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Three new compounds from the plant *Lippia alva* as inhibitors of chemokine receptor 5 (CCR5)

Vinod R. Hegde,* Haiyan Pu, Mahesh Patel, Pradip R. Das, Julie Strizki, Vincent P. Gullo, Chuan-Chu Chou, Alexei V. Buevich and Tze-Ming Chan

Schering Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

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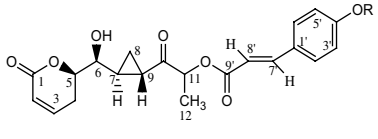
Abstract—The 70% aqueous methanol extract of the Peruvian plant *Lippia alva* (Verbenaceae) was found to contain three novel compounds, **1**, **2**, and **3**, which were identified as inhibitors of the chemokine receptor CCR5. The structures of **1**–**3** were established based on extensive NMR studies. Compounds **1**–**3** inhibited CCR5 receptor signaling as measured by a calcium mobilization assay with IC_{50} values of 5.5, 6.0, and 7.2 μg/mL, respectively.

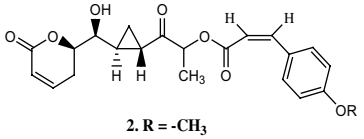
Chemokine receptors are defined by their ability to signal upon binding to one or more members of the chemokine superfamily of chemotactic cytokines. In addition to their role in regulating cell trafficking, several chemokine receptors have been identified to act as cofactors for HIV-1 attachment and entry into target cells.[1] The chemokine receptors CCR5 and CXCR4, and possibly CCR3, are the principal human immunodeficiency virus type 1 (HIV-1) co-receptors, apparently interacting with HIV-1 envelope, in association with CD4.[2] CCR5 mediates migration of T lymphocytes and macrophages by its conjugate chemokine ligands (MIP)-1α, MIP-1β, and RANTES, and is a major co-receptor for cellular entry of monocyte-tropic (R5) strains of human immunodeficiency virus (HIV) type 1, which has been implicated as the predominant phenotype of HIV in early infection.[3] Recently, much attention has been focused on targeting these receptors for antiviral therapy. CCR5 has been particularly attractive since it is the most commonly used receptor by HIV-1[4] strains and is thought to be important in viral transmission. Furthermore, in vivo evidence from both animal knockout models and humans suggests that functional CCR5 is not essential for immune competence and that blockade of these receptors by a specific antagonist will not severely affect normal immune function. Several small molecule antagonists of CCR5 are being developed for HIV therapy, one of which, SCH-C,[5] is currently in clinical trials.

As part of our continuing investigation of natural products as leads for inhibiting HIV infection, we have screened aqueous methanol extracts of different plants utilizing a CCR5 binding assay.[5] One of the fractions derived from a plant identified as *Lippia alva* sp (Verbenaceae), was found with inhibitory activity in the CCR5 binding assay. A semi-purified fraction from that plant showed 92% inhibition at 10 μg/mL concentration. Bioassay-guided fractionation of this extract led to the isolation of three compounds **1**–**3**.

The detanninized aqueous methanol extract (2.0 g) of the plant *L. alva* was loaded as a plug (with 15 mL



1. R = -CH_3
3. R = -H



2. R = -CH_3

* Corresponding author. Tel.: +1 908 820 3871; fax: +1 908 820 6772; e-mail: vinod.hegde@spcorp.com

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CHP-20 gel) on a CHP-20 (2 × 12 cm) column equilibrated with water and chromatographed using a water and methanol gradient system collecting 25 semi-purified fractions. These fractions were screened for CCR5 activity in a high throughput binding assay. The active fractions were collected and dried to yield 83.6 mg of enriched complex. Separation of the active compounds from the active semi-purified fraction was achieved by reverse phase preparative HPLC on a Phenomenex Luna C-18 silica column (21.2 × 250 mm), eluting with a gradient mixture of acetonitrile and 0.05% trifluoroacetic acid (0 → 50% v/v). Acetonitrile was removed from the active fractions, and the aqueous solution was freeze-dried to yield two fractions of 3.5 and 8.9 mg, containing **3** and a mixture of **1** and **2**, respectively. Purification of the later fraction on a Nomura Chemical's Develosil RPAqueous C-30, 5 μm column eluting with a mixture of 0.05% trifluoroacetic acid and acetonitrile (60:40) yielded 5.9 and 1.2 mg of **1** and **2**, respectively. The first fraction enriched with **3** was purified on a Phenomenex Luna C-18, 5 μm 2.1 × 250 mm column eluting with a mixture of 0.05% trifluoroacetic acid and acetonitrile (70:30) gave 1.1 mg of compound **3**.

