively). The relatively noncytotoxic compound UC781 provides the widest TI of the compounds evaluated (EC₅₀ = 0.008 μM, IC₅₀ = >500 μM, TI₅₀ = 62,500). Toxicity values for each of the UC compounds were determined to be similar in each of the human cell lines reported in Table 1 (data not shown). For comparative purposes, data obtained with the reference compound nevirapine (EC₅₀ = 0.1 μM, IC₅₀ > 100 μM, TI > 1,000) are also presented in Table 1.

Range of activity and mechanisms of action of UC compounds. The activities of the four UC analogs were evaluated against a range of laboratory HIV-1 isolates, against primary HIV-1 isolates (including isolates with syncytium-inducing and non-syncytium-inducing phenotypes), and against HIV-1 isolates in a variety of established human cells (Table 1). Each of the UC compounds was active against all of the HIV-1 isolates evaluated in each of the tested cell lines. Protection from HIV-induced cytopathic effects was directly correlated with a reduction in virus replication as quantified by RT assay, p24 enzyme-linked immunosorbent assay, and infectious virus assay. Comparative evaluation of the four UC compounds demonstrated the higher levels of activity of UC781 and UC82. Each of the compounds was inactive when evaluated against HIV-2 ROD and simian immunodeficiency virus (SIV) DeltaB670. As would be expected for compounds which inhibit an early step in virus reproduction, none of the compounds was

able to inhibit virus production from cells already infected with HIV (chronically infected cells).

Mechanistic evaluations performed with the UC compounds indicated that the analogs inhibited reverse transcription when tested by a homopolymeric poly(rC)-oligo(dG) template-primer assay (see activity versus wild-type enzyme in Table 5). Biochemical and cell-based inhibition assays demonstrated that the UC compounds did not inhibit virus attachment or virus-cell or cell-cell fusion, protease or integrase activity, or late-stage events (data not shown). High-multiplicity-of-infection, single-step-of-replication assays demonstrated that each of the UC compounds had to be added to the infected cell cultures within 8 h of infection, indicating a mechanism of action similar to that of zidovudine (AZT) and dideoxycytosine (ddC) and distinct from that of the sulfonated, cell surface-active agent Chicago Sky Blue (10) and the protease inhibitor KNI 272 (17). In the high multiplicity-of-infection assay, attachment inhibitors must be added prior to 4 h postinfection, RT inhibitors lost activity at approximately 8 h, and protease inhibitors remained active for 12 to 18 h (data not shown).

Combination interaction of UC compounds with other anti-HIV agents. Combination anti-HIV assays were performed with the four UC analogs in order to evaluate the interactions between these compounds and other agents active against HIV and to aid in efforts to prioritize the compounds for further development. These additional agents included the nucleoside analogs AZT, dideoxyinosine (ddI), ddC, and lamivudine (3TC) and the NNRTI costatolide (an analog of calanolide A). A summary of the combination anti-HIV data obtained from the MacSynergy II evaluations is presented in Table 2. Data obtained from the MacSynergy II evaluations are presented in synergy volume units (square micromolar percent) at the 95% confidence interval as described previously (26). Table 2 presents synergy volume values for two replicate combination assays for each drug combination. The MacSynergy II program presents the data from the combination assay as a three-dimensional surface. Synergy is represented by areas extending above the plane of additivity, and antagonism is represented by surfaces extending below the plane of additivity. The calculated synergy volume values provide a quantitative measure of the volume of the peak of synergy (or trough of antagonism) in the three-dimensional surface generated by MacSynergy II and allows a direct comparison of the level of synergy between different combination assays. Each of the four UC analogs interacted in a highly synergistic fashion with AZT. In these

TABLE 2. Activities of UC compounds in combination with other anti-HIV agents

Agent used in combination	Replicate synergy volumes ($\mu\text{M}^2\%$) ^a			
	UC10	UC040	UC781	UC82
AZT	254, 195	164, 443	232, 113	105, 152
ddI	74, 0	82, 150	47, 36	1, 13
ddC	14, 23	57, 10	132, 120	75, 28
3TC	49, 10	35, 13	81, 93	58, 40
Costatolide	30, 12	97, 103	152, 123	39, 1

^a Synergy volumes were calculated as described previously (26). Combinations of compounds with synergy volumes greater than 100 $\mu\text{M}^2\%$ are considered synergistic, those with volumes ranging from 50 to 100 or 0 to 50 $\mu\text{M}^2\%$ are considered slightly synergistic or additive, respectively, and those with volumes of less than 0 $\mu\text{M}^2\%$ are considered antagonistic. High test concentrations were used for each compound, as follows: UC10, 240 nM; UC040, 150 nM; UC781, 50 nM; UC82, 50 nM; AZT, 100 nM; ddI, 20 μM ; ddC, 160 nM; 3TC, 1.4 μM ; costatolide, 2.5 μM . Synergy volume values represent the results of two individual replicate assays.

