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Suksdorfin: an Anti-HIV Principle from *Lomatium suksdorfii*, its Structure–Activity Correlation with Related Coumarins, and Synergistic Effects with Anti-AIDS Nucleosides†

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Abstract—Suksdorfin (**1**), which is isolated from the fruit of *Lomatium suksdorfii*, was found to be able to inhibit HIV-1 replication in the T cell line, H9, with an average EC_{50} value of 2.6 ± 2.1 μM. In addition, suksdorfin was also suppressive during acute HIV-1 infections of peripheral blood mononuclear cells, monocyte/macrophages and the promonocytic cell line, U937. Combinations of **1** and the anti-HIV nucleosides ddI and ddC demonstrated statistical synergy in inhibiting HIV-1 replication (ddC > ddI). However, the viral inhibition mediated by combining **1** with AZT was not statistically synergistic. Furthermore, the presence of suksdorfin did not antagonize the suppression mediated by the three nucleoside reverse transcriptase inhibitors. Comparison of the structure and activity of **1** with those of ten related compounds indicated that the dihydroseselin type of pyranocoumarin possessing a 4'-isovaleryl group is important to suksdorfin's enhanced anti-HIV activity.

Introduction

Lomatium suksdorfii (Watson Coult. and Rose (Umbelliferae)) is distributed on the United States western coast. The roots of several *Lomatium* species were used medicinally by the Gosiute Indians who called the plant "pia-a-na-tsu" or "great medicine."[2] The oil and a crystalline substance obtained from *L. suksdorfii* were previously found to exhibit antispasmodic and antibacterial activities.[2] In the course of our continuing search for natural products as anti-AIDS agents, the methanolic extract of the fruit of *L. suksdorfii* was tested for inhibition of HIV replication and showed significant activity. Bioactivity-directed fractionation of this extract has led to the isolation and identification of suksdorfin (**1**), a dihydroseselin type angular pyranocoumarin, as the active principle.

Chemistry

Suksdorfin (**1**) was obtained as colorless needles (mp 140–141 °C) by silica gel chromatography of the active hexane fractions. Its molecular formula was determined to be $C_{21}H_{24}O_7$ by high resolution mass spectroscopy, and a comparison of its UV, IR, and ^1H NMR spectral data with those described in the literature identified **1** as suksdorfin, which had been previously isolated from this same plant by Willette and Soine.[3] Suksdorfin is a 3'-*O*-acetyl-4'-*O*-isovaleryl-(+)-*cis*-khellactone.[4] It was also isolated previously from the roots of *Angelica morii* Hayata (Shan Du Huo), a drug of folk remedy in Taiwan.[5]

Biological Results

Suksdorfin (**1**) suppressed virus replication in eleven separate acute HIV-1 (IIIB isolate) infections of H9 cells. The average concentration of suksdorfin from these experiments which inhibited 50 % of virus replication (EC_{50}) was 2.6 ± 2.1 μM as determined by a p24 antigen ELISA assay. In order to inhibit H9 cell growth by 50 % (IC_{50}) a 21-fold increase in the concentration of suksdorfin (53.8 ± 30.9 μM, Table 1) was necessary. The average therapeutic index (TI), which is the ratio of the IC_{50} value to the EC_{50} value, was 30.6 ± 22.4 for **1**. Compound **1** also suppressed acute HIV-1 replication in fresh peripheral blood mononuclear cells (a T cell infection, EC_{50} value of 3.9 μM), in monocyte/macrophages (EC_{50} value of 3.9 μM), and in U937 cells (a promonocytic cell line, EC_{50} value of 1.5 μM).

