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ORIGINAL ARTICLE

**Antonio R.T. Gagliardi · Matthias Kassack
Annett Kreimeyer · Guido Muller · Peter Nickel
Delwood C. Collins**

Antiangiogenic and antiproliferative activity of suramin analogues

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Abstract The purpose of this study was to test the ability of 70 polyanionic analogues of suramin to inhibit angiogenesis. The ID_{50}, the dose that produced 50% inhibition of angiogenesis, was determined for suramin and each of the analogues by measuring the ability of various amounts to inhibit angiogenesis in vivo in the chick egg chorioallantoic membrane (CAM) assay. Of the 70 analogues, 11 had antiangiogenic activities similar to suramin and an additional 7 were significantly more potent than suramin. All seven of these analogues were from the naphthalenetrisulfonic acid group and contained large urea groups. The benzene sulfonic and disulfonic acid analogues were less active inhibitors of angiogenesis than the naphthalenetrisulfonic acid analogues. Replacement of the naphthalenetrisulfonic acid groups by aliphatic carboxylic acids or benzoic acid gave analogues with very little antiangiogenic activity. In subsequent experiments, the antiproliferative activity of selected analogues on basic FGF (bFGF)-stimulated growth of immortalized human microvascular endothelial cells in vitro was determined. Analogues that inhibited angiogenesis to a greater extent than suramin in the CAM assay generally showed a greater antiproliferative effect on bFGF-induced growth of human microvascular endothelial cells. These results suggest that some of the polyanionic analogues may be potent therapeutic agents for cancers and angiogenesis-dependent diseases.

A.R.T. Gagliardi · D.C. Collins
VA Medical Center and Departments
of Obstetrics/Gynecology and Physiology,
University of Kentucky College of Medicine,
Lexington, KY, USA

M. Kassack · A. Kreimeyer · G. Muller · P. Nickel
Pharmaceutical Institute, University of Bonn, Bonn, Germany

D.C. Collins (✉)
204 HSRB, UK Medical Center, Lexington,
KY 40536-0305, USA
Tel. +1 606-323-5293; Fax +1 606-257-9700

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Introduction

We have previously reported for the first time [10] that suramin alone is an effective inhibitor of angiogenesis in the chick chorioallantoic membrane (CAM) assay. Our results showed that suramin inhibits angiogenesis in a dose-dependent manner. An important finding was the antagonism between suramin and heparin. These results support the hypothesis that suramin may interfere with the effects of heparin-binding growth factors, such as basic FGF (bFGF), VEGF and PDGF, on angiogenesis. Suramin has been recently shown to inhibit endothelial cell binding of bFGF, endothelial cell migration and bFGF induction of urokinase-type plasminogen activator [23]. A major problem encountered with suramin during clinical trials has been the adverse neurotoxic side effects. These effects are partly related to the prolonged half-life in vivo (45–55 days). This prolonged half-life is a consequence of being tightly bound to serum proteins, mainly albumin [2], and limited metabolism [22]. The narrow margin between the dose for antitumor activity and toxic effects prompted us to look for suramin analogues with similar or more potent antiangiogenic activity and for reduced protein binding and toxicity than suramin.

It has been known for many years that a small variation in the structure of suramin leads to great changes in the trypanocidal activity. For example, replacement of the two methyl groups of suramin by hydrogen reduces the trypanocidal activity by 95% [8]. In contrast, inhibition of HIV-1 reverse transcriptase is less sensitive to structural modifications and the structure–activity relationships are completely different from those of its trypanocidal or antifilarial activity [11]. Braddock et al. [3] investigated the structure–activity relationships for antagonism on the growth factor and angiogenic activity of bFGF by suramin and a limited number of related

polyanions. They examined 16 polyanionic analogues and found that four express bFGF-blocking activity equipotent to that of suramin in vitro. However, compounds with two bridging aromatic groups are less toxic than suramin in mice, suggesting a potential for an improved therapeutic ratio. These observations suggest that the study of a large number of suramin analogues could substantially widen the therapeutic opportunities for this class of compounds.

