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O. Hnatyszyn, A.M. Broussalis, G. Herrera, Liliana Muschietti ...+10 more authors

Institutions: National Scientific and Technical Research Council, University of Navarra

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Argentine Plant Extracts Active Against Polymerase and Ribonuclease H Activities of HIV-1 Reverse Transcriptase

Oksana Hnatyszyn[1], Adriana Broussalis[1], Gilda Herrera[1], Liliana Muschietti[1], Jorge Coussio[1], Virginia Martino[1], Gabriela Ferraro[1], María Font[2], Antonio Monge[2,*], Juan José Martínez-Irujo[2], Marcos Sanromán[2], M. Teresa Cuevas[2], Esteban Santiago[2] and Juan José Lasarte[2]

[1]IQUIMEFA (Instituto de Química y Metabolismo del Fármaco) UBA-CONICET, Cátedra de Farmacognosia, Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, República Argentina
[2]CIFA (Centro de Investigación en Farmacobiología Aplicada) Universidad de Navarra, 31080, Pamplona, España

Lipophilic and hydrophilic extracts of four Argentine plants (*Gamochaeta simplicaulis* Cabr. 1, *Achyrocline flaccida* Wein. D. C. 2, *Eupatorium buniifolium* H. et A. 3, and *Phyllanthus sellowianus* Muell. Arg. 4) were examined *in vitro* for their ability to inhibit the polymerase and ribonuclease H (RNase H) activities of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) (wild and Y181C mutant types). The active extracts were also examined as inhibitors of viral replication in HLT4LacZ-1_{IIIB} cell cultures, evaluating their cytotoxicity in parallel. Infusions 2I and 4I, among the crude extracts, showed the highest activity. These extracts were refractioned into four fractions; $2I_4$ and $4I_4$ were active as inhibitors of DNA-polymerase (wild and Y181C types) and RNase H activities. These fractions were potent as inhibitors of viral replication and were not cytotoxic. Refractionation of $2I_4$ yielded five new fractions, two of which, $2I_4$-4 and $2I_4$-5, showed notable activity. Refractionation of $4I_4$ yielded four new fractions; of these, $4I_4$-3 and $4I_4$-4 were active. The marked biological activity found in the infusion of *A. flaccida* and *P. sellowianus* makes them sufficiently attractive to be considered in the combined chemotherapy of the disease.

Keywords: Gamochaeta simplicaulis Cabr.; *Achyrocline flaccida* Wein. D. C.; *Eupatorium buniifolium* H. et A.; *Phyllanthus sellowianus* Muell. Arg.; HIV-1 RT; RNase H.

INTRODUCTION

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is a multifunctional enzyme exhibiting both RNA and DNA dependent DNA-polymerase activities as well as an inherent ribonuclease H (RNase H) activity. The enzyme is considered an ideal target for the chemotherapeutic intervention of AIDS and, in fact, compounds, such as 3′-azido-2′,3′-dideoxythymidine, that inhibit its function are used in the treatment of the disease. However, their clinical use is limited due to their toxicity and the emergence of resistant viral strains.

Extracts from four selected Argentine medicinal species have been examined *in vitro* for their ability to inhibit the HIV-1 RT DNA-polymerase and RNase activities.

Gamochaeta simplicaulis Cabr. 1, common name ‘plan cachu’, is used in folk medicine against pock, measles and varicella; and it is used externally for lavages of sores and pimples (Zardini, 1984). Infusions of *Achyrocline flaccida* Wein. D. C. 2 (common name: ‘marcela’, ‘marcela macho’) are used as an antispasmodic, febrifuge, stimulant, emenagogue, excitant and antihelmintic (Zardini, 1984). It is also used in external preparations for healing cuts, lacerations and ulcerations of the skin. Extracts of this plant have been reported to have antimicrobial activity (Gutkind *et al.*, 1984). Investigations of the antiherpetic activity of both species has been previously reported (Cavallaro *et al.*, 1995; García *et al.*, 1995). *Eupatorium buniifolium* H. et A. 3, common name ‘romero’, ‘romerillo’, ‘romero colorado’, is used as a tincture for its hepatoprotective and disinfectant properties (Zardini, 1984). *Phyllanthus sellowianus* Muell. Arg. 4 (Euphorbiaceae), commonly known as ‘sarandi blanco’, is a shrub native to South America, widely distributed in Paraguay, Northeastern Argentina, Southern Brazil and Uruguay. It is a well-known Argentine medicinal plant and it is quoted in the Argentine Pharmacopeia (1978) as a hypoglycaemic, diuretic, laxative and antiseptic in folk medicine (Hieronymus, 1882; Sorarú and Bandoni, 1978). This species was selected based on the activity reported for other species of this genus against hepatitis B virus (Unander *et al.*, 1995).