When compound **1** was added for 72 h to the chronically HIV-1 infected T cell line, ACH-2, and to the chronically HIV-1 infected promonocytic cell line, U1, there was no increase in the induction of virus expression from either cell line. Even when both chronically HIV-1 infected cell lines were cultured in the presence of a known virus inducer such as the phorbol ester, PMA (phorbol 12-myristate 13-acetate), there was no alteration in the level of virus expression (Table 2). In addition, the ability of

†Anti-AIDS Agents 13. For paper 12 in this series, see Ref. 1.

compound 1 at 10.3 μM to inhibit HIV-1 replication was found to statistically synergize (Table 3) with the suppression mediated by ddI at 1 ng/mL and ddC at 0.1 ng/mL. Based on 3-D computer analysis,[5,6] compound 1 in the presence of ddI mediated a minor but significant level of synergy (24.12 $\mu M^2\%$) whereas in the presence of ddC a moderate level of synergy (54.7 $\mu M^2\%$) was detected that may be important *in vivo*. However, suksdorfin's suppressive activity was not statistically synergistic with AZT (9.98 $\mu M^2\%$). Furthermore, suksdorfin did not antagonize the suppressive effects of the three nucleoside reverse transcriptase inhibitors when tested at the various concentrations.

Table 1. Summary of suksdorfin data

Assay Run	$IC_{50}(\mu M)$[a]	$EC_{50}(\mu M)$[b]	Therapeutic Index
1	103.0	1.3	79.2
2	46.3	1.7	27
3	90.2	2.1	43
4	72.2	3.9	18.5
5	52.0	7.2	7.2
6	16.8	0.4	42
7	21.9	0.4	54.8
8	20.6	2.1	9.8
9	64.4	2.1	30.7
10	21.9	2.1	10.4
11	82.5	5.7	14.5
Average	**53.8 ± 30.9**	**2.6 ± 2.1**	**30.6 ± 22.4**

[a]Concentration which inhibits uninfected cell growth by 50 %.
[b]Concentration which inhibits viral replication by 50 %.

Table 2. Inhibition of HIV-1 replication in ACH-2 and U1 cells by suksdorfin (1)

Suksdorfin Concentration	ACH-2 Cells[a] -PMA[c]	ACH-2 Cells[a] +PMA[d]	U 1 Cells[b] -PMA	U 1 Cells[b] +PMA
0 μM	3,676 pg/ml	52,122 pg/ml	0 pg/ml	6,963 pg/ml
52 μM	4,541 pg/ml	49,914 pg/ml	0 pg/ml	5,096 pg/ml
10.3 μM	4,723 pg/ml	61,235 pg/ml	0 pg/ml	9,728 pg/ml
2.1 μM	3,821 pg/ml	55,910 pg/ml	0 pg/ml	7,360 pg/ml
0.4 μM	3,688 pg/ml	50,775 pg/ml	0 pg/ml	6,611 pg/ml

[a]Chronically HIV-1 infected T cell line
[b]Chronically HIV-1 infected promonocytic cell line
[c]p24 antigen level after 72 hours in culture
[d]PMA 10^{-8} M

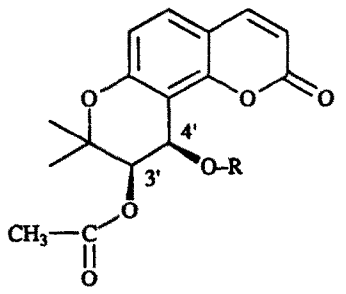
Table 3. The ability of suksdorfin to synergize with known anti-AIDS nucleosides

Nucleoside tested with Suksdorfin[a]	95 % Confidence μM^2 % Value[b]	Synergy Significance Interpretation[c]
AZT[d]	9.98	Insignificant
ddI	24.12	Minor but Significant
ddC	54.70	Moderate with possible *in vivo* importance

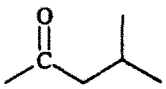
[a]Suksdorfin (10.3, 2.1, and 0.4 μM) was tested both separately and in combination with the following nucleoside reverse transcriptase inhibitors: AZT, ddI and ddC.
[b]Is the mathematical calculation for the volume (μM^2 %) of synergy produced by the drug combination outside the 95 % confidence level at a suksdorfin concentration of 10.3 μM. These values are analogous to the units for area under a dose–response curve in the two dimensional situation (μM %).
[c]Guidelines provided with the computer program to aide investigators in interpreting their results. The guidelines are intended only to serve as a rough estimate of signifance.
[d]The nucleoside reverse transcriptase inhibitors were tested at five 10-fold serial dilutions (from 1 μM/mL) separately and in matrix-combination with suksdorfin.