Microvascular endothelial proliferation is postulated to be a key event in the complex process of tumor angiogenesis [5]. Other steps include endothelial cell migration, secretion of metalloproteinases, the formation of capillaries and anastomosis [1]. Considering our findings that suramin can inhibit angiogenesis in the CAM assay and the report by Pesenti et al. [20] that suramin can inhibit tumor-induced angiogenesis, we examined some structurally related analogues of suramin for their ability to inhibit angiogenesis in the CAM assay and bFGF-stimulated human microvascular endothelial cell proliferation. A total of 70 suramin analogues synthesized by Nickel and coworkers [11, 16, 17] were purified and examined for their ability to inhibit angiogenesis in the CAM assay. Selected analogues were also tested for their ability to inhibit bFGF-stimulated endothelial cell growth in vitro. Structural features of the suramin analogues important for the expression of antiangiogenic activity were identified.

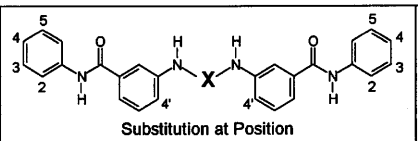
Material and methods

Structure of the analogues

The purity of the 70 suramin analogues studied was determined by high-pressure liquid chromatography before use [12]. These analogues were derivatives of naphthalenetrisulfonic acids, naphthalenedisulfonic acids, benzene sulfonic acids, benzoic acids and aliphatic carboxylic acids. The chemical structures, molecular formulae and molecular weights are shown in Figs 1–4.

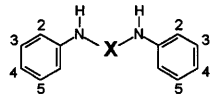
Chorioallantoic membrane assay

This assay determined the ability of the suramin analogues to inhibit angiogenesis in vivo [9, 10]. Specific pathogen-free fertile eggs (Sunrise Farms, Catskill, N.Y.) were incubated for 72 h in a horizontal position in a humidified Petersine Hatching Incubator at 37 °C. After 72 h, the egg shells were broken and the egg contents were placed in 20 × 100 mm plastic petri dishes (Falcon #1005). The petri dishes containing the egg contents were then placed in a Forma Scientific incubator at 37 °C in 3% CO_2/air and 98% humidity. Each compound to be tested was dissolved in 0.45% methylcellulose in water and a 10 µl aliquot of this solution was air dried on a Teflon-coated metal tray (forming a disk around 2 mm diameter) and implanted on the outer third of a 6-day CAM where capillaries were intensively growing. The zone around the methylcellulose disk was examined 48 h after implantation with a Wild M8 stereomicroscope. A positive inhibition of angiogenesis was indicated by an avascular area of ≥ 4 mm. At least 20 embryos were measured for each amount of analogue tested and the results are expressed as the percentage of embryos that showed inhibition. The methylcellulose disk only and suramin (70 nmol/disk) were used as



