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Short Communication

Anthraquinones as a new class of antiviral agents against human immunodeficiency virus

Raymond F. Schinazi[1*], Chung K. Chu[2], J. Ramesh Babu[2], Barbara J. Oswald[1], Vanessa Saalmann[1], Deborah L. Cannon[1], Bertil F.H. Eriksson[1] and Mohamed Nasr[3]

[1]*Veterans Affairs Medical Center, and Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, U.S.A.,* [2]*Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Georgia, Athens, Georgia, U.S.A. and* [3]*AIDS Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Services, Bethesda, Maryland, U.S.A.*

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Summary

Various anthraquinones substituted with hydroxyl, amino, halogen, carboxylic acid, substituted aromatic group, and sulfonate were tested to determine their activity against human immunodeficiency virus type 1 (HIV-1) in primary human lymphocytes. Among the compounds tested, polyphenolic and/or polysulfonate substituted anthraquinones were found to possess the most potent antiviral activity. Hypericin, an anthraquinone dimer previously shown to have activity against nonhuman retroviruses also exhibited anti-HIV-1 activity in lymphocytes. Some of the active anthraquinones inhibited HIV-1 reverse transcriptase. However, this enzyme inhibition was selective only for 1,2,5,8-tetrahydroanthraquinone and hypericin. Hypericin interacts nonspecifically with protein suggesting that this effect may dictate its inhibitory activity against the viral reverse transcriptase.

Anthraquinone; Anti-HIV-1 activity; Reverse transcriptase; Hypericin

A number of nucleosides have been found to possess antiviral activity against human immunodeficiency virus type 1 (HIV-1). These include 3′-azido-3′-deoxy-

Correspondence to: R.F. Schinazi, Veterans Affairs Medical Center, 1670 Clairmont Road, Decatur, GA 30033, U.S.A.

TABLE 1

Effect of various anthraquinones on HIV-1 replication, PBMC growth, HIV-1 reverse transcriptase (RT), and PBMC-derived DNA polymerase α

Compound	Anti-HIV-1 activity: EC_{50} (μM)	Cytotoxicity in PBMC: IC_{50} (μM)	Inhibition of HIV-1 recombinant RT: EC_{50} (μM)	Inhibition of DNA polymerase α: EC_{50} (μM)
Anthraquinone [1]	> 100	> 100		
1-Chloroanthraquinone [2]	> 100	> 100		
1-Aminoanthraquinone [3]	> 100	> 100		
1-(Methylamino)anthraquinone [4]	> 100	> 100		
2-Methylanthraquinone [5]	44.4	> 100	> 100	> 100
Anthraquinone-2-sulfonic acid sodium salt monohydrate [6]	97.7	> 100		
Anthraquinone-2-carboxylic acid [7]	> 100	> 100		
1,5-Diaminoanthraquinone [8]	> 100	> 100		
Alizarin (1,2-Dihydroxyanthraquinone) [9]	> 100	> 100		
Quinizarin (1,4-Dihydroxyanthraquinone) [10]	> 100	> 100		
1,8-Dihydroxyanthraquinone [11]	> 100	> 100		
Anthrarufin (1,5-Dihydroxyanthraquinone) [12]	> 100	> 100		
Anthraflavic acid (2,6-Dihydroxyanthraquinone) [13]	21.0	25.7	92.5	> 100
Oil Blue N [14]	> 100	> 100		
9,10-Dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracene carboxylic acid [15]	36.9	15.9	> 100	> 100
Acid Blue 80 [16]	63.1	> 100		
Acid Green 25 [17]	3.1	> 100	18.5	5.5
Anthraquinone-1,5-disulfonic acid disodium salt dihydrate [18]	8.3	> 100	> 100	> 100
Alizarin Red S Monohydrate (3,4-Dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt) [19]	3.5	> 100	10.0	25.8
1-Amino-4-bromo-2-methylanthraquinone [20]	83.3	> 100		
Acid Blue 25 [21]	> 100	> 100		
Acid Blue 40 [22]	> 100	> 100		
Purpurin (1,2,4-Trihydroxyanthraquinone) [23]	3.4	27.4	7.7	1.0
Alizarin Complexone dihydrate [(3,4-Dihydroxy-2-anthraquinonyl)-methyliminoacetic acid)] [24]	17.6	78.0	45.8	50.0
1,2,5,8-Tetrahydroxyanthraquinone [25]	3.3	87.8	3.1	60.0
Acid Blue 45 [26]	55.8	> 100		
6,7-Dichloro-1,4-dihydroxyanthraquinone [27]	> 100	> 100		
Emodine (6-Methyl-1,3,8-trihydroxyanthraquinone) [28]	36.3	> 100	> 100	> 100
(±)1-Acetoxy-8-hydroxy-1,4,4a,9a-tetrahydroanthraquinone [29]	10.3	> 100	> 100	> 100
Hypericin [30]	0.44	> 100	0.77	14.7
3′-Azido-3′-deoxythymidine (AZT)[a] [31]	0.002	> 100	0.007	> 200
Phosphonoformate (PFA) [32]	22.4	> 100	0.21	248