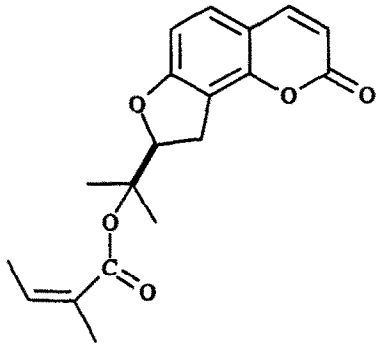
In order to elucidate structure–activity relationships, the HIV-replication inhibitory effects of ten **1**-related coumarins, which were isolated from various plant sources,[7–13] were determined and compared with that of **1**. The compounds included an additional dihydroseselin type angular pyranocoumarin, **2** (pteryxin), a dihydroangelicin type angular furanocoumarin, **3** (columbianadin), three dihydropsoralen linear furanocoumarins, **4** (nodakenetin), **5** (nodakenin), and **6** (acetyl nodakenin), four psoralen type linear furanocoumarins, **7** (imperatorin), **8** (bergapten), **9** (isoimperatorin), and **10** (oxypeucedanin), and a dicoumaryl ether, **11** (daphnoretin).

As shown in Table 4, only **1** showed potent anti-HIV-1 activity at nontoxic concentrations. All other compounds (**2–11**) were either inactive or were less active and more toxic. The furanocoumarins (**3–10**) were inactive or active only at toxic concentrations. The dicoumaryl ether (**11**) showed no activity.



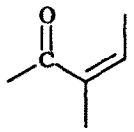
1 (suksdorfin) R =

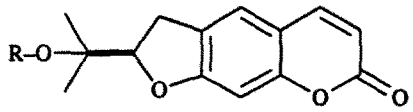




3 (columbianadin)

2 (pteryxin) R =





4 (nodakenetin) R = H

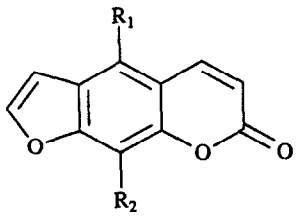
5 (nodakenin) R = Glucose

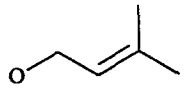
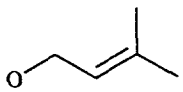
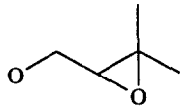
6 (acetylnodakenin) R = Tetraacetyl glucose

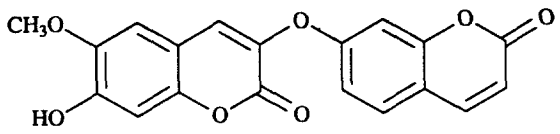
Table 4. HIV inhibition of HIV-1 replication in H9 cells by suksdorfin (**1**) and related compounds (**2–11**).

Compound		IC 50(μM)[a]	EC 50(μM)[b]	Therapeutic Index
1	Suksdorfin	103.0	1.3	79.2
2	Pteryxin	20.7	4.6	4.5
3	Columbianadin	13.1	4.6	2.8
4	Nodakenetin	>203.2	Inactive[c]	ND[d]
5	Nodakenin	>122.5	Inactive	ND
6	Acetyl Nodakenin	>86.8	Inactive	ND
7	Imperatorin	35.2	11.1	3.2
8	Bergapten	208.3	30.1	6.9
9	Isoimperatorin	> 185.2	40.7	> 4.6
10	Oxypeucedanin	139.9	31.5	4.4
11	Daphnoretin	22.7	Inactive	ND

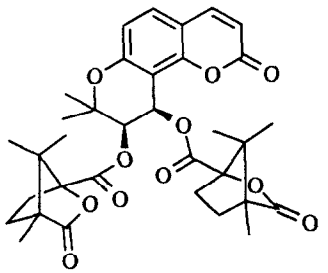
[a]Concentration which inhibits uninfected cell growth by 50 %.
[b]Concentration which inhibits viral replication by 50 %.
[c]No suppression of HIV-1 replication in H9 cells.
[d]ND = not determined.