MATERIAL AND METHODS

Plant Material. *Gamochaeta simplicicaulis* aerial parts were collected in Buenos Aires Province, November 1991; *Achyrocline flaccida*, aerial part in Entre Ríos

* Correspondence to: Prof. Dr. D. Antonio Monge, CIFA, Universidad de Navarra, 31080 Pamplona, Spain.

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Table 1. Activity of crude extracts

	HIV-1 DNA-polymerase				HIV-1 ribonuclease H	Antiinfective activity	
	Wild type		Y181C				
Extract	% Inh.†[a] (100 μg/mL)	IC_{50}‡[b] (μg/mL)	% Inh.[a] (100 μg/mL)	IC_{50}‖[b] (μg/mL)	% Inh.[a] (100 μg/mL)	IC_{50}[b] (μg/mL)	HLT4LacZ-1_{IIIB} IC_{50}[a] (μg/mL)
1I	99 ± 0.14	36.0 ± 5	90 ± 12.0	nd[c]	49 ± 6.4	-	-
1M	92 ± 3.0	20.0 ± 1	90 ± 1.1	nd	61 ± 1.9	nd	>2.0
1C	15 ± 6.2	-	5 ± 3.5	-	26 ± 5.4	-	-
2I	93 ± 9.5	3.9 ± 0.1	82 ± 18.0	nd	97 ± 1.6	10.0 ± 2.5	1.1
$2I_1$	11 ± 9.9	-	19 ± 3.1	-	26 ± 10.7	-	-
$2I_2$	9 ± 14.3	-	18 ± 1.2	-	52 ± 9.8	-	-
$2I_3$	17 ± 9.3	-	16 ± 5.0	-	53 ± 12.0	-	-
$2I_4$	98 ± 1.9	1.0 ± 0.1	92 ± 0.3	1.8 ± 0.6	96 ± 0.3	3.9 ± 0.9	0.25
$2I_4$-1	4 ± 4.7	-	2 ± 7.8	-	nd	nd	-
$2I_4$-2	25 ± 1.1	-	17 ± 4.5	-	76 ± 3.1	nd	-
$2I_4$-3	85 ± 4.9	43.0 ± 4.0	85 ± 5.5	26.0 ± 2.5	63 ± 2.7	nd	-
$2I_4$-4	98 ± 1.1	17.0 ± 5.0	100 ± 0.9	1.2 ± 0.5	72 ± 3.0	12.8 ± 6.8	nd
$2I_4$-5	99 ± 1.1	12.0 ± 0.8	100 ± 0.9	7.3 ± 0.2	100 ± 0.9	5.4 ± 1.8	1.4
2M	36 ± 1.6	-	31 ± 14.0	-	40 ± 3.1	-	-
2C	17 ± 3.2	-	8 ± 2.5	-	31 ± 3.2	-	-
3I	96 ± 0.2	16.0 ± 1.0	90 ± 5.9	nd	100 ± 0.8	3.6 ± 2.8	5.0
3M	35 ± 9.9	-	21 ± 3.8	-	37 ± 0.4	-	-
3C	4 ± 5.6	-	1 ± 1.4	-	19 ± 4.4	-	-
4I	99 ± 0.1	4.1 ± 0.5	96 ± 3.8	nd	94 ± 6.2	17.0 ± 3.2	1.6
$4I_1$	11 ± 11.0	-	17 ± 9.0	-	27 ± 1.0	-	-
$4I_2$	8 ± 4.2	-	4 ± 6	-	32 ± 5.7	-	-
$4I_3$	57 ± 9.6	-	50 ± 6.8	-	46 ± 14.0	-	-
$4I_4$	100 ± 0.9	2.4 ± 0.8	91 ± 0.3	3.7 ± 0.6	96 ± 1.7	5.9 ± 1.4	3.0
$4I_4$-1	10 ± 5.6	-	6 ± 0.6	-	31 ± 4.5	-	-
$4I_4$-2	21 ± 0.6	-	11 ± 3.2	-	42 ± 8.3	-	-
$4I_4$-3	100 ± 0.9	2.4 ± 0.2	100 ± 0.3	1.7 ± 0.2	95 ± 2.4	3.0 ± 0.4	1.2
$4I_4$-4	100 ± 0.7	1.7 ± 0.1	100 ± 0.3	1.7 ± 0.6	95 ± 0.1	2.6 ± 0.5	1.8
4M	77 ± 1.4	52.0 ± 2.0	75 ± 1.4	nd	62 ± 0.7	nd	-
4C	22 ± 1.9	-	14 ± 1.3	-	33 ± 7.0	-	-
AZT	-	-	-	-	-	-	1.21[e]
U-90152s	-	0.21 ± 0.03[d]	-	4.5 ± 0.2[d]	nd	-	14.0[e]
L-697661	-	0.19 ± 0.05[d]	-	28.3 ± 4.3[d]	nd	-	20.0[e]

[a] Mean ± SEM (n = 3–5)
[b] Concentration-activity curves were carried out with four or more concentrations of test extracts; IC_{50} values were calculated from log curve.
[c] nd, no data;
[d] μM;
[e] nM.

