Structure-Activity and Cross-Resistance Evaluations of a Series of Human Immunodeficiency Virus Type 1-Specific Compounds Related to Oxathiin Carboxanilide

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A series of compounds related to the nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) oxathiin carboxanilide (UC84) were evaluated for activity against the human immunodeficiency virus (HIV) to determine structural requirements for anti-HIV activity. Twenty-seven compounds representative of the more than 400 Uniroyal Chemical Company (UC) compounds were evaluated for structure-activity relationships. Several of the compounds evaluated were highly active, with 50% effective concentrations in the nanomolar range and therapeutic indices of >1,000. Highly synergistic anti-HIV activity was observed for each compound when used in combination with 3'-azido-3'-deoxythymidine; additive to slightly synergistic interactions were observed with the compounds used in combination with dideoxycytidine. In combination with the NNRTI costatolide, only UC38 synergistically inhibited HIV type 1. Residues in the RT which, when mutated, impart resistance to the carboxanilide compounds were defined by evaluation of the UC compounds against a panel of NNRTI-resistant virus isolates selected in cell culture, against virus variants with site-directed mutations, and against RTs containing defined single amino acid changes. The mutations included changes in RT amino acids 100, 101, 103, 106, 108, and 181. The results with isolates selected in cell culture indicate that the carboxanilide compounds interact with the RT at two vulnerable sites, selecting UC-resistant virus isolates with the Y-to-C mutation at position 181 (Y181C) or the L100I substitution. A resistant virus isolate containing both Y181C and K101E amino acid changes and another with both Y181C and V106A mutations were isolated. In combination with calanolide A, an NNRTI which retains activity against virus isolates with the single Y181C mutation, UC10 rapidly selected a virus isolate with the K103N mutation. The merits of selecting potential candidate anti-HIV agents to be used in rational combination drug design as part of an armamentarium of highly active anti-HIV compounds are discussed.

The serious toxicities associated with the administration of nucleoside reverse transcriptase (RT) inhibitors (42, 53, 54), as well as the rapid emergence of viruses resistant to these drugs (30, 47), have emphasized the urgent need to identify and develop new chemical and pharmacologic classes of anti-human immunodeficiency virus (anti-HIV) agents for clinical evaluation, alone or in combination with other active anti-HIV agents. The highly diverse pharmacologic class of nonnucleoside RT inhibitors (NNRTIs) has provided a variety of drug candidates which possess great potency and wide therapeutic indices (1, 9, 10, 12, 27, 28, 31, 32, 36, 39, 44, 52). The NNRTIs inhibit HIV type 1 (HIV-1) RT by a highly specific interaction with the enzyme at the NNRTI binding site in the palm domain of the p66 subunit of the RT. The high degree of specificity and potency of the NNRTIs, however, are compromised by the rapid appearance of drug-resistant viruses containing single amino acid changes which confer insensitivity to the antiviral

effects of the compounds (for a review, see reference 34). Viral isolates cross-resistant to the NNRTIs have been obtained by tissue culture or site-directed mutagenesis (3, 4, 20, 35, 37, 41, 51) as well as from patients (43, 45). Sequence analysis of a large number of virus isolates has identified resistance-engendering mutations at amino acid positions 98, 100, 103, 106, 108, 138, 179, 181, 188, 190, and 236 (35). In the crystal structure of the RT (26, 29), these amino acid changes correspond to the deep hydrophobic pocket near the polymerase active site in the RT p66 palm subdomain. This is the site at which the different HIV-1-specific inhibitors interact (17, 18, 24, 46). The effects of some of these resistance-engendering mutations on the efficacy of both HIV-1-specific and nucleoside RT inhibitors have been recently reported (15, 16).

The NNRTI compound oxathiin carboxanilide (UC84) (1) and an analog, UC38 (32), were synthesized by the Uniroyal Chemical Company, Ltd. (UC), Guelph, Ontario, Canada, and identified as potent anti-HIV agents through the high-capacity anti-AIDS screening program of the National Cancer Institute (NCI) (7, 50). The significant activity of these two compounds against HIV-1 led to the synthesis of a large number of analogs

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in an effort to improve on the antiviral activity and in vivo stability of the compounds. A recent report probed the structure-activity relationships of a subset of the Uniroyal compounds which possessed ester linkages at one of the substitution sites (2). The data presented in this report reflect the structural requirements of the compounds which are essential for anti-HIV potency, including substitutions at four different positions (see Fig. 1). These substitutions involved (i) methyl and benzyl derivatives in the R_1 position, (ii) sulfur in the R_2 position, (iii) four different derivatives with changes at the R_3 position (oxathiin, thiocarbamate, furan, and thiophene analogs), and (iv) ether, ester, and oxime derivatives with changes at the R_4 position. In addition, the interactions of the UC compounds with other nucleoside and nonnucleoside anti-HIV agents were explored in combination drug studies. Finally, the effects of structural modifications in the compounds on the activity against viruses and RTs with specific NNRTI resistance-engendering amino acid changes have been evaluated. The potential use of UC analogs as part of an armamentarium of compounds against HIV is discussed below.

