Antiviral Research, 22 (1993) 107–119
 / 0166-3542/93/$06.00

AVR 00650

Antiviral Research

Chemical modifications of aminonaphthalenesulfonic acid derivatives increase effectivity and specificity of reverse transcriptase inhibition and change mode of action of reverse transcriptase and DNA polymerase alpha inhibition

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(Received 21 December 1992; accepted 28 June 1993)

Summary

The reverse transcriptase (RT) inhibition and the specificity of 15 aminonaphthalenesulfonic acid derivatives were examined with RT of a simian immunodeficiency virus derived from an African green monkey (SIVagmTYO-7). The two compounds with the strongest RT inhibition (NF415) or the highest specificity (NF345), together with suramin, were evaluated against polymerase alpha-primase complex from calf thymus. We have also compared the kinetics of inhibition of the viral and the cellular polymerase by these three compounds. While RT inhibition followed a mixed competitive and non-competitive mechanism, inhibition of the DNA polymerase alpha was competitive for suramin and non-competitive for NF415 and NF345. Certain structural characteristics appeared to be common for specific RT inhibitors.

Inhibition kinetics; Aminonaphthalenesulfonic acid derivatives; RT specificity

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Introduction

Simian immunodeficiency viruses (SIVs) are related to the human immunodeficiency viruses HIV-1 and HIV-2 which have been identified as the cause of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983; Popovic et al., 1984). Some SIVagm isolates obtained from African green monkeys including SIVagmTYO-7 are able to infect rhesus monkeys and induce pathological changes in the infected monkeys (Herchenröder et al., 1989). Therefore, the infection of monkeys with SIV provides an animal model for studying vaccines and drugs against AIDS.

The enzyme reverse transcriptase (RT) plays an essential role in retroviral replication. Hence, several attempts to develop an antiviral therapy for AIDS have concentrated on inhibiting the RT. Suramin (Germanin^{R}), a derivative of aminonaphthalenetrisulfonic acid, which has long been used in the treatment of trypanosomiasis and onchocerciasis (Nickel et al., 1986), is a potent RT inhibitor (De Clercq, 1979) and has been shown to inhibit HIV-1 replication in cell culture (Mitsuya et al., 1984). When administered in high doses, however, suramin is toxic and was abandoned as a potential drug against AIDS (Levine et al., 1986; Cheson et al., 1987). In 1987 we published the anti-HIV screening results of more than 90 suramin analogues (Jentsch et al., 1987). Meanwhile more than 100 further analogues have been screened (unpublished results). Some of these compounds are less toxic in vitro and more potent in inhibiting HIV RT than suramin.

Here, we propose models for the inhibition mechanism of suramin and the two newly synthesized suramin analogues NF345 and NF415, with regard to SIVagmTYO-7 RT as well as with an immunaffinity-purified DNA polymerases alpha-primase complex from calf thymus. Since part of suramin's toxicity may result from concomitant inhibition of cellular DNA polymerases the effect of suramin and suramin analogues on the viral and the cellular enzyme was compared. In addition, we have tried to correlate particular structural and physicochemical properties of the inhibitors with their in vitro effects on both enzymes. The understanding of enzyme–inhibitor interactions should facilitate systematic modifications of the inhibitor molecules leading to more potent but less toxic inhibitors.

Materials and Methods

Cell culture assay

The in vitro toxicity and the therapeutic efficiency (Jentsch et al., 1987) of the compounds were determined in an assay using MT-4 cells (Harada et al., 1986). The III_B strain of HIV-1 (Popovic et al., 1984) was grown in Jurkat cells (Wendler et al., 1987), whereas SIVagmTYO-7 (kindly provided by Prof. Dr. M. Hayami, Kyoto) was propagated in CEM cells (Foley et al., 1965) as described (Jurkiewicz et al., 1992). The MT-4 cell assay was performed in 96-

well microtiter plates. Serial dilutions of the compounds were incubated with 3 $\times$ 10^4 MT-4 cells per well, in the presence or absence of virus. The final virus concentration was 100 $TCID_{50}$ of HIV-1 or SIVagmTYO-7, respectively. Fresh medium was added to each well 3 days after infection. Four days after infection 0.1 μCi of [^{3}H]thymidine with a specific activity of 25 Ci/mmol (Amersham-Buchler, Braunschweig) was added per well. After 20 h the cellular DNA was harvested on glass fiber filters and [^{3}H]thymidine incorporation was determined by liquid scintillation counting. The log of incorporated [^{3}H]thymidine in counts/min was plotted against log of concentration of the compounds tested.