Province, February 1991; *Eupatorium buniifolium* aerial parts and *Phyllanthus sellowianus*, stem bark were collected in Entre Ríos Province, February, 1988. Voucher samples are deposited in the Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

Preparation of extracts. Hot water infusions (I) were prepared according to the Argentine Pharmacopoeia VI and lyophilized. Dichloromethane (C) and methanol extracts (M) were also prepared and taken to dryness together under reduced pressure. The marc from the CH_2Cl_2 extraction was extracted twice with methanol (100 mL each), following the same procedure as for the dichloromethane extract.

The infusion of *P. sellowianus* (4I) was extracted successively with CH_2Cl_2, Et_2O and AcEt. The aqueous remaining fraction ($4I_4$) was chromatographed on a Sephadex LH20 column (15 × 2 cm). Four fractions were eluted using MeOH, MeOH–H_2O 7:3, MeOH–H_2O 5:5 and H_2O. The infusion of *Achyrocline flaccida* (2I) was extracted in a liquid/liquid extractor successively with CH_2Cl_2, Et_2O and AcEt. The aqueous remaining fraction ($2I_4$) was lyophilized, redissolved in water and centrifuged. The supernatant was precipitated by adding a $BaCl_2$ saturated solution. The precipitate was centrifuged and the pellet discarded. The supernatant was chromatographed on a Sephadex LH20 column (15 × 2 cm) eluted with MeOH, MeOH–H_2O 5:5 and H_2O, affording five fractions.

Enzyme assays. Wild type and mutated (Y181C) recombinant reverse transcriptases (p66/p66) were purified by immobilized metal affinity chromatography (Imac) as described (Sharma *et al.*, 1991). RNA directed DNA polymerase activity was measured as previously described (Font *et al.*, 1995). RNase H activity was measured as described by Zhan *et al.*, (1994). Infusion extracts were solubilized in water whereas methanol and dichloromethane extracts were solubilized in DMSO, diluted in water and assayed, maintaining the final concentration of DMSO at 0.5% (v/v).

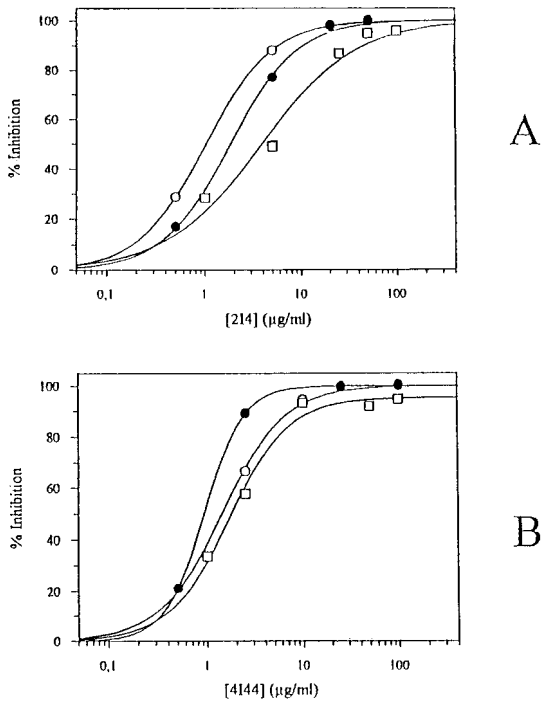

Figure 1. Dose-response curves of inhibition of the catalytic functions of HIV-1 RT by (A) $2I_4$ and (B) $4I_4$-4 fractions. (○) wild type RNA-dependent DNA polymerase activity; (□) wild type RNAse H activity; (●) Y181C RNA-dependent DNA polymerase activity.

Cell cultures. HIV-1_{IIIB} chronically infected Molt-3 cells (Molt-3/HIV-1_{IIIB}), kindly provided by F. Barin and B. Janvier from Université François Rabelais (Tours, France) and HLT4LacZ-1 cells, kindly provided by S. Saragasti from Hôpital Cochin (Paris, France), were cultured according to previously described methods (Font *et al.*, 1995).