MATERIALS AND METHODS

Compounds. Antiviral compounds were prepared by chemists at the UC (25) and provided to the NCI. Each compound structure is identified by a unique UC number (see Tables 1 and 2).

Cells and viruses. The CEM-SS cells used in these evaluations were obtained from the NIAID AIDS Research and Reference Reagent Program. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml). The HIV-1 isolate IIIB and the nevirapine-resistant isolate N119 were also obtained from the NIAID AIDS Research and Reference Reagent Program (41), while the pyridinone-resistant isolate A17 was obtained from E. Emini at Merck Sharp & Dohme Laboratories (West Point, Pa.) (37). Virus isolates resistant to a variety of NNRTIs were selected in cell culture (10) or were constructed by site-directed mutagenesis (15).

Materials. All experimental antiviral agents were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, NCI, or from the NIAID AIDS Research and Reference Reagent Program. Crystalline stock materials were stored at -70°C and solubilized in 100% dimethyl sulfoxide. The reference anti-HIV compounds used in these studies were 3'-azido-2',3'-dideoxythymidine (AZT) (NSC 602670), 2',3'-dideoxycytidine (ddC) (NSC 606170), and nevirapine (NSC 641530). Materials required for the performance of RT inhibition assays and anti-HIV assays and for the growth and maintenance of established cells have been previously described (9). RTs with single amino acid changes introduced by site-directed mutagenesis were obtained from P. Boyer and S. Hughes at the ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center.

Anti-HIV assays. The HIV-inhibitory activity of the compounds was evaluated as described previously (9) in a microtiter anti-HIV assay which quantitates the ability of a compound to inhibit HIV-induced cell killing or HIV replication. 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 [EC_{50}]) and the quantity of drug required to reduce cell viability by 50% (50% inhibitory concentration [IC_{50}]).

Combination antiviral analysis. Analysis of drug combinations was performed by the anti-HIV assay method described above with statistical evaluations performed according to the method of Prichard and Shipman (40). Combination antiviral assays were performed with CEM-SS cells, utilizing the IIIB strain of virus as previously described (14). Five concentrations of the UC compounds were tested in all possible combinations with eight concentrations of AZT, ddC, or costatolide. Effects of the drug combination were calculated on the basis of the activities of the two compounds when tested alone. The expected additive antiviral protection was subtracted from the experimentally determined antiviral activity at each combination concentration, resulting in a positive value (synergy), a negative value (antagonism), or zero (additivity). Data were analyzed by the most stringent statistical means by assuming that the compounds inhibited HIV replication by action at the same site (mutually exclusive), although it is known that the nucleoside RT inhibitors and NNRTIs bind at different sites on the RT enzyme and do not compete with one another for binding to the RT enzyme. The results of the combination assays are presented three-dimensionally for 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. For these studies, synergy is defined as drug combinations yielding synergy volumes of $>50 \mu\text{M}^2\%$. Slightly synergistic activity and highly synergistic activity have been defined as yielding

FIG. 1. (A) Chemical structure of oxathiin carboxanilide (UC84; NSC 615985). (B) Basic structure of UC analogs.

synergy volumes of 50 to 100 and $>100 \mu\text{M}^2\%$, respectively. Synergy volumes between 0 and $50 \mu\text{M}^2\%$ are considered additive, and synergy volumes of $<0 \mu\text{M}^2\%$ are considered antagonistic.

Generation of drug-resistant virus strains and analysis of cross-resistance. Resistant virus isolates were generated 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 EC_{50} 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. Each resistant virus isolate was obtained in three to five passages. Upon attainment of a drug-resistant virus strain, cross-resistance testing was performed according to the methods described for the performance of antiviral assays. Resistance has been defined in this study as a ≥ 5 -fold increase in EC_{50} in comparison with the wild-type (IIIB) isolate.

RT enzyme inhibition assays. Evaluation of the activity of compounds using homopolymer and heteropolymer templates was performed as previously described (8).

RESULTS

Structural requirements for anti-HIV activity. Oxathiin carboxanilide (UC84; NSC 615985), an analog of the fungicide Vitavax and a potent inhibitor of HIV-1 replication, was the prototype of over 1,200 compounds chemically synthesized by the UC. Structurally, UC84 consists of an oxathiin-3-carboxylic acid coupled to an *m*-amidobenzoate by an amide linkage (Fig. 1A). UC84 has an EC_{50} of $0.29 \mu\text{M}$, an IC_{50} of $180 \mu\text{M}$, and a respectable 50% therapeutic index (TI_{50}) of 620 (Table 1).