Reverse transcriptase assay

The SIVagmTYO-7 RT was purified as described (Lüke et al., 1990). Virus was harvested from 3 l of cell culture supernatant and suspended in 1.5 ml 10 mM Tris-HCl (pH 8.0), 1% Triton X-100 for preparation. RT was adjusted to an enzyme activity of 0.6 pmol dGMP incorporation per microliter in 1 min.

The reaction mixtures (50 μl) for the RT assay contained 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 0.6% Triton X-100, 30 mM KCl, 15 mM $MgCl_2$, 9 μM dGTP, 1–5 μCi of alpha-[^{32}P]dGTP, 1 μl of the RT sample and the indicated concentrations of the template-primer poly(rC):oligo$(dG)_{10}$. Template-primers were activated by heating poly(rC) and oligo$(dG)_{10}$ at 96°C for 3 min and slowly cooling to room temperature over 30 min. The molar ratio of poly(rC) to oligo$(dG)_{10}$ was 5:1. Templates and primers were purchased from Pharmacia-LKB, Freiburg, dGTP from Boehringer-Mannheim and alpha-[^{32}P]dGTP with a specific activity of 3000 Ci/mmol from Amersham-Buchler, Braunschweig.

The reaction mixtures were incubated for the indicated time at 42°C. The reactions were terminated by the addition of 20 μl of 10% SDS. Thereafter the samples were adsorbed to DEAE filters (Whatman, UK) and dried for 15 min at 80°C. The filters were extensively washed with 5% Na_2HPO_4 and once with water, then soaked in ethanol and dried at 80°C. The radioactivity was measured by liquid scintillation counting in a Packard Minaxi Tri-Carb Series 4000.

DNA polymerase alpha-primase complex

The immunaffinity-purified DNA polymerase alpha-primase complex from calf thymus was a kind gift from Dr. F. Grosse, Göttingen. The enzyme is closely related to human DNA polymerase alpha, e.g., it reacts with anti-human DNA polymerase alpha monoclonal antibodies (Nasheuer and Grosse, 1987).

The reaction mixtures (50 μl) for the DNA polymerase assay contained 20 mM Tris-acetate (pH 7.3), 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 9 μM dGTP, 1–5 μCi alpha-[^{32}P]dGTP, 1 μl of the DNA polymerase alpha sample with an enzyme activity of 6 pmol dGMP incorporation per microliter in 1 min and the indicated concentrations of poly(dC):oligo$(dG)_{10}$. The template-primer was activated and the assay was

performed as described for the RT, except that the mixture was incubated at 37°C.

Inhibitors

Suramin was purchased from Bayer, Leverkusen. The suramin analogues were synthesized by the group of Prof. Nickel using methods similar to those described earlier (Nickel et al., 1986). The synthetic procedures will be described elsewhere. Purity of the compounds was determined by HPLC and was found to be more than 98% with the exception of NF415 which had a purity of about 75%. Chemical structures of the compounds are shown in Fig. 2 and are published (Jentsch et al., 1987), respectively. The substances were dissolved in bidistilled water and dilutions from freshly prepared stock solutions were added to the reaction mixtures at 0°C.

Most of the compounds like NF032, suramin (NF060), NF070, NF150, NF156, NF171, NF201, NF212, NF213, NF252, NF260 and NF415 have a central urea group. In NF346 the central group is an isophthalic acid diamide, in NF345 and NF348 it is a terephthalic acid diamide.

NF070 is a derivative of benzenephosphonic acid. All other compounds are derivatives of the following aminonaphthalenetrisulfonic acids: 8-aminonaphthalene-1,3,5-trisulfonic acid (suramin/NF060, NF150, NF156, NF171, NF212, NF213, NF260, NF415), 8-aminonaphthalene-1,3,6-trisulfonic acid (NF032, NF345, NF346), 7-aminonaphthalene-1,3,5-trisulfonic acid (NF348), 7-aminonaphthalene-1,3,6-trisulfonic acid (NF252). NF345, NF346 and NF348 are isomers with only slight variations of the substitution pattern.

