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Anti-HIV-1 and HIV-2 activity of naphthalenedisulfonic acid derivatives. Inhibition of cytopathogenesis, giant cell formation, and reverse transcriptase activity

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Since the causative agent of the acquired immunodeficiency syndrome (AIDS) was identified as a human retrovirus [1, 2], a number of compounds have been reported as active anti-human immunodeficiency virus type-1 (anti-HIV-1) agents *in vitro* [3, 4]. So far, among these compounds, 3'-azido-2',3'-dideoxythymidine (AZT) is the only antiviral drug that has been licensed for clinical use in patients. Although AZT does prolong survival in AIDS patients [5], its use has been linked with toxicity [6], resumption of viral replication during therapy [7], and the emergence of AZT-resistant HIV-1 mutants [8]. Many other dideoxynucleoside analogs have been claimed as potential anti-HIV agents. These agents, like AZT, are essentially prodrugs because they must be converted to their active 5'-triphosphate form by cellular enzymes before acting on the target reverse transcriptase enzyme [9]. However, this class of compounds has not been able to completely halt virus replication *in vivo*.

Being cognizant of these facts, many workers have looked elsewhere for leads, in particular, *non-nucleoside* agents. One such approach is the investigation of the vast reservoir of natural products [10–13], and another the evaluation of synthetic chemical entities [14, 15]. Since none of these agents has so far demonstrated the ability to cure AIDS, there still remains a compelling need to search for new agents with innovative mechanisms of action against HIV-1 replication. In this context, we recently extended studies in the naphthalenedisulfonic acid area. Although the anti-HIV activity of the related azo naphthalenedisulfonic acid dyes has been reported [16], the dye properties of these agents and the possible *in vivo* carcinogenicity of their amine metabolites will hamper their use as therapeutic agents. To circumvent these problems, we have designed several derivatives that lack the azo functionality but still keep the approximate distance between the naphthalene units. In this paper, we report the potent and selective inhibition of HIV-1 and HIV-2 replication, giant cell formation, and reverse transcriptase activity by novel naphthalenedisulfonic acid derivatives.

Materials and Methods

Chemicals. All compounds (Fig. 1) were synthesized from the respective naphthalenedisulfonic acid and the acyl or sulfonyl chloride. These synthetic procedures are reported elsewhere [17]. 3'-Azido-2',3'-dideoxythymidine (AZT) and 2',3'-didehydro-2',3'-dideoxythymidine (D4T) were provided by Dr. E. De Clercq (Rega Institute, Katholike Universiteit, Leuven, Belgium). 2',3'-Dideoxyadenosine (DDA) was purchased from Pharmacia PL-Biochemicals (Uppsala, Sweden).

Cells. MT-4 [18] and MOLT-4 (clone No. 8) [19] cells were used for the anti-HIV assays. The cells were mycoplasma-negative. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, and 20 μg/mL gentamicin (culture medium).

Viruses. HIV-1 (HTLV-III_B) and HIV-2 ($LAV\text{-}2_{ROD}$) were used in the anti-HIV assays. Both HIV strains were obtained from the culture supernatant of MOLT-4 cells chronically infected with the virus. HIV stocks were titrated in MT-4 cells and stored at −80° until used.

Antiviral assays. Activity of the compounds against the replication of HIV-1 and HIV-2 was based on the inhibition of virus-induced cytopathogenesis in MT-4 cells as previously described [20]. The number of viable MT-4 and MOLT-4 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method [21].

Giant cell formation assay. The assay procedure for giant cell formation has been described previously [22]. MOLT-4 cells were cultured with an equal number of MOLT-4/HTLV-III_B for 24 hr, and the number of giant cells was determined microscopically.

Reverse transcriptase assay. Purified recombinant HIV-1 RT (930 units/mL) was used in the assay.

Results

Anti-HIV activity. Compounds **1–4** were evaluated for their inhibitory effects on cytopathogenesis in MT-4 cells and demonstrated anti-HIV-1 activity at non-toxic concentrations. Compound **3** was the most active, and it almost completely protected the cells against virus-induced cell destruction at a concentration of 20 μM (Fig. 2). Its 50% antiviral effective concentration (EC_{50}) was 8.3 μM (Table 1). Compound **3** was not inhibitory to the viability of mock-infected MT-4 cells at 100 μM, and the 50% cytotoxic concentration (CC_{50}) was 320 μM. Consequently, the compound has an *in vitro* therapeutic index of 38.6 (Table 1). When the activity and cytotoxicity of compound **3** were compared with those of suramin, the compound was as active against HIV-1 as suramin, but slightly less toxic (Table 1). Compound **3** also inhibited HIV-1 replication in MOLT-4 cells, and exhibited an EC_{50} value slightly higher than that in MT-4 cells. HIV-2 proved sensitive to all the compounds. Again, compound **3** was the most active (Table 1).