The structure of the UC compounds related to UC84 can be divided into five distinct substitutable regions (R_1 through R_5), as shown in the generic structure presented in Fig. 1B. Each of these regions can be analyzed to determine structure-activity relationship, as shown for the 27 selected compounds representing the five series of UC analogs presented in Tables 1 and 2. In this study, we investigated over 400 UC compounds with substitutions at R_1 , R_2 , R_3 , and R_4 ; chlorine at R_5 was *para* to the nitrogen and remained constant in all of the compounds evaluated except for two, UC39 and UC40. Table 1 summarizes the structure-activity relationship based on the substitution of the nitrogen, R_1 . These substitutions affected the steric features and hydrogen-bonding capability of the molecules, which significantly affected the inhibitory efficacy of the respective analogs. UC31, having a hydrogen at the R_1 site and a methoxy benzene substitution at R_3 , was an active anti-HIV agent, exhibiting an EC_{50} of $1.3 \mu\text{M}$, an IC_{50} of $380 \mu\text{M}$, and a TI_{50} of 292 (Table 1). However, replacing the R_1 hydrogen at the nitrogen with either a methyl (UC34 and UC62) or a benzyl (UC800) group abolished all anti-HIV activity.

In another series of analogs, substituting the oxygen at R_2 with a sulfur generally enhanced the anti-HIV activity (Table 2, compare UC84 with the thiolated UC23). Hence, a series of several hundred analogs bearing the thiolated R_2 but differing in R_3 and R_4 substitutions were synthesized. Most of these

TABLE 1. Anti-HIV activities of UC compounds bearing substitutions at the nitrogen, R₁, and R₃ of the amidobenzene

UC compound (NSC no.)	Substitution ^a		Anti-HIV activity		
	R ₁	R ₃	EC ₅₀ (μM)	IC ₅₀ (μM)	TI ₅₀
UC84 (615985)	H		0.29	180	620
UC34 (632780)	CH ₃		None (inactive)	72	ND ^b
UC31 (624575)	H		1.3	380	292
UC62 (628536)	CH ₃		None (inactive)	38	ND
UC800 (630922)			None (inactive)	17	ND

^a R₂, R₄, and R₅ remain the same in this series of UC compounds.^b ND, not determined.

analogues were found to be inhibitors of HIV replication. Substitutions at the R₃ position (Table 2) with an ether group in the thiocarbamates (UC32, UC33, UC38, and UC581), with a methyl furanyl group in the furans (UC05, UC10, UC20, UC25, UC39, UC40, UC57, UC68, UC70, UC80, and UC538), or with a sulfur in the thiophenes (UC30, UC69, UC71, UC77, and UC939) increased the inhibitory efficacy to a significant extent. Among these select thiolated UC analogues having EC₅₀s in the 20 to 45 nM range and TI₅₀s of >1,000 (Table 2), UC05, UC20, UC23, UC25, UC40, UC57, UC70, and UC80 are considered highly potent anti-HIV agents. Substitutions at the R₄ position of these compounds may include ethers, oxime ethers, esters, or hindered esters. UC compounds carrying the R₄ ester substitution (UC20, UC25, UC68, UC70, UC71, UC79, UC84, and UC538) were highly active, a finding that is in agreement with a recent report on other oxathiin compounds with ester linkages (2). Modifications at the R₄ with a halogen substitution (UC80), with cyclopropylethyl derivatives (UC20 and UC70), or with oxime derivatives (UC30, UC32, UC57, UC77, and UC81), as well as other substitutions not shown in Table 2, enhanced the anti-HIV activity.

After analysis of the anti-HIV activity of over 400 UC compounds, it became evident that substitutions at R₁, R₂, R₃, and R₄ positions in the basic structure (Fig. 1B) created diverse analogues with various degrees of antiviral activity. Substitution of the hydrogen at the R₁ position resulted in complete loss of anti-HIV efficacy (Table 1, UC34, UC62, and UC800). Substitution of the oxygen with sulfur at the R₂ position enhanced anti-HIV activity (Table 2). A 2-methyl-3-furanyl substitution at the R₃ position enhanced the anti-HIV activity of the compound. Some of these furanyl UC compounds (UC05, UC10, UC20, UC25, UC39, UC40, UC57, UC70, and UC80) with diverse substitutions at R₄ were found to be potent NNRTIs. The length and complexity of the substitution at R₄ did not affect the inhibitory activity but, rather, tended to increase the therapeutic index of the compound. Substitution of the oxygen in the furan ring of R₃ with a sulfur (i.e., the thiophenes: UC30,

UC69, UC71, UC77, and UC989) slightly increased the EC₅₀s. Substitution of the oxygen of the furan with a methylated nitrogen (i.e., a methyl pyrrole), as shown for UC79, decreased the potency to a degree similar to that observed with the thiophenes. However, all of these UC compounds showed effective inhibition of HIV-1, with EC₅₀s of approximately 100 nM.

All of the UC compounds evaluated here were inactive against HIV-2 and simian immunodeficiency virus.