Syncytia formation assay. According to previously described methods (Font *et al.*, 1995) in HLT4LacZ-1 cells.

Cell toxicity. According to previously described methods, in HLT4LacZ-1 cells (Aggarwal *et al.*, 1985; Font *et al.*, 1995).

RESULTS

Among the crude extracts the highest activity was observed in the hydrophilic extracts, especially in the infusions **2I** and **4I**. Among the methanol extracts, only **1M** and **2M** presented slight activity. All the lipophilic extracts (CH_2Cl_2) were inactive (Table 1).

Extracts 2I and 4I were refractioned into four fractions. The fractions $2I_4$ and $4I_4$ showed activity in the inhibition of the DNA-polymerase activity of wild and mutant types as well as in the inhibition of the ribonuclease activity (Fig. 1a); in addition, these fractions were potent as inhibitors of viral replication and were non cytotoxic (Fig. 2).

Refractionation of $2I_4$ yielded five new fractions, two of which ($2I_4$-4 and $2I_4$-5) showed activity. Refractionation of $4I_4$ yielded four new fractions; of these four, $4I_4$-3 and $4I_4$-4 were active (Fig. 1b and Fig. 2). U-90152s, L-697661 and AZT were used as reference patterns.

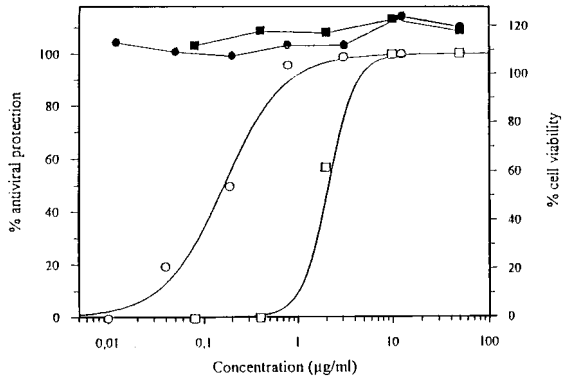

Figure 2. Effect of selected fractions on virus replication and cytotoxicity on HLT4LacZ-1 cells. (○) $2I_4$ antiviral activity; (□) $4I_4$-4 antiviral activity; (●) $2I_4$ cytotoxicity; (■) $4I_4$-4 cytotoxicity.

DISCUSSION

Many of the RT inhibitors obtained from synthetic or natural sources have been reported to affect the DNA polymerase activity of RT, but there are only a limited number of compounds that have been found to inhibit both polymerase and RNase H activities. It has been shown that the monophosphorylated form of AZT can inhibit RNase, but at much higher concentrations than that needed to inhibit the polymerase activity (Tan *et al.*, 1991). On the other hand, nevirapine, a nonnucleoside RT inhibitor, has been found to alter the cleavage specificity of RNase but it was unable to inhibit this activity (Palaniappan *et al.*, 1995). It has been reported that small naphthalenesulfonic acid derivatives were able to inhibit both activities, but their potency as RNase H inhibitors was over tenfold to 100-fold lower compared with the polymerase activity (Mohan *et al.*, 1994). These derivatives showed poor activity when tested in the whole virus assay, probably due to a lack of cellular entry.

A number of sustances have been isolated from natural sources that inhibit the RNase H of RT. Of these, suramin and illimaquinone have been shown to specifically inhibit this activity, whereas 3,5,8-trihydroxy-4-quinolone, avarol and avarone derivatives, obtained from sponges from the Red Sea, preferentially inhibited the polymerase activity (Mitsuya *et al.*, 1984; Loya *et al.*, 1990, 1994). However, these compounds turned out to be either insufficiently potent or too toxic to be considered as potential anti-HIV drugs.

In this paper we describe the inhibition of DNA polymerase and RNase H activity of HIV-1 RT mediated by extracts obtained from four Argentine medicinal plants. Infusions of *Achyrocline flaccida* and *Phyllanthus sellowianus* were the most active. More interestingly, they were active when tested on a mutated enzyme, in which a cysteine substitutes a tyrosine in position 181 (Y181C); this form of mutation confers cross resistance to the majority of the nonnucleoside inhibitors. In addition, these aforementioned extracts are also capable of inhibiting the RNase H activity, they are active in

antiviral assays and do not show cytotoxicity in the range of concentrations tested. The correlation between their potency and the antiviral assay reinforces the idea that the mechanism of action of these extracts is due to RT inhibition. The marked biological activity found in the infusions of *A. flaccida* and *P. sellowianus* makes them sufficiently attractive to be considered in the combined chemotherapy of the disease. Bioguided fractionation of the active extracts is being carried on and studies to characterize the active principles and to determine the mechanism of these inhibitors are currently in progress.

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