Inhibitory effect on giant cell formation. When uninfected MOLT-4 cells were cocultured with MOLT-4/HTLV-III_B in the absence of test compound, the formation of giant (multinucleated syncytial) cells was observed after 24 hr of incubation. Such giant cells were hardly detected when the four test compounds were added to the cocultures at a concentration of 100 μM. Compound **3** emerged as the most active derivative. At 17 μM, it achieved approximately 50% reduction (IC_{50}) in the number of giant cells. On the other hand, a concentration of 32 μM was required for suramin to suppress giant cell formation by 50%. Compounds **1**, **2** and **4** exhibited IC_{50} values of 37, 30 and 27 μM, respectively, in the same assay (data not shown).

Inhibitory effect on reverse transcriptase. Since it is well known that suramin is a potent inhibitor of retroviral RT [23], we evaluated the compounds for their inhibitory effects on purified recombinant HIV-1 RT. All of the compounds exhibited concentration-dependent inhibition of HIV-1 RT activity in the cell-free system (Fig. 3). Compound **1** ($IC_{50} = 2.8$ μM) was almost as potent as suramin, and compound **3** was even more active. The 50% inhibitory concentration (IC_{50}) of compound **3** was 2.5-fold

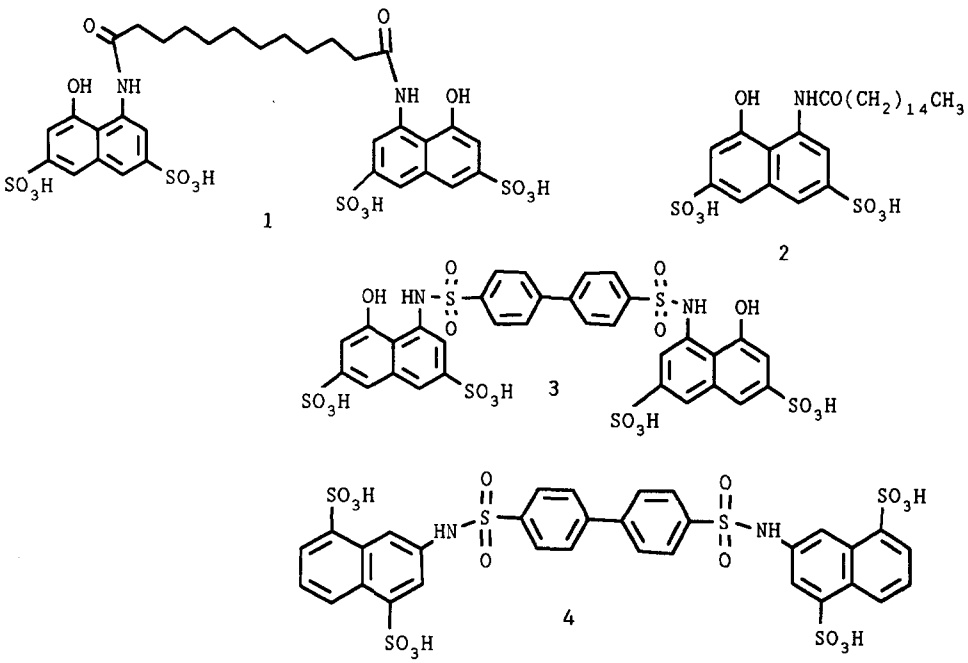

Fig. 1. Structures of naphthalenedisulfonic acid derivatives tested for anti-HIV activity.

Table 1. Inhibitory effects of naphthalenedisulfonic acid derivatives on HIV-induced cytopathogenesis in MT-4 and MOLT-4 cells

Compound	Virus	Cell	EC_{50}* (μM)	CC_{50}† (μM)	T.I.‡
1§	HIV-1	MT-4	44	430	9.8
		MOLT-4	47	>500	>10.6
	HIV-2	MT-4	52	—	—
2‖	HIV-1	MT-4	37	250	6.8
		MOLT-4	53	230	4.3
	HIV-2	MT-4	30	—	—
3¶	HIV-1	MT-4	8.3	320	38.6
		MOLT-4	35	>500	>14.3
	HIV-2	MT-4	23	—	—
4¶	HIV-1	MT-4	18	>500	>27.8
		MOLT-4	37	>500	>13.5
	HIV-2	MT-4	61	—	—
Suramin	HIV-1	MT-4	8.2	250	30.5
AZT	HIV-1	MT-4	0.0030	7.8	2600
D4T	HIV-1	MT-4	0.034	15	441.2
DDA	HIV-1	MT-4	6.8	>500	>73.5

All data are mean values for at least two separate experiments.