Activity of compounds in combination with other anti-HIV agents. A subgroup of compounds which were evaluated in the structure-activity relationships study was selected for further biological anti-HIV analysis. Initially, the ability of these selected compounds to inhibit HIV-1 when used in combination with nucleoside RT inhibitors and NNRTIs was evaluated. A summary of the results of these assays is presented in Table 3. These combination anti-HIV activity results confirm previously reported observations that the use of NNRTIs in combination with AZT results in synergistic inhibition of HIV-1 replication. The synergy volumes obtained in these assays vary from compound to compound, but nearly all of the drug combinations were characterized as significantly synergistic (>100 μM²%). UC10, UC38, UC40, UC84, and UC57 yielded the greatest synergy volumes when evaluated in combination with AZT (>250 μM²%). Further evaluation of each of these selected compounds in combination with the nucleoside analog ddC resulted in only additive to slightly synergistic anti-HIV activity. Similar results were obtained with dideoxyinosine (ddI) (data not shown). In these assays, the synergy volumes obtained rarely were >100 μM²%. The compound UC10, which was also highly synergistic with AZT, yielded the greatest level of synergy when used in combination with ddC. Only UC38 and UC69 exhibited synergistic anti-HIV activity when used in combination with the NNRTI costatolide (200 to 300 μM²%). UC38 also exhibited slightly synergistic anti-HIV activity (synergy volume of 61 μM²%) when used in combination with UC84 (data not shown). In combination with costatolide, most of the compounds exhibited additive anti-HIV activity with synergy volumes of <50 μM²%. Antagonism was not detected in any of the combination anti-HIV assays involving the carboxanilide derivatives. In the control assays performed in parallel, AZT was found to synergize with ddC and costatolide (Table 3).

Activity of compounds against drug-resistant virus isolates. Initial evaluation of activity in the primary anti-HIV screen involved identification of EC₅₀s and IC₅₀s for each of the compounds against wild-type virus, as well as evaluation of activity against resistant virus isolates possessing the Y181C mutation in the RT. The UC compounds which exhibited some level of antiviral activity against the drug-resistant virus isolate were subjected to further evaluation. Studies were performed to analyze the activity of the compounds against diverse NNRTI-resistant virus isolates selected in cell culture (10) as well as against virus isolates with defined single amino acid changes in the RT introduced by site-directed mutagenesis. The data presented in Tables 4 and 5 indicate that a variety of amino acid changes in the RT result in substantial loss of sensitivity to the tested carboxanilide derivatives. The most detrimental mutation (as determined by the fold resistance relative to the activity of the compound against wild-type virus isolate IIIB or NL4-3) was the L100I mutation present in both the UC84-resistant virus isolate (Table 4) and the L100I virus derived by site-directed mutagenesis (Table 5). At the high test concentrations employed (or the greatest nontoxic drug concentrations), antiviral activity of many of the UC compounds against viruses with the L100I mutation was not detectable.

TABLE 2. Anti-HIV activities of UC compounds with substitutions at R₂, R₃, R₄, and R₅^a

UC compound (NSC no.)	Substitution				Anti-HIV activity		
	R ₂	R ₃	R ₄	R ₅	EC ₅₀ (μM)	IC ₅₀ (μM)	TI ₅₀
UC84 (615985)	0			Cl	0.290	180	620
UC23 (638521)	S			Cl	0.024	70	2,938
UC38 (629243)	S			Cl	0.050	16	319
UC33 (650059)	S			Cl	1.180	113	96
UC581 (645727)	S			Cl	0.013	2	156
UC32 (645542)	S			Cl	0.008	2	267
UC05 (645124)	S			Cl	0.018	21	1,167
UC10 (645129)	S			Cl	0.049	20	416
UC57 (647014)	S			Cl	0.011	16	1,420
UC68 (638532)	S			Cl	0.012	0.5	43
UC538 (645548)	S			Cl	0.032	13.2	414
UC80 (639475)	S			Cl	0.011	19	1,770

^a The hydrogen at R₁ remains constant.

Continued on following page

However, UC10, UC25, UC30, UC57, UC68, and UC77 exhibited some residual activity against virus isolates with the L100I amino acid change. Significant loss of activity against viruses with the Y181C mutation, which include the UC38-resistant virus as well as the nevirapine-, diphenylsulfone-, and HEPT (1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine)-resistant virus isolates (Table 4) and the corresponding Y181C virus in Table 5, was also detected. With most of the compounds evaluated, activity against virus isolates with the Y181C mutation was detectable, but at higher effective

concentrations. Mutations A98G, K101E, K103N, V106A, and V179D also resulted in some loss of activity with most of the UC compounds. The presence of both the Y181C and the K103N mutations in the RT of the pyridinone-resistant (A17) virus isolate resulted in complete or partial resistance to the inhibitory activity of the compounds against the virus. Mutation at position 236 (P236L) had no detrimental effect on the activity of the UC compounds. Several compounds exhibited enhanced activity against virus isolates with the P236L mutation (i.e., UC33, UC70, and UC32). Similarly,