Suramin (NF060), NF171, NF212, NF213 are also very similar compounds which differ only in the substitution pattern of the methyl substituents. The molecular dimensions of NF415 are very similar to suramin and NF171.

Permeability of the viral envelope for the compounds

In order to test whether the compounds can penetrate the viral envelope, 100 μg or 500 μg of suramin, NF345 or NF415 were preincubated with a SIVagmTYO-7 pellet in 10 mM Tris-HCl, pH 8 (enzyme activity: 0.18 pmol dGMP incorporation per microliter in 1 min) for 2 h at 39°C. Thereafter, virus was pelleted for 30 min at 30000 × *g* and 4°C. After washing with 10 mM Tris-HCl (pH 8), the viral pellets were resuspended in 20 μl of the same buffer and the RT assay was performed as described.

Results

Enzyme purification and characterization

The SIVagmTYO-7 RT was purified and characterized with poly-(rC):oligo$(dG)_{10}$ as template-primer. In contrast to the respective polyadenylate-oligodeoxythymidylate, this template-primer is stable in low salt solutions (Waters et al., 1974; Modak et al., 1977) which were used because the

compounds showed different inhibition characteristics at higher salt concentrations (Jentsch et al., 1987). The dGMP incorporation rate was linear up to 30 min at 42°C (not shown).

For characterization of the DNA polymerase alpha-primase complex poly(dC):oligo(dG)$_{10}$ was used as template primer. At 37°C the reaction was linear for up to 3 min (not shown). Previous investigations have shown that the highest activity of both enzymes was obtained at a 5:1 ratio (molecules) of template to primer.

Inhibitory dose and inhibition efficiency

The inhibitor concentrations decreasing the polymerase activities by 50% (ID_{50}) were determined for suramin and 14 suramin derivatives with purified RT and viral lysate of SIVagmTYO-7, respectively (Table 1). The inhibition efficiency (IE) has been defined as the ratio of the ID_{50} obtained with viral lysate to the ID_{50} of purified RT (Lüke et al., 1990). The IE is useful to rank RT inhibitors according to their RT specificity, e.g., one would find an IE close to 1 for a specific inhibitor binding to the RT.

Among the inhibitors examined, the suramin analogue NF345 with an IE near 1 appeared to be specific and showed a low ID_{50} value. NF415, on the other hand, proved to be an unspecific but the most potent of the inhibitors. Therefore, these analogues were chosen for further examination along with the well-characterized parental substance suramin.

The ID_{50} of suramin, NF345 and NF415 for the DNA polymerase alpha-

TABLE 1

Inhibition of SIVagmTYO-7 RT by aminonaphthalenesulfonic acid derivatives (NF)

Compound	ID_{50} (lysate)[a,b]	ID_{50} (RT)[a,b]	IE[c]	Molecular weight
NF060[d]	**26**	**4**	**6.5**	**1429.2**
NF032	21	20	1	1401.1
NF070	58	22	2.6	698.4
NF150	9	4	2.2	1247.1
NF156	17	13	1.3	1198.9
NF171	14	7.8	1.8	1429.2
NF201	9	13	0.7	1315.1
NF212	7	10	0.7	1457.3
NF213	18	16	1.1	1429.2
NF252	14	8	1.7	1191.0
NF260	14	3.5	4	1461.2
NF345	**9**	**7**	**1.3**	**1266.2**
NF346	16	5	3.2	1266.2
NF348	25	5	5	1266.2
NF415	**5.7**	**0.66**	**8.6**	**1395.4**

[a]The ID_{50} is given in $\mu g \cdot ml$.

[b]Single ID_{50} values were determined at least five times. The difference between single values and the mean value was in the range of $\pm$ 10%.

[c]The inhibition efficiency IE is defined as the ratio of the ID_{50} (lysate) to the ID_{50} (purified RT).

[d]Suramin.