assays, synergy volumes were routinely greater than 200 $\mu\text{M}^2\%$. Slightly synergistic interactions were detected when the UC analogs were used in combination with other nucleoside analogs such as ddI, ddC, and 3TC. In these assays, synergy volumes ranged from 50 to 100 $\mu\text{M}^2\%$. Variable results were obtained when each of the four UC analogs was evaluated in combination with the NNRTI costatolide. In these assays, UC10 and UC82 exhibited only additive interactions, with synergy volumes of less than 50 $\mu\text{M}^2\%$. UC040 and UC781, however, exhibited synergistic anti-HIV activities in combination with costatolide. In these assays, synergy volumes ranged from 100 to 150 $\mu\text{M}^2\%$.

Sensitivities of UC compounds to NNRTI-resistant virus isolates. The efficacies of the UC compounds were evaluated against a panel of virus isolates selected in cell culture for resistance to a variety of NNRTIs (Table 3). Although many of the amino acid changes in the RT resulted in a reduction in the overall level of anti-HIV activity achieved with the UC compounds, these data demonstrate that the UC analogs, especially UC781, remained active against the resistant viruses at high nanomolar concentrations. In these studies, the activity of each of the four UC compounds was found to be highly sen-

sitive to the amino acid changes L100I, K103N, and Y181C in the RT. A loss of sensitivity of the UC compounds was also observed, but to a smaller extent, when the compounds were evaluated against virus isolates with the amino acid changes A98G, V108I, and Y188H. The amino acid changes T139I, M184I, and P236L did not have any effect on the activities of the four compounds. The sensitivity profile of each of the four UC compounds appeared to be similar in these assays. Further evaluation of the sensitivity of the compounds to viruses with NNRTI resistance-engendering mutations was performed in assays which measured the activities of the compounds against wild-type NL4-3 virus isolates in which single-amino-acid changes were introduced into the RT of the virus by site-directed mutagenesis (Table 4). In these assays, each of the UC compounds was found to be most sensitive to the L100I amino acid change in the RT. The K103N, V106I, Y181C, and Y188C amino acid changes also reduced the inhibitory activities of the UC compounds. Mutations K101E and V179D yielded some loss of activity with UC10 and UC040, but they had less of an effect on the activities of UC781 and UC82. The activity of each of the compounds against viruses with 3TC (M184V), ddI (L74V), and AZT resistance-engendering mutations was similar to that observed against the wild type. Viruses with the AZT resistance mutations and either the L100I or Y181C mutation on the same genome were found to be resistant to the inhibitory activities of the UC compounds. Finally, the activities of the compounds were evaluated against purified RT possessing single amino acid changes which were introduced by site-directed mutagenesis (Table 5). As previously reported for the class of NNRTIs, the inhibitory concentrations of the UC compounds determined in the homopolymer poly(rC)-oligo(dG) template-primer assay system were much greater than those observed in cell culture (14). In these biochemical inhibition assays, the amino acid changes L100I, K103N, and Y181I were found to have the greatest effect on the activities of the UC compounds. Each of the compounds was inactive against the HIV-2 recombinant RT up to a high test concentration of 100 μM . The loss of inhibitory activity with RTs possessing various other amino acid changes was only slight.

Selection and characterization of UC-resistant virus isolates. Virus isolates resistant to the anti-HIV-1 action of the UC compounds were rapidly selected in cell culture by serial

TABLE 3. Activities of UC compounds against viruses resistant to HIV-1-specific inhibitors