TABLE 2—Continued

UC compound (NSC no.)	Substitution				Anti-HIV activity		
	R ₂	R ₃	R ₄	R ₅	EC ₅₀ (μM)	IC ₅₀ (μM)	TI ₅₀
UC20 (647024)	S			Cl	0.020	20	1,000
UC70 (638534)	S			Cl	0.022	54	2,455
UC25 (650058)	S			Cl	0.026	43	1,680
UC39 (650064)	S		-O-CH ₂ -CH=CH ₂	-O-C	0.080	118	1,500
UC40 (650065)	S		-O-CH ₂ -CH=CH-CH ₃	-O-C	0.045	82	1,820
UC69 (646989)	S		-O-CH ₂ -CH=CH ₂	Cl	0.235	22	95
UC77 (668859)	S		-CH=N-O-	Cl	0.071	26	408
UC30 (645540)	S		-CH=N-O-	Cl	0.083	14	172
UC71 (648360)	S			Cl	0.159	24	153
UC939 (668856)	S		-CH=N-O-	Cl	0.095	11	119
UC79 (648344)	S			Cl	0.130	18	132

mutation at position 108 (V108I) had little, if any, effect on the activity of the compounds.

Anti-HIV evaluations were also performed with virus isolates possessing mutations conferring resistance to AZT, with or without the Y181C or L100I mutation in addition to the AZT resistance mutations. The UC compounds were uniformly active against the AZT-resistant virus isolate (Table 5). Addition of the Y181C mutation to the AZT-resistant genetic background resulted in a slight loss of activity of the compounds but not as extensive as that observed with the Y181C mutation alone. The presence of the L100I mutation with the AZT-resistant background still resulted in substantial loss of activity of the UC compounds, similar to that observed with the L100I virus alone.

Activity of compounds against RT with resistance mutations. A subgroup of the carboxanilide compounds selected on the basis of either their high therapeutic index or their residual activity against the resistant virus isolates was further evaluated for activity in biochemical RT inhibition assays. RTs with defined single amino acid changes introduced by site-directed mutagenesis were used to further evaluate the effect of specific mutations on the activity of the compounds. Results obtained with the RT assay (Table 6) were similar to those reported in Tables 4 and 5. RT with the L100I mutation has the greatest level of resistance to the UC compounds. In all of the assays performed, the presence of the amino acid change L100I, Y181C, K103N, or V106A resulted in significant loss of activity against the RT. Variable effects on the activity of the com-

TABLE 3. Activities of UC compounds in combination with other anti-HIV agents

Compound (NSC no.)	Synergy vol ($\mu\text{M}^2\%$) ^a		
	AZT	ddC	Costatolide
UC10 (645129)	254	141	13
UC25 (650058)	79	0	43
UC32 (645542)	141	58	0
UC33 (650059)	207	26	43
UC38 (629243)	274	22	322
UC40 (650065)	281	14	56
UC57 (647014)	523	23	30
UC69 (646989)	90	14	123
UC70 (638534)	212	118	73
UC581 (645727)	194	28	30
UC84 (615985)	254	67	50
AZT	0	219	223

^a Synergy volumes were calculated at the 95% confidence interval as the area under the synergistic peaks in the three-dimensional model of Prichard and Shipman (40) as described in Materials and Methods. In this study, antagonistic, additive, slightly synergistic, and highly synergistic interactions have been defined as yielding synergy volumes of 0, 0 to 50, 50 to 100, and $>100 \mu\text{M}^2\%$, respectively.

pound were observed with the V108I and the Y188H amino acid changes. Mutation P236L had little effect on RT-inhibitory activity. With several compounds, greater inhibition of the P236L RT than of the wild-type enzyme was observed, similar to the enhanced activity against the P236L virus (i.e., UC70) detected in the cytopathic cell killing assay (Tables 4 through 6). In general, results from RT inhibition assays correlated well with the results from cytopathicity assays with all of the selected UC compounds in this study.

Selection and characterization of resistant virus isolates. To further explore the interactions of the carboxanilide compounds with the RT, virus isolates resistant to nine of the UC compounds, including UC84 and UC38, were selected in cell

culture (Table 7). As with other NNRTIs, virus isolates significantly resistant to the selecting compounds were produced after a limited number of passages (three to five) in cell culture. These viruses were examined for cross-resistance to other NNRTIs (i.e., calanolide A and E-BPTU [1-benzylloxymethyl-5-ethyl-6-(α -pyridylthio)uracil]) in order to define the NNRTI-resistant virus subgroup to which they belonged. The properties of the NNRTI-resistant virus subgroups and the mutations responsible for resistance in each group have been reported previously (10). The data obtained (not shown) place the isolates in subgroup I or III, indicating that the mutations selected included Y181C (subgroup III) or L100I (subgroup I). Sequencing of the RT of each resistant virus isolate confirmed the presence of these mutations (Table 7). Of the 11 isolates selected in cell culture, 7 possessed the Y181C amino acid change and 2 possessed the L100I change. Isolates possessing the Y181C change remained sensitive to calanolide A; isolates with the L100I change were resistant to all of the NNRTIs evaluated. Two isolates possessed both the Y181C and the K101E mutations, and a third isolate possessed both the Y181C and the V106A mutations. Both sets of mutations yielded virus which was cross resistant to all of the NNRTI compounds tested, as defined by an increase in EC_{50} of ≥ 5 -fold. Selection with UC84 and UC38 was performed twice in order to evaluate the reproducibility of the selection process. With UC84, one selection resulted in a virus isolate with the L100I mutation while the second selection resulted in a virus isolate with the Y181C mutation. UC38 yielded two virus isolates with the Y181C mutation.