	R_1	R_2
7 (imperatorin)	H	
8 (bergapten)	OCH_3	H
9 (isoimperatorin)		H
10 (oxypeucedanin)		H



11 (daphnoretin)



12 (DCK)

Discussion

The inhibition of virus replication mediated by suksdorfin (**1**) in both T (H9) and promonocytic (U937) cell line acute HIV-1 infections designates this compound as a lead structure in a new class of potential anti-HIV agents. To further demonstrate suksdorfin's broad cellular specificity and potential clinical relevance, HIV-1 replication in fresh PHA-stimulated PBMCs (T cell) and monocyte/macrophages was also found to be suppressed in its presence. Therefore to date, there have been 4 different types of acute HIV-1 cell infections that suksdorfin has been tested against. The EC_{50} value obtained from each is in the same range (2.6 ± 2.1 μM). In addition, the absence of increased levels of viral replication in chronically infected cells treated with **1** suggests that it would not increase the *in vivo* replication of HIV in a patient who is latently infected.

In order to determine whether the anti-HIV activity of suksdorfin synergized with either AZT, ddI or ddC, the 3-dimensional computer model developed by Prichard *et al.*,[6,7] was used. This procedure directly determines the shape of the dose-dependent surface, identifies the regions of statistically significant synergy and antagonism and quantitates these effects. In the case of a two drug combination, a 3-D plot is the most appropriate as it reflects the inherent 3-D nature of the drug interactions. Until recently, due to technical difficulties, most researchers could only present their data either in a 1 or 2-D fashion which gave oversimplified and often misleading interpretation.[6,7]

In the preliminary structure–activity relationship study, the 4'-isovaleryl group of **1** was important for selective HIV-1 inhibition. Replacement of this group with an angeloyl moiety as in pteryxin (**2**) increased the toxicity by 5-fold and slightly reduced anti-HIV-1 activity.

In summary, suksdorfin (**1**) is a promising lead compound for the chemotherapy of AIDS either alone or possibly in combination with the FDA-approved nucleoside reverse transcriptase inhibitor, ddC. Preliminary *in vitro* results have shown good activities in a variety of cell types. Modification of **1** has yielded 3',4'-di-*O*-(–)-camphanoyl-(+)-*cis*-khellactone (DCK) (**12**), which demonstrated extremely potent inhibitory activity against HIV-1 replication in H_9 lymphocyte cells with an EC_{50} value of 0.00041 μM and TI range of > 78,049 but < 390,244.[8] Further studies on its mechanism of action and evaluation of its synthetic analogues, including DCK, as potential anti-AIDS drug candidates are in progress.

Experimental Section

Chemistry

Isolation of suksdorfin. The *Lomatium suksdorfii* used was collected in Washington state. The ground, air-dried fruits (100 g) were extracted with MeOH. The active MeOH extract was partitioned between hexane and 90 % MeOH (1:1). Evaporation of the active hexane extract gave a crystalline residue. Recrystallization of this residue with hexane yielded **1** as colorless needles (1 g, 1 % yield): mp 140–141 °C; $[\alpha]_D^{24}$ + 4 ° (*c* 0.5, EtOH). The IR and NMR data of **1** are identical to those reported[3,4] for suksdorfin, which was previously isolated from this same species.[3]

Suksdorfin-related coumarins. Compounds **2** (pteryxin),[10] **3** (columbianadin),[11,12] **4** (nodakenetin),[13] **5** (nodakenin),[13] **6** (acetyl nodakenin),[13] **7** (imperatorin),[13] **8** (bergapten),[14] **9** (isoimperatorin),[13] **10** (oxypeucedanin),[13] and **11** (daphnoretin)[15] were obtained by methods reported previously.