Compound **1** showed a molecular ion *m/z* 401 $(M + H)^+$ and a sodium adduct at 423 $(M + Na)^+$ in FAB mass spectrum suggesting the molecular weight 400 Da. The molecular formula of **1** was established as $C_{22}H_{24}O_7$ by HRMS (High Resolution Mass Spectrum),[6] indicating 11 degrees of unsaturations in the molecule. The UV spectrum (MeOH) showed absorption maxima at 205, 230, and 314 nm and the IR spectrum in KBr showed peaks at 3320, 1705 cm^{-1}, suggesting the presence of ester functionality.

NMR study of **1**–**3** compounds was carried out on a Varian Inova 500 MHz and on a Varian Unity Plus 600 MHz spectrometers equipped with 3 mm probes. ^1H and ^{13}C NMR chemical shifts and proton–proton couplings of **1** are listed in Table 1. The ^1H NMR indicated the presence of four aromatic protons, four sp^2 protons, one secondary methyl and one $–OCH_3$ methyl group. The ^{13}C NMR also showed 22 carbon signals in agreement with the number of carbons revealed by HRMS. APT ^{13}C NMR identified them as three >C=O, one of which was aliphatic keto functionality, ten olefinic (eight =CH–, two =C<), three >CH–O, two –CH<, two $–CH_2$, and two methyls (one $–OCH_3$ and other $>CH–CH_3$). Considering one aromatic ring, two double bonds and the three carbonyl functionalities accounts for nine degree of unsaturations the other two must be from the presence of two rings. Using 2D NMR techniques (COSY, NOESY, HSQC, HSQC–TOCSY, and HMBC) the structure was established as **1**. Vicinal coupling between C-7′H and C-8′H protons (16.1 Hz) suggested **1** is an *E*-isomer. Relative stereochemistry on C-5, C-6, C-7, and C-9 centers was established based on vicinal proton–proton couplings and NOE. Thus, vicinal couplings between C-5H and C-6H protons and between C-H6 and C-H7 indicated their predominant *gauche* orientations, whereas coupling between C-7H and C-9H indicated their *trans*-configuration (Table 1). This combination of coupling constants and strong

Table 1. ^1H and ^{13}C NMR chemical shifts and coupling constants for **1**–**3**[a]