No	Code	Formula	MW	Substitution at Position -2	-3	-4	-5	-4'	-X-	# Embryos	% Inhibition
Naphthalenetrisulfonic acid derivatives											
1	NF060 Suramin	$C_{51}H_{34}N_6O_{23}S_6Na_6$	1429.2	$-CH_3$	-H	-H	-CO-A01	-H	-B1-	67	64.2
2	NF127	$C_{53}H_{38}N_6O_{23}S_6Na_6$	1457.2	$-C_2H_5$	-H	-H	-CO-A01	-H	-B1-	23	80.0
3	NF151	$C_{55}H_{42}N_6O_{23}S_6Na_6$	1485.3	$-CH(CH_3)_2$	-H	-H	-CO-A01	-H	-B1-	20	89.0
4	NF145	$C_{57}H_{46}N_6O_{23}S_6Na_6$	1513.3	$-C(CH_3)_3$	-H	-H	-CO-A01	-H	-B1-	22	100.0
5	NF157	$C_{49}H_{28}F_2N_6O_{23}S_6Na_6$	1437.1	-F	-H	-H	-CO-A01	-H	-B1-	21	53.0
6	NF171	$C_{51}H_{34}N_6O_{23}S_6Na_6$	1429.2	$-CH_3$	-H	-CO-A01	-H	-H	-B1-	22	59.0
7	NF212	$C_{53}H_{38}N_6O_{23}S_6Na_6$	1457.2	$-CH_3$	-H	-H	-CO-A01	$-CH_3$	-B1-	21	46.0
8	NF280	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-H	-H	-CO-A01	-H	-H	-B1-	21	66.0
9	NF032	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-H	-H	-CO-A02	-H	-H	-B1-	21	66.0
10	NF061	$C_{54}H_{38}N_6O_{24}S_6Na_6$	1485.2	$-CH_3$	-H	-H	-CO-A01	-H	-B2-	21	15.0
11	NF066	$C_{54}H_{36}N_6O_{24}S_6Na_6$	1483.2	$-CH_3$	-H	-H	-CO-A01	-H	-B3-	20	37.0
12	NF299	$C_{56}H_{42}N_8O_{24}S_6Na_6$	1541.3	$-CH_3$	-H	-H	-CO-A01	-H	-B4-	23	35.0
13	NF064	$C_{58}H_{38}N_6O_{24}S_6Na_6$	1535.3	$-CH_3$	-H	-H	-CO-A01	-H	-B5-	22	76.0
14	NF059	$C_{58}H_{40}N_6O_{24}S_6Na_6$	1533.3	$-CH_3$	-H	-H	-CO-A01	-H	-B6-	24	83.0
Benzenesulfonic acid derivatives											
15	NF062	$C_{43}H_{34}N_6O_{11}S_2Na_2$	920.9	$-CH_3$	-H	-H	-CO-A11	-H	-B1-	23	7.0
16	NF250	$C_{29}H_{24}N_4O_9S_2Na_2$	682.6	-H	$-SO_3Na$	-H	-H	$-CH_3$	-B1-	20	39.0
17	NF251	$C_{29}H_{22}N_4O_{15}S_4Na_4$	886.7	$-SO_3Na$	-H	-H	$-SO_3Na$	$-CH_3$	-B1-	22	17.0
Carboxylic acid derivatives											
18	NF041	$C_{37}H_{44}N_6O_7$	684.8	-H	-H	$-CO_2Na$	-H	-H	-B1-	22	16.0
19	NF052	$C_{33}H_{28}N_4O_7Na_2$	638.6	$-CH_3$	-H	-H	$-CO_2Na$	$-CH_3$	-B1-	20	-0-
20	NF054	$C_{29}H_{20}N_4O_7Na_2$	582.5	-H	$-CO_2Na$	-H	-H	-H	-B1-	21	-0-
21	NF055	$C_{31}H_{24}N_4O_7Na_2$	610.5	$-CH_3$	-H	-H	$-CO_2Na$	-H	-B1-	23	-0-
22	NF077	$C_{41}H_{36}N_6O_{13}Na_4$	912.7	$-CH_3$	-H	-H	-CO-A15	-H	-B1-	23	-0-
23	NF092	$C_{39}H_{32}N_6O_{13}Na_4$	884.7	$-CH_3$	-H	-H	-CO-A16	-H	-B1-	20	-0-
24	NF186	$C_{35}H_{28}N_6O_{15}S_2Na_4$	928.7	-H	$-SO_2$-A14	-H	-H	-H	-B1-	21	-0-
25	NF191	$C_{35}H_{28}N_6O_{15}S_2Na_4$	928.7	-H	-H	$-SO_2$-A14	-H	-H	-B1-	24	-0-

Fig. 1 Suramin analogues containing large urea groups. The code number, chemical structures, molecular weight, number of eggs tested and the percent inhibition of angiogenesis in the CAM assay on treatment with approximately 70 nmol/disk of the analogues are shown. The structure of acidic groups -R and central bridges -X- are shown in Fig. 4. The percent inhibition of angiogenesis was determined after implanting approximately 70 nmol of each suramin analogue in 10 µl of 0.45% methylcellulose. Implants were made in day-6 CAM and read 48 h later