[a]AZT-5′-triphosphate was used for the enzyme assays.

thymidine (AZT) (Furman et al., 1986; Mitsuya et al., 1985), 2′,3′-dideoxy- (Dahlberg et al., 1987; Mitsuya et al., 1986; Yarchoan et al., 1989) and 2′,3′-didehydro-2′,3′-dideoxynucleosides (Balzarini et al., 1986, 1987; Hamamoto et al., 1987; Lin et al., 1987a,b), 3′-azido-2′,3′-dideoxyuridine (AzddU, AZDU, CS-87)(Schinazi et al., 1987; Chu et al., 1989), 3′-azido-2′,3′-dideoxy-5-methylcytosine (AzddMeC) (Chu et al., 1989), and carbovir (Vince et al., 1988). Among these compounds, AZT is currently the only drug that has been shown to decrease the mortality and frequency of opportunistic infections associated with acquired immunodeficiency syndrome (AIDS) (Fischl et al., 1987). However, toxic effects of AZT on bone marrow cells can limit its usefulness as a long term chemotherapeutic agent, which is required for the patients with AIDs and AIDS-related complex (Richman et al., 1987). Furthermore, recently it has been reported that AZT-resistant variants of HIV-1 have been isolated from some patients treated with this drug (Larder et al., 1989). Therefore, discovery of new and less toxic nonnucleoside antiviral agents with different modes of action are needed. As a part of our continuing efforts to discover new anti-HIV-1 agents, we have evaluated various classes of compounds in human peripheral blood mononuclear cell (PBMC). From these efforts, we have found anthraquinones to be a new class of antiviral agents against HIV-1 (Table 1).

The anthraquinones used in the experiment were purchased from Aldrich Chemical Co., Milwaukee, WI. Hypericin was prepared from emodine according to a published procedure (Rodewald et al., 1977). The compound obtained was identical to an authentic sample of hypericin (Atomergic Chemtals Corp., Farmingdale, NY). AZT and AZT-5′-triphosphate were prepared by previously published methods (Lin and Prusoff, 1978; Schinazi et al., 1989). The purity of these compounds was confirmed by proton NMR and chemical analysis. Phosphonoformate (PFA) was obtained from Astra Alab, Södertälje, Sweden. Bovine serum albumin, fraction V, was obtained from Sigma Chemical Co., St. Louis, MO. The antiviral assays were conducted in mitogen stimulated human PBMC infected with HIV-1 (strain LAV), as described previously (Chu et al., 1988, 1989; Schinazi et al., 1988). Stock solutions of the drugs were prepared in DMSO and diluted so that the final concentration of DMSO in the medium did not exceed 0.05%. The drugs were added about 45 min after infection. Six days later when virus production was at a maximum, the supernatant was clarified and the virus concentrated by high speed centrifugation. The reverse transcriptase activity associated with the disrupted virus was determined. The virus infected control had about 3×10^5 decompositions per minute per ml of reverse transcriptase activity. The blank and uninfected cell control values were about 500 and 1200 dpm, respectively.