C#	**1** δ_H (ppm), *J* (Hz)	**1** δ_C (ppm)	**2** δ_H (ppm), *J* (Hz)	**2** δ_C (ppm)	**3** δ_H (ppm), *J* (Hz)	**3** δ_C (ppm)
1	—	163.8	—	163.5	—	163.5
2	6.03 (ddd, *J* = 0.9, 2.7, 9.7)	120.8	5.94 (dt, *J* = 9.8, 1.9)	120.0	5.94 (dt, *J* = 9.8, 1.9)	120.0
3	6.98 (ddd, *J* = 2.2, 6.4, 9.7)	145.9	7.05 (ddd, *J* = 3.9, 4.5, 9.7)	147.1	7.05 (ddd, *J* = 4.3, 4.5, 9.8)	147.1
4	2.70 (m, *J* = 2.6, 2.6, 12.3, 18.6) 2.54 (m, *J* = 0.7, 3.8, 6.2, 18.5)	23.3	2.5 (m)	23.9	2.48 (m)	23.9
5	4.52 (dt, *J* = 12.3, 3.7)	80.6	4.40 (ddd, *J* = 4.4, 8.1, 8.1)	80.4	4.40 (ddd, *J* = 4.4, 6.8, 8.8)	80.3
6	3.72 (dd, *J* = 3.3, 5.9)	71.7	3.54 (q, *J* = 4.6)	69.8	3.57 (t, *J* = 4.9)	69.7
7	1.59 (m, *J* = 4, 6.4, 6.4, 8.9)	26.5	1.52 (m, *J* = 4.1, 5.2, 6.6, 8.9)	27.0	1.48 (m)	27.0
8	1.34 (m, *J* = 4.0, 4.9, 9.0) 1.12 (ddd, *J* = 4.0, 6.6, 8.2)	14.3	1.05 (m)	13.8	1.03 (m)	13.7
9	2.28 (dt, *J* = 8.6, 4.6)	21.4	2.22 (m, *J* = 4.1, 5.1, 7.7)	20.1	2.22 (dt, *J* = 8.0, 4.4)	20.0
10	—	206.6	—	206.5	—	206.6
11	5.30 (q, *J* = 7.1)	75.1	5.24 (q, *J* = 7.1)	74.8	5.24 (q, *J* = 7.1)	74.7
12	1.53 (d, *J* = 7.0)	16.0	1.40 (d, *J* = 7.1)	15.9	1.43 (d, *J* = 7.1)	16.1
1′	—	126.9	—	126.8	—	125.0
2′	7.48 (m, *J* = 8)	129.9	7.74 (m, *J* = 8)	132.5	7.60 (m, *J* = 8)	130.5
3′	6.90 (m, *J* = 8)	114.4	6.93 (m, *J* = 8)	113.5	6.96 (m, *J* = 8)	115.8
4′	—	161.6	—	160.3	—	160.0
5′	6.90 (m, *J* = 8)	114.4	6.93 (m, *J* = 8)	113.5	6.96 (m, *J* = 8)	115.8
6′	7.48 (m, *J* = 8)	129.9	7.74 (m, *J* = 8)	132.5	7.60 (m, *J* = 8)	130.5
7′	7.70 (d, *J* = 16.1)	145.9	7.00 (d, *J* = 12.9)	144.2	6.90 (d, *J* = 16.1)	145.6
8′	6.37 (d, *J* = 16.1)	114.3	5.71 (d, *J* = 12.8)	115.6	6.42 (d, *J* = 16.1)	113.4
9′	—	166.7	—	165.1	—	166.0
$–OCH_3$	3.84 (s)	55.4	3.79 (s)	55.2		—

[a] NMR spectra of **1** were acquired in $CDCl_3$ at 25 °C and **2** and **3** in DMSO-d_6 at 25 °C. Spectral multiplicity is denoted as s for singlet, d for doublet, t for triplet, q for quartet, and m for multiplet of higher order.

NOE values between C-5H and C-9H; C-5H and C-7H; C-6H and C-9H; C-7H and C-4H′ (*cis*-proton with regard to C-5H) was consistent with relative stereochemistry of **1** shown in scheme above.

Compound **2** showed mass ion *m*/*z* 401 $(M + H)^+$ in FAB mass spectrum suggesting the molecular weight 400 Da. The molecular formula of **1** was established as $C_{22}H_{24}O_7$ by HRMS indicated that **1** and **2** are structural isomers. The ^1H and ^{13}C NMR chemical shifts and proton–proton couplings of **2** (Table 1), and NOE values (data not shown) revealed close structural similarities between **1** and **2**. However the proton coupling between C-7′H and C-8′H in **1** and **2** were different. The coupling value of 12.8 Hz between these protons suggested **2** is a *Z*-isomer.