Substances in bold are those chosen for kinetic inhibition analysis.

primase complex were evaluated. Again, NF415 turned out to have the highest inhibitory activity with an ID_{50} of <0.1 μg/ml, while inhibition by NF345 with an ID_{50} of 13 μg/ml was about 100 times lower. The ID_{50} of suramin was 1.7 μg/ml.

Toxicity and therapeutic efficiency

To test whether suramin, NF345 and NF415 are inhibitory to SIV replication in cell culture, their in vitro toxicity and their therapeutic efficiency was determined. Different doses of these analogues were added to uninfected and SIVagmTYO-7-infected MT-4 cells. At concentrations of 500 μg/ml no toxicity of the compounds was observed. At concentrations of 500 μg/ml of suramin and NF345, respectively, MT-4 cells were protected against experimental SIV infection, whereas no protective effect could be observed with NF415. However, all three substances were able to protect MT-4 cells against infection with HIV-1; suramin at a concentration of 50 μg/ml, and NF345 as well as NF415 at a concentration of 5 μg/ml.

Permeability of the viral envelope for suramin, NF345 and NF415

At a concentration of 100 μg of the compounds mentioned above for preincubation with pellet of SIVagmTYO-7 no effect was observed with suramin, whereas RT inhibition was 20% with NF345 and 50% with NF415. At a concentration of 500 μg RT inhibition was 54% with suramin and 100% with NF345 and NF415.

Inhibition kinetics

In order to distinguish between different inhibition mechanisms, we analyzed the incorporation kinetics of SIVagmTYO-7 RT and DNA polymerase alpha-primase complex in the presence of the inhibitors suramin, NF345 and NF415. Fig. 1 shows the effect of various concentrations of the inhibitors at a constant dGTP concentration on the initial incorporation velocity with template-primer as the variable substrate.

With SIVagmTYO-7 RT suramin and NF415 showed a non-competitive inhibition pattern at low inhibitor concentrations (up to 20 μM suramin and up to 0.37 μM NF415) whereas the inhibition appeared to be competitive at higher concentrations (50–100 μM suramin and 0.51–0.88 μM NF415) (Fig. 1a,e). These different mechanisms are emphasized in the slope and intercept replots (slope or intercept, respectively, against inhibitor concentrations) of the primary double-reciprocal plots (not shown). In comparison, competitive inhibition of DNA polymerase alpha-primase complex by suramin and non-competitive inhibition by NF415 were observed at all concentrations (Fig. 1b,f). The intercept and slope replots were linear.

With NF345 as inhibitor, a different pattern of RT inhibition was observed. The inhibition appeared to be competitive at low concentrations (0–5.5 μM) but non-competitive at high concentrations (15.8–23.6 μM) (Fig. 1c). In contrast, DNA polymerase alpha-primase complex inhibition was non-