* Fifty percent antiviral effective concentration, based on the inhibition of HIV-induced cytopathogenesis.

† Fifty percent cytotoxic concentration, based on the reduction of viability of mock-infected cells.

‡ T.I. = *in vitro* therapeutic index.

§ Disodium salt.

‖ Monosodium salt.

¶ Tetrasodium salt.

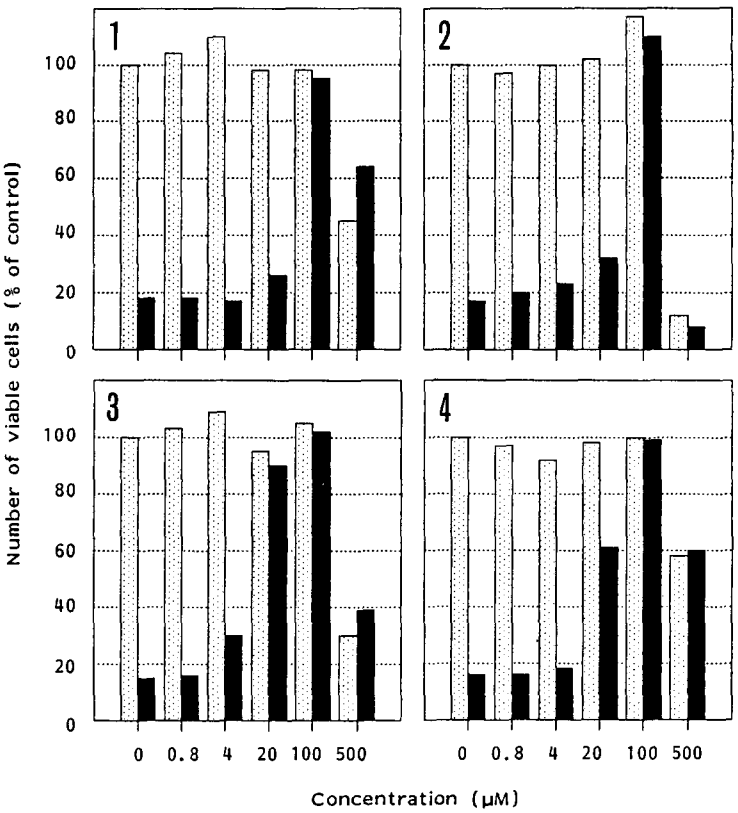

Fig. 2. Inhibitory effects of naphthalenedisulfonic acid derivatives on HIV-1 replication in MT-4 cells. These experiments were based on the inhibition of virus-induced cytopathogenesis in MT-4 cells as previously described [21]. Briefly, MT-4 and MOLT-4 cells were suspended in culture medium at 1×10^5 cells/mL and infected with HIV at a multiplicity of infection of 0.02 and 0.2 respective (50% cell culture infective dose per cell). Immediately after virus infection, 100 μL of the cell suspension was brought into each well of a microtiter tray containing various concentrations of the test compounds. After a 4-day incubation at 37°, MOLT-4 cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the compounds and further incubated. The number of viable MT-4 and MOLT-4 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method [22], on days 4 and 8 after virus infection respectively. The cytotoxicity of the compounds was determined by measuring the viability of mock-infected MT-4 and MOLT-4 cells by the MTT method. Virus infected (■) and mock-infected (▨) MT-4 cells were incubated at 37° with compounds **1–4** (see Fig. 1). The viability of MT-4 cells was measured by the MTT method on day 4 after infection.

lower than that of suramin (0.62 vs 1.6 μM). Compounds **2** and **4** demonstrated IC_{50} values of 14 and 6.8 μM respectively.

Discussion

The first drug to be used for patients with AIDS was the symmetrical bis-naphthalenetrisulfonic acid compound suramin [24]. However, suramin was not pursued any further as a mode of therapy for AIDS due to its inability to improve clinical or immunological parameters, and the appearance of considerable toxicity in patients [25]. On the other hand, on chemical and biological grounds, suramin is an intriguing molecule. It has been used for trypanosomiasis for over 50 years, and is now being studied for the treatment of cancer [26]. Suramin exhibits a half-life of 44–54 days, amongst the longest for a therapeutic drug. Furthermore, in spite of containing six amide bonds, suramin undergoes little or no metabolism *in vivo* [27].

The long half-life of suramin, contributed by its strong protein-binding property [27], may be beneficial when one considers that AZT has a half-life of approximately 1 hr [28]. However, as discussed above, suramin cannot be used as it is, and will have to be modified structurally. Indeed, it was shown that fifty-seven out of ninety suramin analogs had an inhibitory effect on HIV-1 RT, and that twenty-four of these derivatives were superior to suramin [29]. Keeping these facts in mind, we have pursued several sulfonated compounds other than suramin to investigate their potential as anti-HIV-1 agents.

