Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 370-378

HIV-1 AND HIV-2 REVERSE TRANSCRIPTASES: A COMPARATIVE STUDY OF SENSITIVITY TO INHIBITION BY SELECTED NATURAL PRODUCTS

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Received April 13, 1992

SUMMARY: One hundred and fifty six pure natural products, which had previously been tested against HIV-1 reverse transcriptase, were evaluated for HIV-2 reverse transcriptase inhibitory activity. Compounds that lacked effect in the HIV-1 reverse transcriptase system were found also to be inactive against HIV-2 reverse transcriptase. However, compounds belonging to the benzophenanthridine and protoberberine classes of alkaloids, certain flavonoids, the iridoid, fulvoplumierin, and the ansamycin antibiotic, daunomycin, exhibited similar potencies in both enzyme systems. In contrast, HIV-2 reverse transcriptase was observed to be four-fold more sensitive toward the inhibitory effects of the ipecac alkaloids, *O*-methylpsychotrine sulfate heptahydrate and psychotrine dihydrogen oxalate. Such differences in susceptibilities to inhibitors may indicate subtle dissimilarities in enzyme structure and function.

Human immunodeficiency virus type 1 (HIV-1) is responsible for the current pandemic of acquired immunodeficiency syndrome (AIDS) (1). A second immunodeficiency virus (designated HIV-2), which is found predominantly in West Africa, was subsequently isolated from patients with AIDS and AIDS-related complex (2). The genome of HIV-2 has

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ABBREVIATIONS: RT, reverse transcriptase; MP, *O*-methylpsychotrine sulfate heptahydrate; PDO, psychotrine dihydrogen oxalate.

0006-291X/92 $4.00

been cloned and sequenced (3). This virus is serologically distinct from HIV-1 and appears to be more closely related to simian immunodeficiency virus (SIV) (4). In addition, HIV-2 is similar to HIV-1 in morphology, ultrastructure and genomic organization, with both viruses possessing homologous structural and replicative proteins including the reverse transcriptase (RT) (3,5,6).

Genetic comparison of HIV-1 and HIV-2 revealed significant sequence divergence at both the nucleotide and amino acid levels. The predicted amino acid sequence homology of the *pol* gene of HIV-1 and HIV-2 is only about 60% (3). The RTs of HIV-1 and HIV-2 also show substantial sequence divergence, having approximately 60% of their amino acids in common. Despite these differences, the DNA polymerase activities associated with the two enzymes share similar biochemical properties such as ion and template-primer preferences (7,8). Most genetic manipulations also produced similar effects on both enzymes, although several important differences were noted (9). As in the case of HIV-1 RT, genetic data also revealed strong interactions between the two functional domains of HIV-2 RT (9).

At present, limited studies have been conducted to compare the genetic, structural and biochemical properties of the two enzymes. HIV-1 and HIV-2 RTs have been investigated and found to demonstrate similar susceptibilities to RT inhibitors such as foscarnet and azidothymidine triphosphate (10). On the other hand, certain synthetic compounds have been shown to be specific HIV-1 RT inhibitors (11-13). In order for AIDS therapy to be effectively applied, it will be important to identify the functional similarities of the two RTs by comparing the effect of inhibitors on their polymerization activities. We currently present inhibition data for HIV-2 RT utilizing selected natural products that had previously been evaluated against HIV-1 RT (p66) (14). In addition, comparisons of sensitivities to inhibitors were achieved in experiments whereby assays for HIV-1 RT (p66/p51) and HIV-2 RT (p68/p55) were carried out simultaneously, i.e., utilizing identical reaction mixtures and test compound dilutions.

MATERIALS AND METHODS

Test and reference compounds. *O*-Methylpsychotrine sulfate heptahydrate (MP), cephaeline hydrochloride and dehydroemetine were purchased from or donated by Hoffmann-La Roche, Inc. (Nutley, NJ). Emetine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Psychotrine free base, psychotrine dihydrogen oxalate (PDO) and additional test compounds were obtained from the natural product compound repository at the College of Pharmacy, University of Illinois at Chicago. The identity and purity of all compounds were reported previously (15). Fagaronine chloride was isolated as described by Messmer *et al.* (16).

Reagents and template-primers. [*Methyl*-^{3}H]TTP (15 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). TTP, poly (rA), oligo $(dT)_{12-18}$, dithiothreitol, glutathione and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-cellulose filter discs (Whatman DE 81) were obtained from VWR Scientific (Batavia, IL). All other reagents were of analytical grade.