Isolate or inhibitor to which isolate is resistant (mutation) ^a	EC ₅₀ (μM) (fold resistance) ^b				
	UC10	UC040	UC781	UC82	Nevirapine
IIIB (control)	0.2	0.2	0.01	0.008	0.01
Oxathiin carboxanilide (L100I)	2.0 (10)	>2 (>10)	0.5 (50)	0.3 (37)	0.1 (10)
UC10-costatolide (K103N)	7.3 (36)	>2 (>10)	0.9 (90)	1.0 (125)	ND ^c
Thiazolobenzimidazole (V108I)	1.2 (6)	0.9 (S)	0.1 (10)	0.06 (8)	0.3 (30)
TIBO-R82150 (A98G/V108I)	1.1 (5)	1.2 (6)	0.3 (30)	0.2 (25)	0.6 (60)
Calanolide A (T139I)	0.3 (S)	0.4 (S)	0.04 (S)	0.03 (S)	0.01 (S)
Diphenylsulfone (Y181C)	1.1 (5)	>2 (>10)	0.9 (90)	0.2 (25)	5.9 (590)
UC38 (Y181C)	1.3 (6)	>2 (>10)	0.9 (90)	0.2 (25)	ND
Pyridinone (Y181C/L103N)	>100 (>500)	>2 (>10)	>2 (>200)	>2 (>250)	>38 (3,800)
E-BPTU (Y181C)	0.06 (S)	>2 (>10)	0.4 (40)	0.2 (25)	1.9 (190)
3TC (M184I)	0.2 (S)	0.1 (S)	0.03 (S)	0.008 (S)	0.01 (S)
Costatolide (Y188H)	1.6 (8)	1.0 (S)	0.1 (10)	0.07 (8)	ND
HEPT (P236L)	0.2 (S)	0.4 (S)	0.02 (S)	0.02 (S)	0.02 (S)

^a TIBO, tetrahydroimidazo-[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione; E-BPTU, 1-benzylloxymethyl-5-ethyl-6-(α -pyridylthio)uracil; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine.

^b Fold resistance values were calculated by determination of the ratio of the activity of each compound against the drug-resistant virus isolate to the activity against the IIIB isolate (wild-type) control determined in parallel. Fold resistance values indicated as "greater than" indicate that the compound was inactive at the greatest nontoxic concentration tested. Fold resistance values indicated by an S indicate that the virus remained sensitive to the compound.

^c ND, not determined.

TABLE 4. Cross-resistance of viruses with defined amino acid changes against UC analogs

Isolate or mutation	EC ₅₀ (μM) (fold resistance ^a)				
	UC10	UC040	UC781	UC82	Nevirapine
NL4-3	0.08	0.05	0.006	0.006	0.02
L74V	0.07 (S)	0.02 (S)	0.006 (S)	0.006 (S)	0.02 (S)
A98G	0.7 (9)	0.1 (S)	0.02 (S)	0.009 (S)	0.2 (10)
L100I	2.2 (28)	>2 (>40)	0.4 (67)	0.2 (33)	0.1 (5)
K101E	1.0 (12)	0.9 (18)	0.04 (7)	0.006 (S)	0.3 (15)
K103N	4.8 (60)	0.8 (16)	0.1 (17)	0.07 (12)	>2 (>100)
V106I	2.7 (34)	0.7 (14)	0.1 (17)	0.08 (13)	>2 (>100)
V108I	0.2 (S)	0.2 (S)	0.01 (S)	0.006 (S)	0.06 (S)
V179D	0.7 (9)	0.3 (6)	0.008 (S)	0.006 (S)	0.03 (S)
Y181C	0.5 (6)	0.8 (16)	0.08 (13)	0.03 (5)	1.3 (65)
M184V	0.1 (S)	0.1 (S)	0.02 (S)	0.01 (S)	0.1 (S)
Y188C	0.4 (5)	0.6 (12)	0.01 (S)	0.006 (S)	3.2 (160)
4× AZT	0.04 (S)	0.02 (S)	0.006 (S)	0.006 (S)	0.02 (S)
4× AZT + Y181C	0.2 (S)	0.9 (18)	0.2 (33)	0.1 (17)	0.8 (40)
4× AZT + L100I	1.0 (12)	1.0 (20)	0.2 (33)	0.1 (17)	0.03 (S)

^a Fold resistance values were calculated by determination of the ratio of the activity of each compound against the drug-resistant virus isolate to the activity against the NL4-3 isolate (wild-type) control determined in parallel. Fold resistance values indicated as "greater than" indicate that the compound was inactive at the greatest nontoxic concentration tested. Fold resistance values indicated by an S indicate that the virus remained sensitive to the compound.

passage of the IIIB isolate of HIV-1 in CEM-SS cells in the presence of gradually increasing concentrations of the compounds. Within five passages, highly resistant virus isolates were selected. Molecular characterization of the four distinct isolates determined that each possessed the Y181C amino acid change. The UC10-resistant virus isolate also possessed a second amino acid change, K101E, in the RT. As expected, biological cross-resistance analysis of each of the four virus isolates determined that each belonged to subgroup III (6). These viruses were resistant to all of the other NNRTIs evaluated with the exception of the calanolide A analog, costatolide (Table 6). Costatolide was found to exhibit enhanced activity against each of the Y181C-containing, UC-resistant virus isolates. The UC10-resistant virus isolate exhibited a slight loss of sensitivity to costatolide, consistent with the presence of a second mutation (K101E) in the RT.

