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IN VITRO ANTI-HUMAN IMMUNODEFICIENCY VIRUS (HIV) ACTIVITY OF THE CHROMANONE DERIVATIVE, 12-OXOCALANOLIDE A, A NOVEL NNRTI

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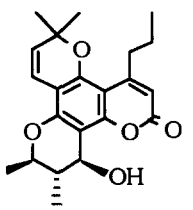
Abstract: The three chromanone derivatives, (+)-, (-)-, and (±)-12-oxocalanolide A (**2**), were evaluated for *in vitro* antiviral activities against HIV and simian immunodeficiency virus (SIV). The compounds were determined to be inhibitors of HIV-1 reverse transcriptase (RT) and exhibited activity against a variety of viruses selected for resistance to other HIV-1 nonnucleoside RT inhibitors. They are the first reported calanolide analogues capable of inhibiting SIV.

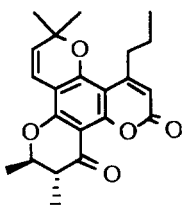
Introduction

It has become increasingly apparent that the HIV-1-specific nonnucleoside reverse transcriptase inhibitor (NNRTI) class of compounds is structurally quite diverse, but that the therapeutic utility of these anti-HIV compounds is compromised by the rapid appearance of drug-resistant virus isolates both in patients and in cell culture. Even though the use of NNRTIs alone is not warranted, the structural diversity of NNRTIs may allow the identification of therapeutically beneficial combinations of compounds which will prevent or retard the selection of drug-resistant viruses, 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. It has been reported that NNRTIs such as nevirapine, delavirdine and efavirenz, when used in combination with additional anti-HIV agents in patients, possess sustained antiviral activity, demonstrating the possible use of NNRTIs as a first line therapeutic option to treat patients without eliminating future therapy options. Therefore, there are benefits for the development of additional novel or more potent NNRTIs.

A series of polycyclic coumarins, originally isolated from several tropical plants of the genus *Calophyllum*, have exhibited activity against HIV-1.[1-3] (+)-Calanolide A (**1**), the most potent compound in the series, was select-



(+)-Calanolide A (**1**)



12-Oxocalanolide A (**2**)

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ed for further development. Evaluation of the activity of (+)-calanolide A against RT and NNRTI-resistant viruses,[4-10] as well as detailed enzyme kinetic studies for RT inhibition,[11] has suggested that it may interact with the HIV-1 RT in a fashion mechanistically different than other known NNRTIs. As such, (+)-calanolide A represents a novel class of HIV-1 specific NNRTI. (+)-Calanolide A (**1**) is currently in clinical trials to evaluate its safety and pharmacokinetics of single and multiple doses in normal healthy volunteers. After oral administration, the drug was generally well tolerated and no patterns indicative of a safety concern were observed.[12] Its plasma concentrations in humans were higher than anticipated from animal data. The AUC and C_{max} values increased with increasing dose, and it appeared that therapeutic levels can be achieved in humans.

In an effort to identify the structural features of naturally occurring (+)-calanolide A necessary for its anti-HIV activity as well as to develop synthetic analogues, 12-oxocalanolide A (**2**), a synthetic intermediate of calanolide A, was discovered to be active against HIV-1.[13] Not only does 12-oxocalanolide A (**2**) have one less chiral center than calanolide A, but, in contrast to calanolide A, both enantiomers of **2**,[14] namely, (+)-**2** and (-)-**2**, are active against HIV-1. This unique feature makes **2** a very attractive candidate for drug development because it can be more conveniently manufactured at a relatively low cost, especially in larger quantities. The cost for treatment of HIV infections with commercially available drugs is high, due in part to difficult and lengthy manufacturing processes. Herein we wish to communicate the characterization of anti-HIV properties of (±)-, (+)-, and (-)-12-oxocalanolide A, **2**.[15]

Materials and Methods

Materials: The (+)- and (±)-**2** were synthesized as previously described,[16,17] while (-)-**2** was separated from (±)-**2** using a semi-preparative chiral HPLC column packed with amylose carbamate (Chiralpak AD, 250 mm x 20 mm i.d., 10 μm particle size). The mobile phase for the HPLC was 10% ethanol in hexane at a flow rate of 6 mL/min, and the UV detector was set at a wavelength of 254 nm. The enantiomeric purities of both (+)- and (-)-**2** were 94% *ee*, as determined by chiral HPLC.