Preparation of HIV-1 (p66/p51) and HIV-2 (p68/p55) RTs. Dimeric HIV-1 RT was purified by modification of the procedures of Clark *et al.*(19). HIV-2 RT is a recombinant enzyme consisting of two polypeptide subunits (68 and 55 kDa). It is synthesized in an *Escherichia coli* expression system using a genetically engineered plasmid (9). The enzyme possesses both RNA-dependent DNA polymerase and ribonuclease H activities typical of retroviral RTs.

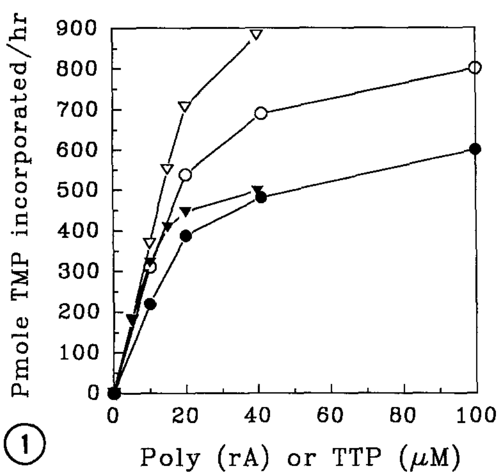
HIV-1 and HIV-2 RT assays. Identical conditions were utilized for the HIV RT assays in view of the conservation of biological properties in both enzymes (17). The standard assay mixture was adapted from the optimal conditions developed for the detection of RT in AIDS associated virions (18) and contained the following: 50 mM Tris•HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethylene glyco-*bis*-(β-amino-ethylether) *N*, *N'*-tetraacetic acid (EGTA), 5 mM dithiothreitol, 0.3 mM glutathione, 2.5 μg/ml bovine serum albumin, 41 μM poly (rA) [ϵ_{260}(mM) = 7.8], 9.5 μM oligo $(dT)_{12-18}$ [ϵ_{265} (mM) = 5.6], 20 μM TTP, 0.5 μCi of [methyl-^{3}H]TTP, and test substance (dissolved in 10 μl of DMSO or H_2O) or solvent only. The reaction was initiated by the addition of 10 μl (0.08 μg) of HIV-1 or HIV-2 RT and the mixture (final volume, 100 μl) was incubated at 37°C for 1 hr. Reactions were terminated by the addition of 25 μl of 0.1 M EGTA followed by chilling in ice. Aliquots of each reaction mixture (100 μl) were then spotted onto circular 2.5 cm DE-81 (Whatman) filters, and washed four times with 5% aqueous Na_2HPO_4 and twice with H_2O. Finally, the filters were dried and subjected to liquid scintillation counting. With these reaction conditions, the uninhibited rates of incorporation were 2.5 and 1.3 μmol TMP/mg protein/10 min for HIV-1 and HIV-2 RTs, respectively.

Based on activity cut-offs established previously with HIV-1 RT (p66) (14), an initial prescreen was conducted utilizing two concentrations of the test compounds (50 and 200 μg/ml). Compounds demonstrating >50% inhibition at 200 μg/ml were then subjected to complete dose-response evaluations. The median inhibitory concentration (IC_{50}) was calculated from a linearly regressed dose-response plot of % control activity versus concentration or log concentration of compound, utilizing at least five concentrations of each compound. Each data point represents the average of duplicate tests. The antileukemic alkaloid fagaronine chloride (16) was used as the positive-control substance in routine testing. All data were confirmed by performing two separate experiments. In addition, both HIV-1 and HIV-2 RT assays were performed simultaneously in experiments to compare the sensitivities of these enzymes to inhibitors.

Polymerization assay conditions. Preliminary experiments were conducted to ensure that product incorporation was linear with respect to incubation time, and that each set of assay conditions used represented subsaturation levels with respect to the template, substrate and enzyme concentrations. The effect of inhibitors were evaluated at the same region on the saturation curves of HIV-1 and HIV-2 RT as an attempt to standardize the polymerization activity of each system and hence their susceptibility to inhibitors.

RESULTS AND DISCUSSION

The standardization of experimental parameters is an important prerequisite for a valid comparison of the effect of inhibitors on the activity of two or more enzymes. As described previously (15), the HIV-1 RT (p66) assay was established utilizing subsaturation conditions with respect to the template-primer, substrate and enzyme concentrations.