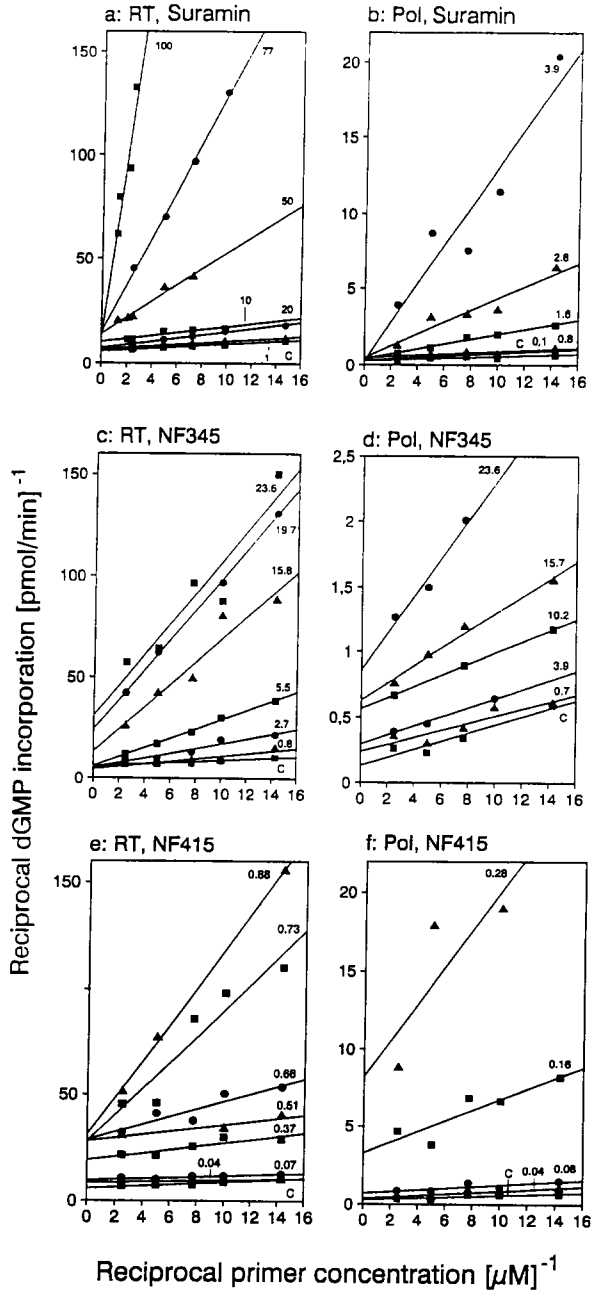


Fig. 1. Lineweaver-Burk plots of dGMP incorporation kinetics of SIVagmTYO-7 RT (RT) and DNA polymerase alpha-primase complex from calf thymus (Pol) vs. primer concentration for different inhibitor concentrations. The concentrations (μM) of the inhibitors are indicated (C [control], no inhibitor). Primers used were poly(rC):oligo(dG)$_{10}$ with RT and poly-(dC):oligo(dG)$_{10}$ with polymerase alpha activity. The molar ratio of template to primer was 5:1 (molecules). The reactions were terminated after 20 (RT) or 4 min (DNA polymerase alpha), respectively. Single values were obtained from at least six independent measurements. The difference between single values and the mean value plotted was in the range of $\pm$ 10%.

TABLE 2

Inhibition constants of suramin, NF345 and NF415 with SIVagmTYO-7 RT and DNA polymerase alpha-primase complex

Enzyme	Inhibitor	Competitive	Non-competitive	
		k_i[a] (μM primer)	k_i[a] (μM primer)	k_{ii}[b] (μM primer)
SIVagmTYO-7 RT				
	NF060	0.4	15.3	29.4
	NF345	0.9	1.1	2.6
	NF415	0.02	0.2	0.2
DNA polymerase alpha				
	NF060	0.09	–	
	NF345	–	5.7	7.6
	NF415	–	0.01	0.01

[a]The inhibition constant k_i is defined as the ratio of [E][I] to [EI] (E = enzyme; I = inhibitor). It was determined according to $k_i = k_m / v_{max}$ · slope (of intercept/[I] replot).
[b]The inhibition constant k_{ii} is defined as the ratio of [ES][I] to [ESI] (S = substrate). It was determined according to $k_{ii} = 1/v_{max}$ · slope (of slope/[I] replot)

competitive at all inhibitor concentrations examined (Fig. 1d).

Inhibition constants were derived from the intercept replots (in case of a non-competitive mechanism) and the slope replots (in case of a competitive mechanism), respectively (Table 2). Whereas RT inhibition by suramin and NF415 appeared to be lower than inhibition of the DNA polymerase alpha activity, the opposite could be shown for NF345. Therefore, among the examined substances NF345 seemed to have the highest specificity for RT.

Discussion

Earlier investigations (Jentsch et al., 1987) have shown, that among the investigated suramin analogues only symmetric polyanionic compounds are potent HIV-RT inhibitors. Our findings have been confirmed by Mohan et al. (1991a,b) who describe the anti-HIV screening of some symmetric naphthalene disulfonic acid derivatives. They find that only rigid molecules are active. A compound in which two 1-hydroxynaphthalene-3,6-disulfonic acid residues are attached to – NH-CO-$(CH_2)_{10}$-CO-NH- – is completely inactive, whereas an analogue a NH-SO_2-C_6H_4-C_6H_4-SO_2-NH bridge shows inhibitory activity