Compound **3** showed mass ion *m*/*z* 387 $(M + H)^+$ in FAB mass spectrum suggesting the molecular weight 386 Da, 14 units less than **1** and **2**. The molecular formula of **3** was established as $C_{21}H_{22}O_7$ by HRMS, revealing **3** is a lower molecular weight homolog of **1**. The ^1H NMR spectrum of **3**, indicated that the $-OCH_3$ peak at δ 3.8 ppm had disappeared. The ^{13}C NMR spectrum confirmed the absence of an $-OCH_3$ peak at 55.2 ppm. These data suggest that the methoxy group of *p*-methoxy cinnamic acid has been replaced by a phenolic –OH. This has been established by preparing a *p*-bromobenzyl derivative by stirring compound **3** with *p*-bromobenzyl bromide in DMF, in presence of anhydrous K_2CO_3 overnight at room temperature and under nitrogen atmosphere. The proton coupling between C-7′H and C-8′H protons (16.1 Hz) suggested **3** is an *E*-isomer. This was also confirmed by the methylation of the phenolic –OH group of **3** using diazomethane, which gave the product identical to **1**.

These compounds have interesting structures with a cyclopropane ring moiety, and to the best of our knowledge are the first examples in the literature of plant natural products containing a cyclopropane ring structure.

Compounds **1**–**3** were found to be active in a high throughput screen for antagonists of the CCR5 receptor with inhibitory IC_{50} of 5.5, 6.0, and 7.2, respectively. All three compounds were then subsequently evaluated in a calcium mobilization assay, which measures the ability of compounds to block intracellular calcium mobilization following activation of the receptor by ligand. The assay was performed in accordance with recommendations of the manufacturer of the fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). 15,000 U-87 astroglioma cells expressing the human CCR5 receptor were loaded with the calcium sensitive dye Fluo 4 for 1 h at 37 °C, followed by washing to remove excess dye. Test compounds were serially diluted and briefly incubated with the cells prior to addition of 10 nM ligand (MIP-1α, MIP-1β, or RANTES). Changes in intracellular calcium induced following ligand addition were measured using the FLIPR. The results of studies performed with MIP-1β are shown in Figure 1 and Table 2 summarizes the IC_{50} values obtained for

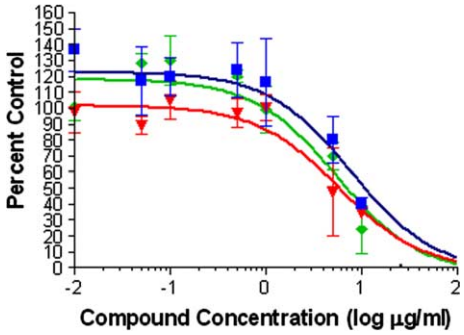

Figure 1. Inhibition of MIP-1β induced calcium signaling in U-87-CCR5 cells. ▼—Compound **1**; ◆—Compound **2**; ■—Compound **3**.

Table 2. Inhibition of chemokine induced calcium mobilization in U-87-CCR5 cells by compounds **1**–**3**

Compd	MIP-1α	IC_{50} (μg/mL) MIP-1β	RANTES
1	>10	5.5	8.7
2	8.8	6.0	>10
3	10	7.2	>10

each of the ligands tested. The data is expressed as concentration of compound required to inhibit 50% of the ligand induced calcium signal in the cells. All three compounds showed modest inhibitory activity against the MIP-1β ligand with IC_{50} values ranging from 5.5–7.0 μg/mL. Activity against the other CCR5 ligands, MIP-1α and RANTES was less potent or not detectable at concentrations below 10 μg/mL. The biological activity of compound **1** was further confirmed by inhibition of MIP-1β (1 nM) – mediated migration of recombinant Ba/F3 cells expressing CCR5 with an approximated IC_{50} of 3 μM. These results suggest that these compounds may overlap with the binding site of the MIP-1β ligand to a greater extent than the MIP-1α and RANTES sites. We also assessed the antiviral activity of these compounds in viral infection assay to determine their effect on infection of CCR5 positive cells by HIV-1. Although some antiviral activity was observed in these studies, the antiviral effects could not be distinguished from cellular cytotoxicity, which was apparent at concentrations >1 μg/mL (data not shown).

Previously we had reported triterpenes showing weak activity in CCR5 assay.[7] Natural products **1**–**3** are interesting compounds, containing unique structural features such as a γ-lactone and a cyclopropyl ring system, with CCR5 activity. Natural products containing a cyclopropyl ring system are not common in nature. Several marine natural products have been reported with cyclopropane ring.[8–11]

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