Cells and Viruses: The established human cells used in these evaluations, as well as the laboratory-derived and low-passage clinical isolates of HIV-1 and HIV-2, have been described in the literature.[7] Established cell lines were obtained from the NIAID AIDS Research and Reference Reagent Program. Fresh human cells were obtained from the American Red Cross (Baltimore, MD, USA) and were cultured as previously described.[7-10]

Antiretroviral Activity and Cytotoxicity: The methodology of antiviral assays has been described previously.[7] Briefly, the HIV inhibitory activity of the compounds was evaluated in a microtiter anti-HIV assay which quantifies the ability of a compound to inhibit HIV-induced cell killing or HIV replication. Quantitation was performed using the tetrazolium dye 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 (EC_{50}) and the quantity of drug required to reduce cell viability by 50% (IC_{50}).

Inhibition of Reverse Transcriptase Assays: Analysis of the drug sensitivity of RT containing defined amino acid substitutions was performed as previously described.[7]

Biological Results And Discussion

The synthetic (±)-**2**, (+)-**2** and resolved (-)-**2** were initially assayed in CEM-SS T-cells infected with both laboratory-derived and clinical isolates of HIV-1, HIV-2, and SIV. The results, summarized in Table 1, indicate that all three entities are active against both HIV-1 and SIV, but inactive against HIV-2. Both (±)- and (+)-**2** exhibited the same level of antiviral activities, while (-)-**2** seemed to be somewhat less active. This is in stark contrast to other calanolide analogs, for example, calanolide A and calanolide B. Only (+)-calanolide A (**1**) and (-)-calanolide B account for the anti-HIV activity,[1] while the other enantiomers are inactive.[3] It is also worthwhile to note that compound **2** is the first reported calanolide analogue capable of inhibiting SIV.

Table 1. Antiviral Activity of (±)-, (+)-, and (-)-12-Oxocalanolide A (**2**) against Various Laboratory and Clinical Isolates in CEM-SS Cells

Virus (Isolate)	Activity	(±)-2	(+)-2	(-)-2	ddC[a]	(+)-Calanolide A
HIV-1 (R_F)	EC_{50} (μM)	0.40	0.90	3.41	0.05	0.27 (ref. 3)
	IC_{50} (μM)	5.8	> 10.0	> 10.0	> 5.0	
	TI (IC_{50}/EC_{50})	14	> 11	> 3	> 100	
HIV-1 (III_B)	EC_{50} (μM)	0.51	1.0	1.88	0.02	0.17 (ref. 4)
	IC_{50} (μM)	5.6	> 10.0	> 10.0	> 5.0	
	TI (IC_{50}/EC_{50})	11	> 10	> 5	> 250	
HIV-1 (SK1)	EC_{50} (μM)	0.17	0.17	0.27	0.05	0.14 (ref. 4)
	IC_{50} (μM)	6.3	> 10.0	> 10.0	> 5.0	
	TI (IC_{50}/EC_{50})	37	> 58	> 37	> 100	
SIV (Delta)	EC_{50} (μM)	1.24	1.66	6.12	0.19	inactive
	IC_{50} (μM)	6.6	> 10.0	> 10.0	> 5.0	
	TI (IC_{50}/EC_{50})	5	6	2	> 26	
HIV-2 (ROD)	EC_{25} (μM)	5.57	15.90	ND[b]	0.03	inactive
	IC_{25} (μM)	13.8	34.8	ND	> 10.0	
	TI (IC_{25}/EC_{25})	2	2	ND	> 333	

[a] ddC was used as a positive control drug; [b] ND: not determined.

Due to their availability and ease of manufacture, (±)- and (+)-**2** were chosen for further evaluation of their anti-HIV activity in fresh human peripheral blood leukocytes and monocyte-macrophages infected with a variety of low passage clinical virus isolates (Table 2). Both compounds exhibited anti-HIV activity against clinical isolates in different cells, with the EC_{50} values ranging from 0.3 to 3.0 μM and 0.6 to 3.0 μM, respectively. Depending on the cell lines and virus isolates used, the antiviral activity of (±)- and (+)- **2** varied. For instance, in PBMC cells,

Table 2. Range of Anti-HIV Activity of (±)- and (+)-12-Oxocalanolide A (**2**)

Cell Line[a]	Virus Strain	EC_{50} (μM)	
		(±)-**2**	(+)-**2**
CEM-SS	III_B	0.52	1.09
CEM-SS	SK1	0.27	0.62
MT2	III_B	0.56	2.62
174 x CEM	III_B	> 10	> 10
Macrophage	ADA	0.33	1.76
Macrophage	BaL	0.51	2.19
PBMC	ROJO	2.18	2.66
PBMC	TEKI	> 10	> 10
AA5	III_B	2.80	> 10
U937	III_B	3.0	> 10

[a] Cellular phenotypes: CEM-SS and MT2, T-cells; 174 x CEM, T-cell:B-cell fusion; PBMC, fresh peripheral blood mononuclear cell; AA5, EBV-infected B-cell; U937, monocytic cell.

both (±)- and (+)- **2** were inactive against the clinical isolate TEKI, while decreased activity was observed for (+)-**2** against the laboratory strain III_B in both AA5 and U937 cells. However, the racemic form, (±)-**2**, was consistently more active than the pure enantiomer (+)-**2**, implying a possible synergistic effect in the combination of (+)- and (-)-**2**.