In the present study, we have found that the introduction of a spacer unit consisting of a biphenyldisulfonyl or a decamethylene unit does retain anti-HIV activity in these compounds. It is noteworthy that the naphthalenesulfonic acid unit in analogs **1–3** is different from those in the anti-HIV dyes [16]. However, compound **4** has the naphthalenesulfonic acid fragment present in the anti-HIV-1 dye Direct Yellow 50 [16]. Substituting the biphenyldisulfonyl spacer (as in **3**) for the decamethylene spacer (as in **1**) increases potency against both HIV-1 and HIV-2 (Table 1). Such an increase of activity is also evident in the giant cell formation assay (data not shown) and the reverse transcriptase assay (Fig. 3).

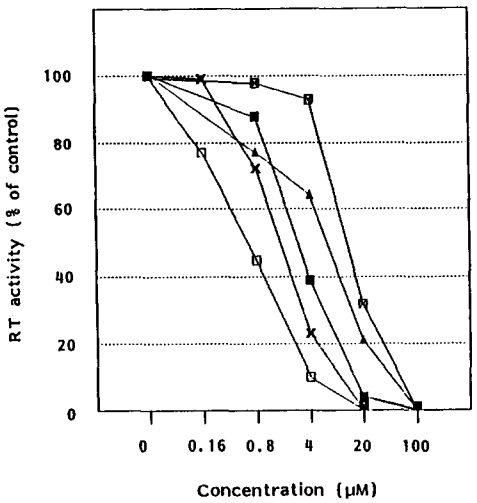

Fig. 3. Concentration–response effect for inhibition of HIV-1 reverse transcriptase activity by naphthalenedisulfonic acid derivatives. Purified recombinant HIV-1 RT (930 units/mL) was used in the assay. The assay was performed at 37° for 30 min with 50 μL reaction mixture containing 50 mM Tris–HCl (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 μCi of [*methyl*-^{3}H[dTTP (30 Ci/mmol), 0.01 units of poly(Ra)·oligo(Dt), test compound, and 0.01 units of enzyme. The reaction was quenched with 200 μL of 5% trichloroacetic acid, and the precipitated material was analyzed for radioactivity. The reaction was carried out in the presence of various concentrations of compounds **1** (■), **2** (⊠), **3** (□), **4** (▲), and suramin (×).

The detailed mechanism of action of these agents remains to be elucidated. However, their mechanism of action against reverse transcriptase may be related to the metal binding properties of these compounds. It is known that the anti-HIV dyes, Congo Red and Evans Blue, form metal complexes [30, 31]. Careful study of the naphthalenesulfonic acid moiety of compounds **1–3** shows that they also have the potential of forming metal complexes via the 4-amido-5-hydroxy functionality. This becomes pertinent when one considers that HIV-1 RT is a zinc metalloenzyme requiring magnesium for optimum activity [32].

The utility of these compounds as practical anti-HIV agents may be argued on the basis of their expected difficulty in entering cells. If this is so, prodrug modifications may enable the compounds to enter cells more easily. Keeping in mind the difficulty of cell penetration, the mechanism of action that is responsible for the inhibition of cytopathogenesis may be due to the inhibition of viral adsorption. This mechanism has been demonstrated for suramin [33] and the sulfated polysaccharides, dextran sulfate and heparin [33, 34]. Therefore, in such a case, cell penetration may not be needed to exhibit anti-HIV activity. Experiments to decipher the mechanisms for these new agents are currently being pursued.

In summary, certain naphthalenedisulfonic acid analogs have been evaluated for their inhibitory effects on the replication of human immunodeficiency virus type-1 (HIV-1) and type-2 (HIV-2), giant cell formation and HIV-1 reverse transcriptase (RT) activity. Among the evaluated compounds a bis derivative of 4-amino-5-hydroxy-2,7-naphthalenedisulfonic acid containing a semi-rigid biphenyl spacer emerged as the most potent and selective inhibitor of virus-induced cytopathogenesis in MT-4 cells. The 50% antiviral effective concentration was 8.3 and 23 μM for HIV-1 and HIV-2 respectively. The 50% cytotoxic concentration was 320 μM. The anti-HIV-1 activity of the compound was further confirmed in MOLT-4 cells. This compound also inhibited giant cell formation induced by cocultivation of uninfected MOLT-4 cells with HIV-1 chronically infected MOLT-4 cells (MOLT-4/HTLV-III_B). In the cell-free RT assay, the compound proved to be a more potent inhibitor of purified recombinant HIV-1 RT than suramin. This class of compounds represents new leads for the development of new anti-HIV agents.

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