No	Code	Formula	MW	Substitution at Position -2	-3	-4	-5	-X-	# Embryos	% Inhibition
Naphthalenetrisulfonic acid derivatives										
26	NF023	$C_{35}H_{20}N_4O_{21}S_6Na_6$	1162.9	-H	-CO-A01	-H	-H	-B1-	25	36.0
27	NF103	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-CO-A01	-H	-H	-B1-	20	10.0
28	NF150	$C_{41}H_{32}N_4O_{21}S_6Na_6$	1247.0	$-CH(CH_3)_2$	-H	-H	-CO-A01	-B1-	22	44.0
29	NF170	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-CO-A01	-H	-B1-	23	29.0
30	NF192	$C_{47}H_{28}N_4O_{21}S_6Na_6$	1315.1	-H	$-C_6H_4$-4-CO-A01	-H	-H	-B1-	21	47.0
31	NF201	$C_{47}H_{28}N_4O_{21}S_6Na_6$	1315.1	$-C_6H_4$-3-CO-A01	-H	-H	-H	-B1-	26	62.0
32	NF013	$C_{35}H_{20}N_4O_{21}S_6Na_6$	1162.9	-H	-H	-CO-A02	-H	-B1-	23	19.0
33	NF248	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-H	-CO-A02	-B1-	24	11.0
34	NF249	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-H	-CO-A03	-B1-	21	12.0
35	NF252	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-H	-CO-A04	-B1-	22	22.0
Naphthalenedisulfonic acid derivatives										
36	NF289	$C_{35}H_{22}N_4O_{15}S_4Na_4$	958.8	-H	-H	-CO-A05	-H	-B1-	20	50.0
37	NF290	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A05	-B1-	22	11.0
38	NF298	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A07	-B1-	20	-0-
39	NF326	$C_{35}H_{22}N_4O_{17}S_4Na_4$	990.8	-H	-CO-A09	-H	-H	-B1-	22	19.0
40	NF340	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A06	-B1-	23	41.0
41	NF291	$C_{35}H_{22}N_4O_{15}S_4Na_4$	958.8	-H	-H	-CO-A08	-H	-B1-	23	30.0
42	NF294	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A08	-B1-	21	20.0
43	NF338	$C_{42}H_{26}N_4O_{16}S_4Na_4$	1062.9	-H	-H	-CO-A06	-H	-B5-	20	13.0
44	NF339	$C_{42}H_{26}N_4O_{16}S_4Na_4$	1062.9	-H	-H	-CO-A06	-H	-B6-	21	41.0
45	NF341	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A06	-B5-	22	28.0
46	NF342	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A06	-B6-	23	30.0
47	NF383	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A07	-B6-	20	58.0
48	NF293	$C_{42}H_{26}N_4O_{16}S_4Na_4$	1062.9	-H	-H	-CO-A08	-H	-B6-	24	75.0
49	NF324	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A08	-B6-	23	58.0
Benzenesulfonic acid derivatives										
50	NF110	$C_{41}H_{28}N_6O_{17}S_4Na_4$	1096.9	-H	-CO-A11	-H	-CO-A11	-B1-	32	65.0
51	NF442	$C_{45}H_{36}N_6O_{17}S_4Na_4$	1153.0	-CO-A12	-H	-H	-CO-A12	-B1-	20	37.0
52	NF109	$C_{20}H_{15}N_3O_8S_2Na_2$	535.4	-H	-H	$-SO_3Na$	-H	-B7-	23	-0-
53	NF241	$C_{18}H_{18}N_4O_8S_2Na_2$	528.5	-H	-H	$-SO_3Na$	-H	-B4-	24	26.0
54	NF440	$C_{52}H_{40}N_6O_{18}S_4Na_4$	1257.1	-H	-CO-A12	-H	-CO-A12	-B6-	23	62.0
55	NF443	$C_{52}H_{40}N_6O_{18}S_4Na_4$	1257.1	-CO-A12	-H	-H	-CO-A12	-B6-	20	20
Carboxylic acid derivatives										
56	NF042	$C_{23}H_{18}N_4O_{11}Na_4$	618.4	-H	-CO-A14	-H	-H	-B1-	23	-0-
57	NF072	$C_{23}H_{18}N_4O_{11}Na_4$	618.4	-H	-H	-CO-A14	-H	-B1-	24	-0-
58	NF076	$C_{27}H_{26}N_4O_{11}Na_4$	674.5	$-CH_3$	-H	-H	-CO-A15	-B1-	22	-0-
59	NF091	$C_{25}H_{22}N_4O_{11}Na_4$	646.4	$-CH_3$	-H	-H	-CO-A16	-B1-	21	-0-
60	NF178	$C_{25}H_{22}N_4O_{11}Na_4$	646.4	$-CH_3$	-H	-H	-CO-A14	-B1-	22	4.0
61	NF230	$C_{23}H_{10}F_2N_4O_{11}Na_4$	654.3	-F	-H	-H	-CO-A14	-B1-	20	23.0