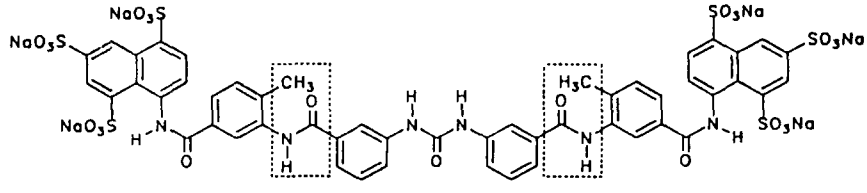
The compounds described in the present investigation are also symmetrical molecules with different degrees of rigidity in which two identical moieties are attached to three different central groups (see Materials and Methods).

Fifteen suramin analogues were tested for their ability to inhibit SIVagmTYO-7 RT. With suramin and the two most promising suramin analogues, NF345 and NF415, we then examined the inhibition of DNA

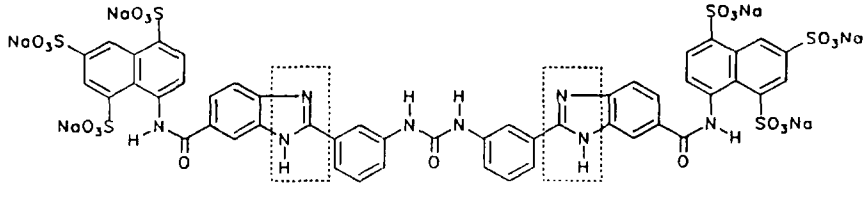
polymerase alpha-primase complex from calf thymus. Suramin and NF415 are unspecific RT inhibitors, i.e., they bind to RT as well as to other proteins and inhibition of DNA polymerase alpha activity is within one order of magnitude. In contrast, NF345 appears to be specific for RT and inhibits host DNA polymerase – judged by its ID_{50} – to a lesser extent than RT.

These three analogues are not toxic for MT-4 cells. In contrast to suramin and NF345 the most unspecific RT inhibitor NF415 showed no therapeutic effect against SIV infection in MT-4 cells.

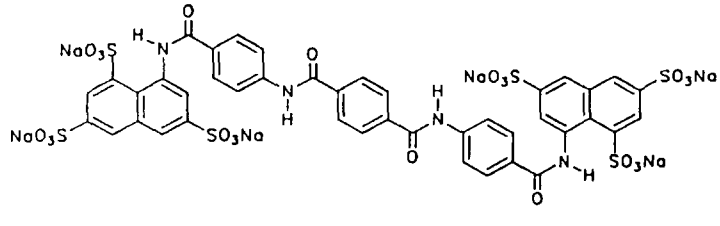
The inhibition mechanisms were characterized with double-reciprocal plots as well as slope and intercept replots. Whereas inhibition of SIVagmTYO-7 proved to follow a mixed mechanism with, depending on the inhibitor concentration, competitive and non-competitive contributions, DNA polymerase alpha activity inhibition was either competitive (suramin) or non-competitive (NF345, NF415). The mixed mechanisms point to several binding



NF060



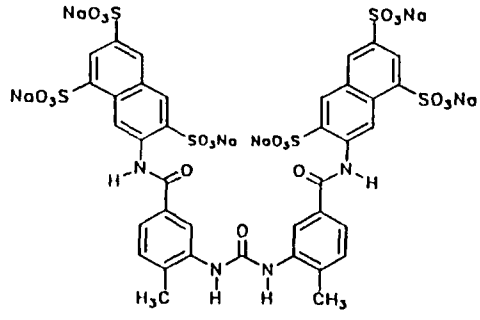
NF415



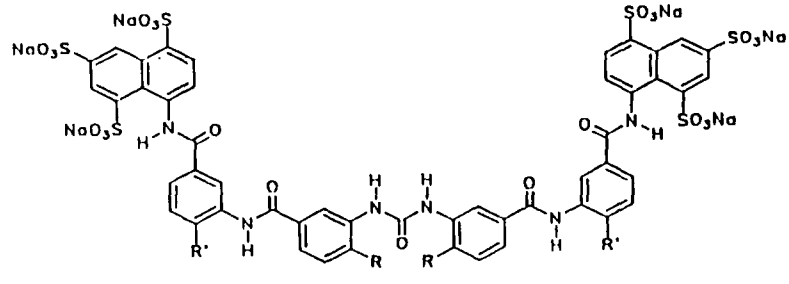
NF345

sites for the inhibitors on the RT.