The effects of drugs on the growth of uninfected human PBMC were also established. Mitogen-stimulated PBMC (3.8×10^5 cells/ml) were cultured in the presence and absence of drugs under the same conditions as those used for the antiviral assays described above. The cells were counted using a hemacytometer 6 days after initiation of treatment using the trypan blue exclusion method.

A recombinant 66-kDa HIV-1 reverse transcriptase, that differs from virion-derived enzyme only in that it has two additional amino terminal amino acids, was

obtained from Dr S. Hughes (National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD). This enzyme was recently reported to have an inhibition profile with various classes of antiviral agents indistinguishable from the virion-derived enzyme (Schinazi et al., 1989). DNA polymerase α was isolated from uninfected phytohemagglutinin-stimulated PBMC. Briefly, the cells were pelleted, resuspended in elution buffer, sonicated, and centrifuged free of cell debris. The extract was then applied to a stepwise purification through ion-exchange column chromatography, as described previously for herpes simplex DNA polymerase (Eriksson et al., 1980). The standard reaction mixture (100 μl) for HIV-1 assays contained 100 mM Tris-Cl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.05 U of $(rA)_n \cdot (dT)_{12-18}$ per ml (equivalent to 3.1 μg/ml), and 1 μM [^{3}H]dTTP (specific activity 82.3 Ci/mmol). PBMC DNA polymerase α activity was assayed in 100 μl reaction mixtures containing 100 mM Tris-Cl (pH 8.0), 6 mM $MgCl_2$, 5 mM dithiothreitol, 1 μM [^{3}H]dTTP (specific activity 82.3 Ci/mmol), 100 μM each of dATP, dCTP, and dGTP, and 200 μg of activated calf thymus DNA per ml. Reactions were started by the addition of 10 μl of enzyme. The reaction mixtures were incubated and processed as described previously (Eriksson and Schinazi, 1989). The median effective (EC_{50}) and inhibitory (IC_{50}) concentrations were determined by the median effect method (Schinazi et al., 1986).

The results of the antiviral and cytotoxicity assays on 29 anthraquinones and hypericin in PBMC are shown in Table 1. For comparison AZT and PFA were included in the assays. Compounds substituted with polyhydroxy groups and/or polysulfonate groups such as compound 17, 19, 23, and 25 exhibited the highest antiviral activity among the anthraquinones studied. Anthraquinones with amino, alkylamino, and arylamino groups (3, 4, 8, 14, 21 and 22) did not exhibit any anti-HIV-1 activity. Halogen substituted compounds (2, 20 and 27) also did not show any significant antiviral activity. Although compound 7 which is substituted with a carboxylic acid did not show any antiviral activity, compound 15, which is substi-

TABLE 2

Effect of hypericin (10 μM) on HIV-1 reverse transcriptase in the presence and absence of bovine serum albumin (BSA)[a]

BSA (μg/ml)	Mean CPM with hypericin	Inhibition (%)	Mean CPM without hypericin	Inhibition (%)
0	1,359	95		
1	1,014	96	24,462	13
5	2,257	92	29,385	−4
10	7,090	75	58,561	−108
50	68,970	−144	65,280	−131
100	50,330	−78	72,194	−156
500	76,489	−171	69,873	−148

[a]The average counts per minute (CPM ± S.D.) for the background and control (no drug) were 327 ± 42 and 28,216 ± 6,640 (N = 8), respectively. CPM values in the presence of hypericin and/or BSA are mean of duplicate samples. The variation between duplicates was less than 10%.

tuted with a combination of carboxylic acid and hydroxy groups, exhibited some activity. It is interesting to compare the antiviral activity of 16 and 17. Compound 17, in which the sulfonate group on the arylamino group is at the ortho position with one methyl group at the para position (relative to amino), exhibited good antiviral activity, while 16, in which the sulfonate group is at the meta position with three other methyl groups on the ring, showed much less activity. There was no clear correlation between the pattern of substitution and the antiviral activity of the molecules.