Fig. 2 Suramin analogues containing small urea groups. The code number, chemical structures, molecular weight, number of eggs tested and the percent inhibition of angiogenesis in the CAM assay on treatment with approximately 70 nmol/disk of the analogues are shown. The structure of acidic groups -R and central bridges -X- are shown in Fig. 4. The percent inhibition of angiogenesis was determined after implanting approximately 70 nmol of each suramin analogue in 10 µl of 0.45% methylcellulose. Implants were made in day-6 CAM and read 48 h later

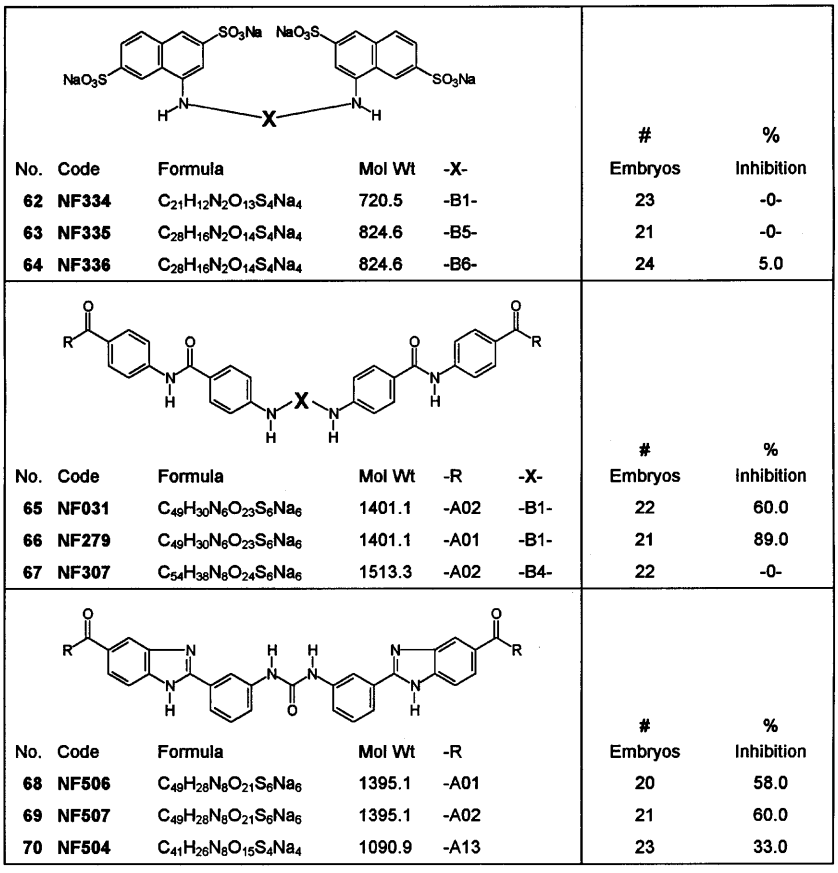
negative and positive controls, respectively. The range of dose levels was chosen to fit a response curve for each suramin analogue that showed inhibition the same or more than suramin at 70 nmol/disk.