Biology

Chronically HIV-1 infected cell lines. HIV-1 chronically infected T cell line, ACH-2,[16] and HIV-1 chronically infected promonocytic cell line, U1,[17] were continuously maintained in RPMI 1640 with 10 % fetal calf serum (FCS). For experiments, the cell lines were only used in log phase of growth. Cells (0.5×10^6 cells/well) and either various concentrations of suksdorfin or medium alone were added to 24-well plates in the presence or absence of PMA (10^{-8} M). After 72 h at 37 °C and 5 % CO_2, an aliquot of the cell-free supernatants were collected and analyzed for p24 antigen by ELISA (see below for details of p24 antigen ELISA).

HIV growth inhibition assay. The T cell line, H9, and the promonocytic cell line, U937, were maintained separately in continuous culture with RPMI 1640 and 10 % FCS at 5 % CO_2 and 37 °C. Cell lines were used in experiments only when in log phase of growth, whereas, uninfected peripheral blood mononuclear cells (PBMCs) were first stimulated with PHA (1 μg/mL) for 3 days. All cell targets were incubated with HIV-1 (IIIB isolate, $TCID_{50}$ 10^4 IU/mL, at a multiplicity of infection of 0.1–0.01 IU/cell) for 1 h at 37 °C and 5 % CO_2. The cell lines and PBMCs were also washed thoroughly to remove unadsorbed virions and resuspended at 4×10^5 cells/mL in complete medium or complete medium with 10 % v/v interleukin 2 (IL-2), respectively. Aliquots of cells (1 mL) were placed in wells of 24-well culture plates containing an equal volume of test compound (diluted in the appropriate culture medium). After incubation for 4 days at 37 °C, cell density of uninfected cultures was determined by counting cells in a Coulter counter to assess toxicity of the test compound. A p24 antigen ELISA assay was used to determine the level of virus released in the medium of the HIV-infected cultures. The p24 antigen assay uses an HIV-1 anti-p24 specific monoclonal antibody as the capture antibody coated-on 96-well plates. Following a sample incubation period, rabbit serum containing antibodies for HIV-1 p24 is used to tag any p24 'captured' onto the microtiter well surface. Peroxidase conjugated goat anti-rabbit serum is then used to tag HIV-1 p24 specific rabbit antibodies which have complexed with captured p24. The presence of p24 in test samples is then revealed by addition of substrate. The lower limit for the p24 ELISA assay is 12.5 pg/mL. p24 In the culture medium was quantitated against a standard curve containing known amounts of p24. The effective (EC_{50}) and inhibitory (IC_{50}) concentrations (for anti-HIV activity and cytotoxicity, respectively) were determined graphically by computer. Both the EC_{50} and IC_{50} values were calculated by plotting drug concentration versus percent inhibition, and then identifying a 50 % inhibition value from the graph.

Acute HIV-1 infections with monocyte/macrophages. PBMCs from an HIV-1 seronegative donor were incubated in 24-well plates in the presence of DMEM and 10 % human AB+ sera for 7–10 days. Nonadherent cells were removed by washing with plain DMEM. The adherent cells that remained were referred to as monocyte/macrophages. They were incubated with a monotropic isolate of HIV-1 (BaL, 15,000 pg of p24/well), overnight at 37 °C, 5 % CO_2. The next day, the cells were washed 3× with plain DMEM to remove unadsorbed virions. One mL of DMEM + 10 % human AB+ sera was added to each well and to the appropriate wells various concentrations of suksdorfin, a nucleoside analog (ddC or AZT), or media was added. Aliquots of the cell free supernatants were collected every 3–4 days post-infection for p24 antigen determination at which time fresh media with the appropriate concentration of drug was added.

Combination study. The experimental design is identical to the growth inhibition assay except that various concentrations of AZT, ddI or ddC were also added to cultures of acutely HIV-1 infected H9 cells that either have or have not received different concentrations of suksdorfin. The concentrations of AZT, ddI and ddC were 5 ten-fold serial dilutions starting at 1 μg/mL. In order to determine whether suksdorfin statistically synergized with any of the 3 nucleoside reverse transcriptase inhibitors, the 3-D computer program that utilized response surface methodology[6,7] was used to analyze the replicate data.

Acknowledgement

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