Selection of virus isolates resistant to two NNRTIs. The compound UC10 was selected on the basis of its antiviral and pharmacologic properties for further evaluation of its abilities to inhibit HIV replication and to limit or retard the appearance of drug-resistant virus isolates in cell culture when used in combination with a second NNRTI. On the basis of cross-resistance evaluations, the second NNRTI chosen was costatolide or calanolide A. In each case, a virus which was resistant

TABLE 4. Activities of UC compounds against NNRTI-resistant virus isolates

Compound (NSC no.)	EC_{50} (μM) with the indicated isolate									
	IIIB (wild type)	Group I			Group II, CALO (T139I)	Group III				Group IV, HEPT (P236L)
		THZ (V108I)	TIBO (A98G/V108I)	OC (L100I)		HEPT (Y181C)	DPS (Y181C)	UC38 (Y181C)	A17 (Y181C/K103N)	
UC05 (645124)	0.14	1.3	0.35	5.4	0.4	1.0	0.4	6.8	>100	0.4
UC10 (645129)	0.2	1.2	1.1	2.0	0.3	0.6	1.1	1.3	>100	0.2
UC25 (650058)	0.01	0.16	2.4	4.9	0.02	1.7	4.8	2.0	>100	0.01
UC30 (645540)	0.1	0.5	0.1	2.5	0.2	0.07	1.4	1.9	>100	0.2
UC32 (645542)	0.4	6.4	6.4	>100	1.6	5.2	19.9	13.0	>100	0.09
UC33 (650059)	0.5	5.5	>100	>100	1.5	>100	>100	>100	>100	0.05
UC38 (629243)	0.2	3.3	8.2	>38	0.6	5.2	8.5	7.3	>38	0.6
UC39 (650064)	0.3	1.7	1.6	>100	0.5	0.8	8.5	11.2	>100	0.5
UC40 (650065)	0.01	0.12	2.2	13.2	0.03	2.1	5.8	17.5	>100	0.01
UC57 (647014)	0.09	0.4	0.5	5.6	0.1	0.8	1.6	2.0	>100	0.1
UC68 (638532)	0.07	0.5	0.4	2.7	0.2	0.4	0.7	0.3	>100	0.1
UC69 (646989)	0.8	6.7	>30	>100	1.7	>30	>100	>100	>100	0.2
UC70 (638534)	0.02	0.35	0.44	7.6	0.04	0.08	0.2	0.3	>100	0.006
UC77 (668859)	0.07	0.4	0.03	1.7	0.1	0.3	0.5	0.6	>100	0.1
UC79 (648344)	0.06	1.4	0.1	>100	0.4	0.06	5.7	>100	>100	0.2
UC80 (639475)	0.05	0.6	0.3	10.7	0.09	0.4	0.4	0.9	>100	0.02
UC84 (615985)	0.8	10.1	>28	>28	1.6	3.9	7.6	12.4	>28	1.4
UC581 (645727)	0.1	2.0	4.4	>100	0.3	1.8	5.3	6.2	>100	0.4
Nevirapine	0.01	0.3	0.6	0.1	0.01	1.9	5.9	ND ^a	>3	0.02
AZT	0.003	0.003	0.003	0.01	0.006	0.003	0.003	0.004	0.003	0.003

^a ND, not determined.

TABLE 5. Activities of UC compounds against panel of virus isolates with site-directed mutations in the RT

Compound (NSC no.)	EC ₅₀ (μM) for the indicated isolate												
	NL4-3 (WT) ^a	L74V	A98G	L100I	K101E	K103N	V106A	V108I	V179D	Y181C	4×AZT ^b	4×AZT/ L100I	4×AZT/ Y181C
UC10 (645129)	0.08	0.07	0.7	2.2	1.0	4.8	2.7	0.2	0.73	0.5	0.04	1.0	0.2
UC25 (650058)	0.03	0.03	0.03	0.6	0.7	5.5	0.6	0.09	0.6	1.2	0.03	0.9	0.4
UC33 (650059)	0.05	0.06	1.7	5.0	7.3	>33	>33	2.0	2.3	>33	0.5	>33	1.3
UC38 (629243)	0.09	0.09	1.3	>16	>16	>16	31.0	0.6	0.9	3.5	<0.1	>16	1.9
UC40 (650065)	0.03	0.03	0.03	0.6	0.6	3.2	0.6	0.09	0.6	1.4	0.03	0.7	4.2
UC57 (647014)	0.007	0.01	0.4	1.3	0.9	6.8	0.8	0.1	0.66	0.4	0.03	1.3	0.3
UC70 (638534)	0.05	0.05	0.1	1.1	0.9	1.4	1.1	0.08	0.5	0.2	0.04	2.0	0.11
UC84 (615985)	0.6	0.6	2.2	17.0	>28	>28	2.0	1.7	2.2	3.4	0.5	6.7	1.7
AZT	0.003	0.03	0.01	0.003	0.004	0.16	0.004	0.01	0.005	0.01	>5.0	0.1	0.1
Nevirapine	0.02	0.02	0.2	0.1	0.3	>2.0	>2.0	0.06	0.03	1.3	0.02	0.03	0.8