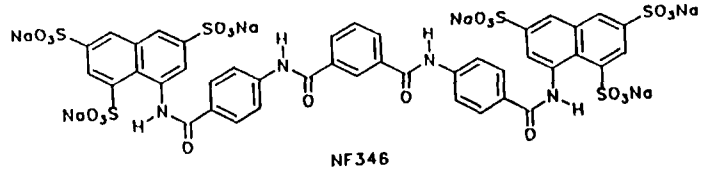
The exchange of a benz(2-methylanilide) group in suramin or NF171 by a 2-



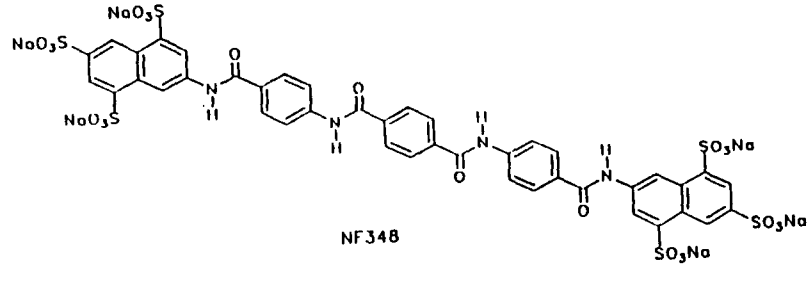
NF252



NF260 R' = $-OCH_3$ R = -H



NF346



NF348

← ↑

Fig. 2. Structural formulas of suramin (NF060), NF415, NF345, NF252, NF260, NF346 and NF348. The framed areas indicate differences between NF060 and NF415.

phenylbenzimidazolyl group at two sites of the molecules leads to NF415. Thereby, intramolecular rotation, which should be possible in suramin to a certain extent, is abolished totally at four bonds within the NF415 molecule (Fig. 2; framed areas). Therefore, NF415 is a still more rigid molecule than suramin. In addition to these stereochemical differences, there are physicochemical differences as well. In contrast to suramin, NF415 contains two basic benzimidazole groups which are partially protonated at physiological pH values. These stereochemical and physicochemical differences should substantially influence the binding of these inhibitors to proteins. This is reflected by their different mode of action with DNA polymerase alpha as well as by the ID_{50} which is considerably lower for NF415 than for suramin with both enzymes.

In comparison with both suramin and NF415, the distance of the two naphthalene rings in the stretched molecule of NF345 is smaller. Furthermore, the substitution pattern of the sulfonic acid substituents in the naphthalene rings is different and the central carbonyl group which is part of the urea group of suramin has been replaced by a terephthaloyl group in NF345. Therefore, differences in the binding of these three compounds to a specific binding site could be expected. A better fitting of NF345 to such a binding site could be one reason for its RT specificity. Since binding of this class of polyanionic inhibitors to proteins is probably achieved by interactions between negatively charged sulfonic groups of the inhibitor molecules and positively charged groups of the enzymes the distance of the sulfonic groups in the inhibitor molecules might be important as well. The investigation of further analogues of NF345 and NF415 should clarify the relationship between structural and inhibitory features.

Suramin and its analogues presented here are very polar substances and it seems questionable whether they can penetrate cell membranes. Further experiments are necessary to address this question. In this paper, however, we show that the examined substances when added to viral pellets are able to enter free virus particles. Therefore, we believe that RT inhibition by these substances might be one of the mechanisms by which they protect cells against infection by immunodeficiency viruses.

Acknowledgements

The authors thank Dr. F. Grosse for the generous gift of the DNA polymerase alpha-primase complex, Drs C. Biebricher and R. Clegg for helpful advice and critical reading of the manuscript and J. Wilde for editorial help. This work was supported by a grant from the Bundesgesundheitsamt (BGA) (III-003-89/FVP).

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