In light of the extremely broad selectivity index of UC781 (>62,000), resistance selection was continued for an additional 10 passages in cell culture with increasing drug concentrations in order to determine if a virus which would be totally resistant to the antiviral effects of UC781 could be selected. As described above, at passage 5 the virus contained the Y181C amino acid change in the RT, was 50-fold resistant to UC781, and grew well in the presence of 0.1 μM UC781 (10 to 20 times the EC₅₀). At passage 10, the virus remained approximately 55-fold resistant to the compound, but a second mutation had been acquired in the RT (V108I). The virus was grown in the presence of 1 μM UC781 (100 to 200 times the EC₅₀). A second major increase in the level of resistance of the virus occurred between passages 10 and 12. At passage 12 the virus was determined to be nearly 500-fold resistant to UC781. At passage 15 the virus was grown in 20 μM UC781 and was greater than 500-fold resistant to UC781. Upon sequencing we determined that the virus had acquired a third amino acid change in the RT (K101E). These data are summarized in Fig. 2.

DISCUSSION

Three new NNRTIs in the oxathiin carboxanilide series with anti-HIV-1 activity in the low nanomolar range have been

described. The UC analogs UC781, UC82, and UC040 possess all of the general properties of the compounds comprising the pharmacological class of NNRTIs, including activity against laboratory and low-passage-number clinical virus isolates; activity in a variety of established human cells, including T cells, B cells, and monocytes-macrophages; activity in fresh human peripheral blood mononuclear cells and monocytes-macrophages; and synergistic inhibition of HIV-1 when used in combination with AZT (1, 5–7, 18, 20–22, 24, 25, 27, 29). The properties of one of these compounds, UC781, in vitro and in vivo suggest its potential as a candidate for clinical use (4). UC781 is distinguished from other members of the UC series by the furanyl side chain at position R-3 and an extended ether side chain at position R-4. The TI of UC781 (approximately 62,000) is among the greatest known for the entire class of NNRTIs.

The biological studies reported here distinguish UC781 from other NNRTIs and provide a rationale for the further clinical development of the compound. Sensitivity testing of the UC compounds against biological virus isolates selected in cell culture for resistance to a variety of NNRTIs or evaluation against virus isolates with single amino acid changes conferring resistance to NNRTIs confirmed that UC781 exhibited properties similar to those described for other UC compounds (8, 19). These properties include sensitivity to amino acid changes L100I, K103N, and Y181C in the RT and slight sensitivity to amino acid changes A98G, V108I, and Y188H. Amino acid changes T139I, M184I/V, and P236L, as well as the amino acid changes present in the RT of AZT- and ddI-resistant viruses, did not have any detectable effect on the UC analogs. However, the extremely wide TI of UC781 allowed the complete inhibition of all tested resistant virus isolates at nanomolar to low micromolar concentrations with the exception of certain virus isolates (A17) possessing multiple amino acid changes in the RT (Y181C and K103N). These results indicate that sufficiently high, nontoxic concentrations of UC781 may be used to prevent or retard the selection of resistant virus isolates in patients. Selection of drug-resistant virus isolates has clearly demonstrated that the wide selectivity index of UC781 can be slowly eliminated by strategies which use gradually increasing drug concentrations with serial passage in cell culture. After 14 passages in cell culture, the TI of UC781 had been reduced from

TABLE 5. Cross-resistance of purified RT with defined amino acid changes against UC analogs

Enzyme	EC ₅₀ (μM) ^a			
	UC10	UC040	UC781	UC82
HIV-1, RT wild type	5.5	7.5	3.2	0.2
L74V	6.1	7.8	2.0	0.2
L100I	25.0	9.5	10.2	1.1
K103N	>100	>100	22.3	10.0
V106A	12.7	10.1	3.2	0.5
E138K	8.5	38.0	2.1	0.2
Y181I	27.8	35.5	9.5	1.2
Y188L	95.0	>100	>100	35.0
M184V	5.6	8.0	4.2	0.8
P236L	4.1	8.5	1.1	0.3
AZT-21	6.2	8.0	5.2	0.2
HIV-2	>100	>100	>100	>100

^a Inhibition of RT was measured with a poly(rC)-oligo(dG) template primer assay system as described in Materials and Methods. As described previously (14), inhibitory concentrations are consistently higher with a homopolymer template assay system compared to the values obtained by cell culture.