^a WT, wild type.^b Mutations present in the 4×AZT virus isolate include D67N, K70R, T215Y, and K219Q.

to both compounds was isolated in a limited number of passages (five). Cross-resistance evaluation of the two-drug-resistant isolate demonstrated resistance to all NNRTIs tested (data not shown). By sequencing of the RTs of these two virus isolates, a single amino acid change at position 100 (L100I) was found (data not shown).

DISCUSSION

Compounds UC84 and UC38 were previously identified through the NCI's high capacity anti-AIDS drug screening program as highly active and specific inhibitors of HIV replication (1, 33) with similarity to a variety of structurally diverse molecules previously reported (1, 9, 10, 12, 23, 27, 28, 31, 32, 36, 39, 44, 52). To improve upon the activity of the two lead compounds, a third generation of analogs was synthesized and evaluated for activity. Structure-activity relationships define the central amidobenzene portion of the molecule, which serves as the backbone of the compounds and acts as a hinge to allow proper fit of the compound into the NNRTI binding pocket. Three-dimensional models of the RT suggest that the leucine at codon 100 protrudes into the entrance of the binding pocket, providing a hydrophobic environment and an opportunity for hydrogen bonding via a water bridge between the amino acid and the inhibitor (38, 49). The active UC compounds presented in Table 2 all behave like UC84, which is capable of forming the appropriate interactions with the NNRTI binding site. Among these active UC compounds, a sulfur substituent at R₂ of the analog caused a significant increase in the inhibitory activity. Furthermore, compounds with a furan substituent at R₃ showed even greater anti-HIV efficacy and wide therapeutic indices. Thiophene and methyl pyroly substitution at R₃ yielded active anti-HIV compounds but with relatively more toxicity than the furan analogs. The complexity of the R₄ group among these UC analogs did not affect the reactivity of the compound. It was observed that compounds such as UC05, UC10, UC20, UC57, UC70, and UC80, which possessed significant anti-HIV efficacy and wide therapeutic indices as well as reasonable stability, have R₄ substituents resistant to tissue esterase enzymatic degradation. Balzarini et al. have recently reported on the anti-HIV-1 activity of a series of UC compounds with ester linkages; their reported anti-HIV activities were in agreement with those reported here (2). The halogenated derivatives, the cyclopropylethyl derivatives, and the oxime derivatives within this series were far more stable UC compounds and equally or more active against HIV, with high therapeutic indices. The com-

pounds reported here may therefore be considered better potential therapeutic candidates than the ester derivatives of the UC compounds reported elsewhere (2).

The compounds selected for evaluation as part of this study were all determined to have some activity, albeit low, against virus isolates possessing the Y181C amino acid change in the RT. Further analysis of the compounds yielded a consistent definition of the effects of various amino acid changes on the activity of the compounds. Amino acid changes L100I and Y181C have major effects on the activity of the UC compounds; amino acid changes K101E, K103N, V106I, and V179D have significant effects on the activity of the majority of the compounds. In a similar study of UC compounds containing ester linkages, it was reported that the activities of UC compounds were highly sensitive to virus isolates possessing mutations at positions 100, 106, 138, and 181 (2). Amino acid changes at positions 108 and 236, as well as the novel mutations V90I and T139I present in the RT of the calanolide A-resistant virus isolate, had no effect on the anti-HIV activity of the carboxanilide compounds. Consistent with these results, the selection of virus isolates resistant to the carboxanilide compounds yielded predominantly virus isolates with mutations Y181C and L100I in the RT. In a separate report, five structurally related thiophene and furan analogs of the UC compounds were found to select for amino acid changes at positions 100, 101, 103, and 141 (6).

Studies with a variety of NNRTIs, including those presented here, clearly demonstrate that the administration of single

TABLE 6. Activities of selected UC compounds against bacterially expressed wild-type or mutant RT

Compound (NSC no.)	EC ₅₀ (μM) for the indicated RT ^a							
	Wild type	L100I	K103N	V106A	V108I	Y181C	Y188H	P236L
UC10 (645129)	13	>50	>50	39	ND	37	17	15
UC38 (629243)	13	>50	>50	38	47	>50	>50	6
UC57 (647014)	6	>50	>50	20	>50	>50	7	6
UC70 (638534)	6	>50	>50	>50	23	>50	>50	12

^a Assay performed by using poly(rC):oligo(dG) template-primer system as described in Materials and Methods. EC₅₀s were determined from log regression curve fitting of the experimental data.