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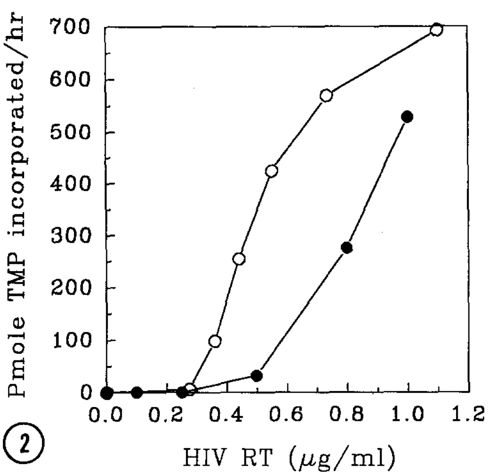

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Figure 1. Effect of variable concentrations of: poly (rA) on HIV-1 RT (○) and HIV-2 RT (●); and TTP on HIV-1 RT (▽) and HIV-2 RT (▼). Standard reaction mixtures incorporating variable concentrations of poly (rA) and TTP were assayed as described in "Materials and Methods".

Figure 2. Dependence of the DNA polymerase activity of HIV-1 RT (○) and HIV-2 RT (●) on enzyme concentration. Standard reaction mixtures were initiated with the indicated concentrations of enzymes.

Similarly, HIV-1 (p66/p51) and HIV-2 RT (p68/p55) assays were performed under identical experimental conditions. Figures 1 and 2 illustrate the saturation curves for HIV-1 and HIV-2 RT obtained with varying poly (rA) and TTP, and enzyme concentrations, respectively.

Benzophenanthridine alkaloids have been shown to inhibit various RNA and DNA polymerizing enzymes via interaction with nucleic acid template-primers (20). Previous studies, however, have not compared the catalytic properties of HIV-1 and HIV-2 RT in the presence of inhibitors known to bind with the template-primer. It has also been suggested that the RT inhibitory property of these alkaloids is in accordance with the postulated active site (the iminium ion, $-C=N^{+}-CH_3$) for antitumor and other biological activities (21); this functionality represents the key structural requirement for the intercalative ability of these alkaloids. However, it is not known if these molecules interact with the RT protein as well. The most potent HIV-2 RT inhibitory activity was demonstrated by fagaronine chloride and nitidine chloride, which exhibited IC_{50} values of 9.5 μg/ml (24.3 μM), and 7.1 μg/ml (18.3 μM), respectively. These values are identical to those obtained with HIV-1 RT (Table 1). The identical sensitivity of the two RTs to the disruption of nucleic acid template functions implies similarities in the nucleotide polymerizing process and possibly the enzyme active sites as well. In addition, since susceptibility of HIV-2 RT to each of these compounds paralleled that obtained for the HIV-1 enzyme, similar structure-activity relationships may be suggested (14).

Table 1. IC_{50} [μg/ml (μM)] values of natural products tested against HIV-1 (p66/p51) and HIV-2 (p68/p55) RT[1]

	HIV-1 RT	HIV-2 RT
Benzophenanthridine alkaloids		
Fagaronine chloride	8.5 (21.8)	9.5 (24.3)
Nitidine chloride	7.4 (19.1)	7.1 (18.3)
Chelerythrine chloride	IA[2]	IA
Chelidonine	IA	IA
N-Demethylfagaronine	IA	IA
Protoberberine alkaloids		
Jatrorrhizine chloride	42.4 (113.4)	39.8 (106.5)
Berberine chloride	60.9 (163.8)	57.8 (155.5)
Coptisine chloride	100.0 (281.1)	117.3 (329.7)
Tetrahydroberberine	IA	IA
Tetrahydropalmatine	IA	IA
Ipecac alkaloids		
O-Methylpsychotrine sulfate heptahydrate (MP)	21.8 (31.0)	6.4 (9.1)
Psychotrine dihydrogen oxalate (PDO)	18.3 (28.4)	6.0 (9.3)
Psychotrine free base	IA	IA
Emetine hydrochloride	IA	IA
Dehydroemetine	IA	IA
Cephaeline hydrochloride	IA	IA
Iridoid		
Fulvoplumierin	97.7 (400.0)	87.4 (357.8)
Plumericin	IA	IA
Plumieride	IA	IA
13-O-t-Coumaroylplumieride	IA	IA
15-Demethylplumieride	IA	IA
Flavonoids		
Kaempferol	91.4 (319.3)	66.8 (233.4)
Quercetin	188.9 (625.0)	195.3 (646.2)
Ansamycin antibiotic		
Daunomycin	125.2 (237.3)	116.0 (219.9)

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[1] Standard assay conditions as described in "Material and Methods" were used.
[2] Inactive ($IC_{50} > 200$ μg/ml).