Of the compounds evaluated, only the anthraquinones 13, 15 and 23 exhibit significant cytotoxicity with IC_{50}s below 30 μM. Several of the compounds that showed significant antiviral activity below 50 μM were examined for their ability to specifically inhibit HIV-1 reverse transcriptase in a cell-free system (Table 1). Hypericin and 1,2,5,8-tetrahydroxyanthraquinone (25) were the only two compounds that appeared to be specific against the viral enzyme having ratios of EC_{50} for DNA polymerase α inhibition to HIV-1 reverse transcriptase inhibition greater than 10. Some compounds such as 17 and 23 inhibited DNA polymerase α to a greater extent than the viral reverse transcriptase. This inhibition pattern for the host cell enzyme compared to the viral enzyme could not be explained. These compounds, and in particular 17, were selective inhibitors of HIV-1 replication, but not of HIV-1 reverse transcriptase. The HIV-1 activity of these compounds could be due to interactions at the cell membrane or at cell surface recognition sites, and not intracellularly where interactions with viral and cellular enzymes takes place. None of the anthraquinones evaluated were more effective or more specific than AZT-5′-triphosphate or PFA (used as positive controls) against the HIV-1 reverse transcriptase (Table 1).

It is known that certain quinones possess interesting biological activities such as antibacterial, antiviral, and antitumor activity. These properties may be related to their ability to form free radicals or to intercalate with nucleic acids. For example, streptonigrin, an antimicrobial and antitumor antibiotic, and its analogues, exhibit a potent inhibitory activity on avian myeloblastosis virus reverse transcriptase (Hafuri et al., 1988). Daunomycin and adriamycin also inhibit the avian myeloblastosis virus reverse transcriptase (Dhananjaya et al., 1987). Recently, it has been reported that adriamycin inhibits HIV-1 infection and replication (Nakashima et al., 1987). Meruelo et al. (1988) reported that hypericin and pseudohypericin, polycyclic quinone analogues, are also highly effective against a variety of nonhuman retroviruses in vitro and in vivo. Initially, these workers reported that these quinones have no effect on the transcription, translation, or transport of viral proteins to the cell membrane. More recently they claimed that hypericin and pseudohypericin probably interfere with viral infections and/or spread by direct inactivation of the virus or by preventing virus shedding, budding, or assembly at the cell membrane. In contrast to the report that hypericin had not effect on avian or murine reverse transcriptase (Lavie et al., 1989; Meruelo et al., 1988), we found that fresh solutions of hypericin inhibited HIV-1 reverse transcriptase at submicromolar concentrations (Table 1). However, this inhibition was dependent on the protein concentration of the radioactive mix. To illustrate the nonspecific protein-

binding effect of hypericin, the inhibitory effect of this compound on HIV-1 reverse transcriptase activity was determined in the absence and presence of bovine serum albumin (Table 2). This effect was negated when bovine serum albumin (50–500 μg/ml) was added to the reverse transcriptase cocktail. This suggests that the enzyme inhibitory activity is dependent on the amount of exogenous protein present. As previously shown (Schinazi et al., 1989), the addition of BSA (10–500 μg/ml) produced a significant stimulatory effect (up to about 150%) of HIV-1 reverse transcriptase activity. This stimulatory effect was not artifactual since no increase in radioactivity was noted when BSA was added to the radioactive cocktail in the absence of the enzyme (data not shown). The effect caused by BSA may be related to its ability to stabilize this enzyme.

In summary, a number of anthraquinones substituted with hydroxyl and/or sulfonate groups were shown to have antiviral activity. In particular, anthraquinones substituted with hydroxyl and/or sulfonate group appear to be the most potent compounds (Table 1). Hypericin 30, which is a dimer of emodine 28, exhibited the most potent activity among the compounds tested (EC_{50} = 0.44 μM). However, none of the synthetic or natural products, including hypericin, were more effective than AZT in this cell culture system. Our results also indicate that hypericin had a selective anti-HIV-1 activity and is a modest inhibitor of HIV-1 reverse transcriptase. The mode of action of the active anthraquinones is under investigation.

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