TABLE 7. Selection of virus isolates resistant to UC compounds

Compound (NSC no.)	Passage no.	Concn (μ M)	Codon change	Amino acid change	Fold resistance
UC84 (615985)	6	2.8	TTA→ATA	L100I	>333
UC84 (615985)	5	28	TAT→TGT	Y181C	>118
UC38 (629243)	6	2.0	TAT→TGT	Y181C	8
UC38 (629243)	5	1.4	TAT→TGT	Y181C	149
UC10 (645129)	6	2.6	TAT→TGT	Y181C	200
			AAA→GAA	K101E	
UC80 (639475)	6	1.1	TAT→TGT	Y181C	18
UC70 (638534)	6	1.4	TTA→ATA	L100I	758
UC57 (647014)	6	2.8	TAT→TGT	Y181C	58
			AAA→GAA	K101E	
UC68 (638532)	6	1.1	TAT→TGT	Y181C	5
UC69 (646989)	6	5.0	TAT→TGT	Y181C	166
			GTA→GCA	V106A	
UC32 (645542)	6	4.0	TAT→TGT	Y181C	38
UC581 (645727)	6	1.5	TAT→TGT	Y181C	53

NNRTIs to patients will not be therapeutically useful because of the rapid selection of drug-resistant virus isolates. Populations of virus which naturally exist both *in vitro* and *in vivo* with amino acid changes allowing replication in the presence of the NNRTIs have been demonstrated (48). The presence of pre-existing populations of drug-resistant virus isolates indicates that therapy with NNRTIs would require their use in combination with other anti-HIV agents, as well as high concentrations to avoid virus breakthrough. The selection of appropriate combinations of NNRTIs might be made by careful analysis of cross-resistance data (10). The results presented here suggest that the UC compounds might be therapeutically useful in combination with the NNRTI calanolide A (or its analog costatolide) (27) or with the HEPT analog NSC 648400 (E-BPTU) (10). *In vitro*, the combination of the carboxanilide UC10 with the NNRTIs costatolide and calanolide A rapidly yielded virus isolates which were resistant to both agents. Similar problems with the combination use of two NNRTIs has been previously reported (5). In our study with a two-drug combination (UC10 plus calanolide A), we observed selection for the amino acid change K103N. The K103N and L100I mutations have yielded cross-resistance to nearly every NNRTI evaluated in our laboratory (11). Thus, the optimal combination of agents to be used must include a compound which remains active against viruses with these mutations. Other NNRTIs that inhibit virus isolates with the K103N and L100I amino acid changes are under examination; preliminary results indicate that both TSAO [2',5' bis-*O*-(tetrabutyltrimethylsilyl)- β -D-ribofuranosyl]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''dioxide)) and α -APA (α -anilinophenylacetamide) can inhibit the L100I mutant virus isolate and that α -APA can inhibit the K103N mutant isolate (11).

The use of multiple NNRTIs in the clinic will require assurance that the use of the compounds will not result in antagonistic anti-HIV interaction or in synergistic toxicity. With the variety of UC analogs reported here, highly synergistic interactions with the nucleoside analog AZT were observed. Only additive to slightly synergistic anti-HIV interactions were observed with the nucleoside analog ddC. These results confirm and expand previous reports of the combination use of NNRTIs with nucleoside analogs (10, 13) and suggest significant differences in the mechanism of action or metabolism of AZT relative to the analog ddC or ddI (13). Combination antiviral therapy results also indicate differences in the mode of action of the NNRTIs. Whereas our results nearly always indicate additive interactions between two NNRTIs,

we have documented two NNRTIs that interact synergistically with the NNRTI costatolide (i.e., UC38 and UC40). UC38 was also found to synergistically inhibit HIV when used in combination with UC84. The potential mechanistic rationale for synergy between two NNRTIs is unclear at present but may involve alternative binding sites of the compound outside the NNRTI binding pocket. A second binding site for the compound calanolide A has been postulated on the basis of kinetic enzymatic data (19). In addition, Fletcher et al. have reported that UC38 and UC84 bind to mechanistically different forms of the RT, yielding synergistic anti-HIV activity in both cell-based and biochemical assays (21, 22).

The potency and wide therapeutic indices of many of the NNRTIs continue to make them attractive therapeutic agents. The utility of these compounds rests on the possibility of delaying or suppressing the development of drug resistance. The possibility of developing an armamentarium of highly active NNRTIs which could be used in combination with each other, as well as with other anti-HIV compounds targeting either RT or other viral structures or functions, should be explored. Careful selection and rational combination drug design with potent NNRTIs, such as UC10 or UC70, plus calanolide A and an L100I and K103N suppressor, such as α -APA, may constitute a plausible anti-HIV therapy.

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