The RT inhibitory properties of the protoberberine alkaloids are similar to those of the benzophenanthridines in that they also interact with the template-primer (22). These alkaloids showed moderate activity in the HIV-2 RT system with the most potent compound being jatrorrhizine chloride [IC_{50} = 39.8 μg/ml, (106.5 μM)] (Table 1). The IC_{50} values of these alkaloids also did not differ from those obtained in the HIV-1 RT system (Figure 3). Implications of such similarities in enzyme sensitivities follow arguments such as those presented for the benzophenanthridine alkaloids.

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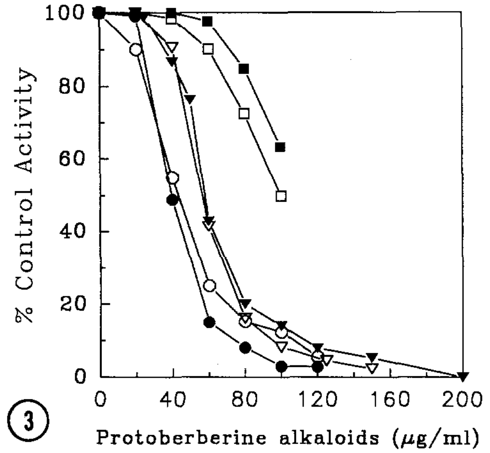

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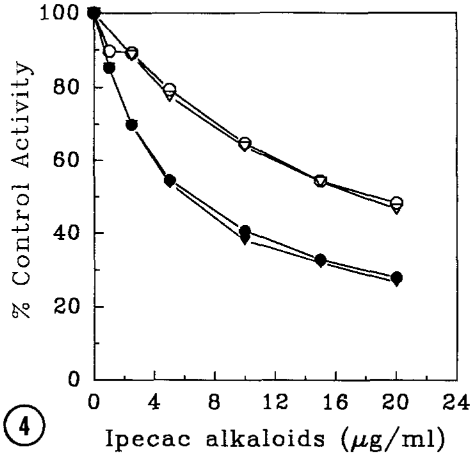

Figure 3. Effect of protoberberine alkaloids on the activity of HIV-1 and HIV-2 RT. Standard conditions were used except that the indicated concentrations of alkaloids were added. HIV-1 RT: jatrorrhizine chloride (○), berberine chloride (▿), and coptisine chloride (□); HIV-2 RT: jatrorrhizine chloride (●), berberine chloride (▾), and coptisine chloride (■).

Figure 4. Effect of MP and PDO on the reactions catalyzed by HIV-1 and HIV-2 RT. Standard assays were performed in the presence of the indicated concentrations of the alkaloids. HIV-1 RT: MP (○) and PDO (▿); HIV-2 RT: MP (●) and PDO (▾).

MP and PDO are minor alkaloids of ipecac, the dried rhizome and roots of *Cephaelis ipecacuanha* (Brotero) A. Richard. We previously reported that MP and PDO were selective inhibitors of both monomeric (p66) and dimeric forms of HIV-1 RT and that the other members of the ipecac alkaloids were inactive (15). It was further concluded that the alkaloids inhibited the elongation stage of the HIV-1 RT polymerization reaction by binding to the enzyme-template-primer-substrate complex. As shown in Figure 4 and summarized in Table 1, MP and PDO were also active against HIV-2 RT with IC_{50} values of 6.4 μg/ml (9.1 μM) and 6.0 μg/ml (9.3 μM), respectively. Notably, these values are approximately four-fold less than those observed with HIV-1 RT [21.8 μg/ml (31.0 μM) and 18.3 μg/ml (28.4 μM) for MP and PDO respectively]. These differences in IC_{50} values indicate that HIV-1 RT is somewhat less sensitive to MP and PDO than HIV-2 RT. Other ipecac alkaloids were tested and found to be inactive in the HIV-1 and HIV-2 RT systems (Table 1). Since MP is believed to bind at a site other than the template-primer or substrate binding sites on the enzyme, this locale is presumeably dissimilar in each of the enzymes and could reflect the involvement of different amino acid residues.

The iridoid, fulvoplumierin, and flavonoids such as quercetin and kaempferol, also demonstrated similar IC_{50} values in both HIV-1 and HIV-2 RT systems, as did the ansamycin antibiotic, daunomycin (Table 1).