TABLE 6. Cross-resistance phenotype of UC-resistant virus isolates in cell culture

Compound ^a	EC ₅₀ (μM) (fold resistance ^b) for:				
	IIIB (wild type)	UC10-R (Y181C+ K101E)	UC040-R (Y181C)	UC781-R (Y181C)	UC82-R (Y181C)
AZT	0.01	0.2	0.008	0.005 (S)	0.01 (S)
Thiazolobenzimidazole	3.4	>69 (>28)	>69 (>20)	>69 (>20)	>69 (>20)
Oxathiin carboxanilide	1.3	>28 (>56)	6.8 (5)	10.1 (8)	14.2 (11)
TIBO	0.1	>35 (>175)	2.0 (19)	11.8 (118)	4.4 (40)
Costatolide	0.5	4.4 (9)	<0.08 (ES)	<0.15 (S)	0.1 (ES)
Diphenylsulfone	7.0	>114 (>36)	>114 (>16)	>114 (>16)	>114 (>16)
TSAO	0.05	>10 (>200)	>10 (>200)	>10 (>200)	>10 (>200)
α-APA	0.01	>1 (>50)	>1 (>100)	>1 (>100)	>1 (>100)
E-BPTU	0.02	>5 (>500)	2.2 (109)	3.1 (155)	4.1 (207)
UC10	0.2	>20 (>100)	0.8 (4)	0.9 (5)	1.1 (6)
UC040	0.06	>2 (>10)	>2 (>33)	1.6 (27)	>2 (>33)
UC781	0.009	>2 (>200)	1.0 (106)	1.0 (111)	1.1 (120)
UC82	0.005	1.5 (183)	0.1 (22)	0.3 (60)	0.2 (46)

^a TIBO, tetrahydroimidazo-[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-thione; TSAO, 2',5'-bis-*O*-(tetrabutyldimethylsilyl)-β-D-ribofuranosyl-3'-spiro-5''-(4'-amino-1'',2''-oxathiole-2'',2''-dioxide); α-APA, α-anilinophenylacetamide; E-BPTU, 1-benzoyloxymethyl-5-ethyl-6-(α-pyridylthio)uracyl.

^b Fold resistance values were calculated by determination of the ratio of the activity of each compound against the drug-resistant virus isolate to the activity against the IIIB isolate (wild-type) control performed in parallel. Fold resistance values indicated as "greater than" indicate that the compound was inactive at the highest concentration tested. Fold resistance values indicated by an S or an ES indicate that the virus remains sensitive or exhibits greater than fivefold enhanced sensitivity to the compound, respectively.

62,000 to approximately 100 with the accumulation of three amino acid changes in the RT (Y181C, V108I, and K101E). The appropriate selection of additional anti-HIV agents with non-overlapping sensitivity profiles to drug-resistant isolates might allow highly effective combination therapy strategies to be used.

Appropriate selection of additional agents for use in combination with UC781 may further preclude the possibility of the rapid selection of resistance. In this regard, use of a compound such as calanolide A or its analog costatolide would suppress virus isolates with the Y181C mutation in the RT.

The results obtained with combinations of drugs against HIV indicate that additive to synergistic inhibition of HIV replication may be achieved with UC781 and one of the nucleoside analogs, AZT, ddI, ddC, or 3TC. The anti-HIV activities of various combinations of the four UC compounds evaluated indicate that the compounds may interact differently with the RT. This is most evident in the combination interactions of each UC analog with costatolide. Our results indicate that two of the compounds interact with costatolide in an additive fashion, while two others interact in a synergistic fashion. Although

FIG. 2. Selection of UC781-resistant virus. The fold resistance of virus pools obtained at each sequential passage in cell culture was determined by evaluation of the EC₅₀ of UC781 for the resistant virus and was compared to the activity of UC781 against wild-type IIIB virus. At passages 5, 10, and 14, the virus was sequenced, and the amino acid changes in the RT are indicated.

we have found that most NNRTI combinations result in additive anti-HIV activity, we have recently shown that combination of two NNRTIs within the Uniroyal series sometimes results in synergistic inhibition (8). Fletcher et al. (13, 14) have suggested that this may occur when the NNRTIs interact with different mechanistic forms of the RT. They proposed that synergistic inhibition of HIV-1 (both virus and RT) was observed due to the interaction of UC84 with the RT-primer-template complex, while UC38 interacted with the RT-primer-template-deoxynucleoside triphosphate ternary complex (14). Identification of NNRTIs that interact synergistically *in vitro* may result in the identification of drug combinations that have more potent anti-HIV activity in patients (13, 14). In addition to its other favorable properties, UC781 interacts synergistically with costatolide, and this combination of anti-HIV agents may therefore have practical therapeutic benefit.

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