Mechanistic assays indicated that (+)- and (±)-**2** inhibited reverse transcriptase when evaluated in a biochemical RT inhibition assay,[13] with IC_{50} values being 10-40 μM. Compared with cytopathic assay, the enzymatic inhibition was less potent. The results are most likely due to the poor solubility of the compounds in the buffer system used for the RT assay. Both (+)- and (±)-**2** did not inhibit virus attachment, integrase, protease, or cell-to-cell fusion. In addition, the compounds did not inhibit late stage virus reproduction events because they did not suppress virus production in chronically infected cells. Limited pretreatment assays demonstrated that compound had to be continuously present in order to be effective.

The compounds (±)- and (+)-**2**, as well as positive control drug AZT, were evaluated for antiviral activity against viruses selected in cell culture for resistance to a variety of antiretroviral agents (Table 3). These assays demonstrated the extreme sensitivity of the compounds to the amino acid changes L100I, V108I, T139I,[8-10] and Y188H. Each of these amino acid changes in the RT resulted in virus strains which were resistant to the antiviral effect of the compounds. The racemate (±)-**2** was also determined to lose activity when challenged with viruses possessing K101E and K103N amino acid changes, while the enantiomer (+)-**2** remained partially active against both mutations. Both (±)-**2** and (+)-**2** remained active against viruses possessing the Y181C, M184I, and P236L amino acid changes as well as AZT-resistant mutations. Interestingly, the presence of both Y181C and K103N changes resulted in a virus which remained sensitive to (+)-**2**.

Table 3. Activity of (±)- and (+)-12-Oxocalanolide A (**2**) against Drug-Resistant Virus Isolates with Amino Acid Changes in the RT

Resistant Isolate	EC_{50}, μM (Fold-Resistant[a])		
	(±)-**2**	(+)-**2**	AZT[b]
III_B (control)	0.32	1.47	0.009
L100I	> 100 (> 313)	> 100 (> 68)	< 0.003
K101E	> 100 (> 313)	26.4 (18)	0.005
K103N	> 100 (> 313)	30.6 (21)	0.004
V108I	> 100 (> 313)	> 100 (> 68)	0.004
T139I[c]	> 100 (> 313)	> 100 (> 68)	0.04
Y181C	0.59 (S[d])	2.1 (S)	0.006
M184I	0.53 (S)	6.47 (S)	0.01
Y188H	> 100 (> 313)	> 100 (> 68)	< 0.003
P236L	2.03 (6)	6.76 (S)	0.02
4 x AZT[e]	3.60 (11)	2.73 (S)	> 1.0 (> 111)
Y181C/K103N	ND[f]	6.19 (S)	< 0.03

[a] Fold-resistant values were calculated by determination of the ratio of the activity of each compound against the drug-resistant virus isolate to the activity against III_B isolate (wild type) control performed in parallel. [b] AZT was used as a positive control drug. [c] Virus isolate with T139I mutation is selected by, and confers resistance to, (+)-calanolide A (refs. 8-10). [d] S indicates that the virus remains sensitive to the compound and the cut-off point is less than sixfold of resistance. [e] Mutations present in the 4 x AZT virus isolate include D67N, K70R, T215Y, and K219Q. [f] ND: not determined.

The sensitivity of (+)- and (±)-**2** to amino acid change at position 188 of HIV-1 RT, but not at 181, may also explain why the compounds are active against HIV-1 and SIV, but inactive against HIV-2. The amino acid alignment of the RT for the three retroviruses indicate that domain 180 to 190 is highly conserved, except for positions 181 and 188.[18] HIV-2 (ROD) and SIV have small aliphatic amino acid residues at 181 (I and V), compared with aromatic residue Y for HIV-1. However, at position 188, HIV-1 and SIV have aromatic amino acid residue Y, W or F, while HIV-2 has aliphatic residue L.

In summary, chromanone derivative 12-oxocalanolide A (**2**), belongs to the calanolide subclass of NNRTIs. Its unique antiviral properties warrant further pharmacological and toxicological studies as well as efficacy evaluation in animal models. Favorable results from these studies would lead to the possible identification of a new drug for inclusion in combination therapy against HIV infections.

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