Table 2. Additional natural products tested for HIV-1 (14) and HIV-2 (p68/p55) RT inhibitory activity

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Alkaloidal amines: colchicine, ephedrine sulfate, phenylephrine
Ansamacrolide: maytansine
Aporphine alkaloids: boldine, magnoflorine chloride, melosmine
Cardiac glycoside: ouabain
Cephalotaxus alkaloids: harringtonine, homoharringtonine
Chromone: khellin
Coumarins: esculin, thamnosmonin
Diterpene alkaloids: cassaine sulfate, taxol
Diterpenes/diterpene glycosides: steviol, stevioside
Flavonoids: 2',4'-dihydroxychalcone, 8-methoxy-5,7-dihydroflavonol, genkwanin, velutin
Furocoumarin: 5-(3'-methyl-2',3'-dihydroxybutyl)-8-methoxypsoralen
Furoquinolines: acetylrobustine, heliparvifoline
Imidazole alkaloid: isopilosine
Indole alkaloids: ajmalicine, ajmaline, alstonine hydrochloride, aspidospermine, aricine, brucine, camptothecine, catharanthine hydrochloride, conoflorine, conopharyngine, corynantheidine, corynantheine, 1,2-dehydroaspidospermine, deserpideine hydrochloride, deserpidine, echitamine chloride, isoschizogamine, isoraunescine, leurosine sulfate, lochnerine, *N*-acetylperivine, narceine, perakine, pericalline, pericyclivine, periformyline, perividine, perivine, rauniticine, raunitidine, sarpagine, schizozygine, tetrahydroalstonine, tubulosine, vinblastine, vincamajine, vincristine
Indolizidine alkaloid: swainsonine
Isoquinoline alkaloids: hernandezine, hydrastine, hydrastine picrate, papaverine, thalicarpine, tubocurarine chloride
Lignan: liriodendrin
Limonoids: limonin, nomilin
Macrocyclic peptide: didemnin B
Piperidine alkaloids: arecoline hydrobromide, trigonelline hydrochloride
γ-Pyrone: *O*-methylkojic acid
Pyrrolizidine alkaloids: indicine *N*-oxide, lasiocarpine, monocrotaline, retrorsine, senecionine, seneciphylline
Quinoline alkaloids: cinchonine, quinidine sulfate
Quassinoid: bruceantin
Quinolizidine alkaloids: nesodine, ormosinine, panamine, panamine diperchlorate, sparteine, sparteine sulfate, leontine
Quinones/quinols: 2,5-dimethyl benzoquinone, 3-*tert*-butyl-4-methoxy-1,6-dione, jacaranone
Rotenoids: 12-hydroxypachyrrhizone, 12a-hydroxyrotenone, rotenone
Sesquiterpene lactone: picrotoxin
Sesquiterpene alkaloid: phyllanthoside
Steroids/steroidal glycosides: 4-androsten-3,17-dione, 4β-hydroxywithanolide, polypodin.
Steroidal alkaloids: tomatine, veratrine
Sterol: stigmasterol
Triterpenes: β-amyrin, aridanin, betulin, 22-hydroxytingenone, primulagenin
Triterpene saponin glycosides: ginsenoside Ra_1, ginsenoside Rb_1, ginsenoside Re
Tropane alkaloids: hyoscine hydrobromide, scopolamine hydrobromide

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However, as summarized in Table 2, 115 other compounds which were previously shown to be inactive against HIV-1 RT (14) were also found to be inactive against the HIV-2 enzyme. Hence, most compounds including those which disrupt enzyme function by interacting with the template-primer, seem to have identical effects on both HIV RTs.

Nonetheless, in spite of a certain degree of amino acid sequence homology, the present results indicate that HIV-1 and HIV-2 RT are functionally dissimilar toward inhibition mediated by certain ipecac alkaloids.

An agent active against both HIV-1 and HIV-2 could potentially have greater clinical utility than a drug specific for only one type of virus. Therefore, the fact that most inhibitors tested were unable to discriminate between the two enzymes may have chemotherapeutic implications. For example, agents that inhibit HIV-1 and HIV-2 RTs presumeably interact with regions on the enzyme that are structurally and/or functionally conserved. It may be significantly more difficult for HIV-1 and HIV-2 to develop resistance to drugs that are able to interact with such fundamental elements of HIV RT. On the other hand, inhibitory results obtained with the ipecac alkaloids indicate that distinct structural regions may exist on both enzymes that are possibly not conserved in each of the two species but are still critical for catalytic activity. This information could be exploited in the design of drugs effective for the treatment of AIDS associated with each type of virus, and may contribute toward elucidating structural features of both HIV-1 and HIV-2 RT.

ACKNOWLEDGMENTS

The authors thank the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, for the provision of daunomycin, and Professor K.L. Rinehart of the Department of Chemistry, University of Illinois, Urbana, Illinois, for a generous gift of didemnin B. Research sponsored in part by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with ABL.

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