Standard statistical software (Procedure Probit on the SAS system) allows fitting parametric families of dose response curves with the normal probit model. The detailed application of the method conforms with that given by Finney [6]. The ID_{50}, the dose that produced 50% inhibition, was calculated by separate response curves to the logarithm of dose. A subsequent plot of the data suggested a parallel lines assay model could be used for comparing the dose response curves. In a parallel lines assay model, the slope of the regression on the log of the dose was assumed to be the same for suramin and its analogues. The response curves differed most in their intercepts. The chi-square goodness of fit test for this model was not significant ($P = 0.30$), indicating that this model is a reasonable fit for the combined data. A statistical comparison of intercepts in this model is equivalent to a comparison of percentiles, such as the ID_{50}, since two response curves have different ID_{50} values only if they have different intercepts under the parallel lines assay model.

In vitro human microvascular endothelial cells

This cell line (a generous gift of Dr. Thomas J. Lawley, Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia) was transfected and immortalized by simian virus 40 larger T antigen and used for in vitro studies of the effect of suramin and selected analogues on bFGF-stimulated growth of these endothelial cells. These cells retained the characteristics of endothelial cells. The culture medium was endothelial basal medium (Clonetics, Santa Anna, Calif.) with 15% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 1 m*M* glutamine, 0.5 m*M* dibutyl cyclic AMP (Sigma Chemical Co., St Louis, Mo.), 1 µg/ml hydrocortisone acetate (Sigma Chemical Co.), 1 ng/ml epidermal growth factor (Clonetics), 100 U/ml penicillin, 100 U/ml streptomycin and 250 µg/ml amphotericin B (Sigma Chemical Co.).

Subconfluent human microvascular endothelial cells were grown in the presence of 20 ng/ml of bFGF with various amounts of each analogue for 72 h at 37 °C in 5% CO_2/air. Stock solutions of the analogues in water were prepared and various aliquots were added to the culture to establish an inhibition curve and to determine the IC_{50}, the concentration of the analogue in the cell culture



No.	Code	Formula	Mol Wt	-X-	# Embryos	% Inhibition
62	NF334	$C_{21}H_{12}N_2O_{13}S_4Na_4$	720.5	-B1-	23	-0-
63	NF335	$C_{28}H_{16}N_2O_{14}S_4Na_4$	824.6	-B5-	21	-0-
64	NF336	$C_{28}H_{16}N_2O_{14}S_4Na_4$	824.6	-B6-	24	5.0

No.	Code	Formula	Mol Wt	-R	-X-	# Embryos	% Inhibition
65	NF031	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-A02	-B1-	22	60.0
66	NF279	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-A01	-B1-	21	89.0
67	NF307	$C_{54}H_{38}N_8O_{24}S_6Na_6$	1513.3	-A02	-B4-	22	-0-

No.	Code	Formula	Mol Wt	-R	# Embryos	% Inhibition
68	NF506	$C_{49}H_{28}N_8O_{21}S_6Na_6$	1395.1	-A01	20	58.0
69	NF507	$C_{49}H_{28}N_8O_{21}S_6Na_6$	1395.1	-A02	21	60.0
70	NF504	$C_{41}H_{26}N_8O_{15}S_4Na_4$	1090.9	-A13	23	33.0

Fig. 3 Miscellaneous suramin analogues with various bridge structures. The code number, chemical structures, molecular weight, number of eggs tested and the percent inhibition of angiogenesis in the CAM assay on treatment with approximately 70 nmol/disk of the analogues are shown. The structure of acidic groups -R and central bridges -X- are shown in Fig. 4. The percent inhibition of angiogenesis was determined after implanting approximately 70 nmol of each suramin analogue in 10 µl of 0.45% methylcellulose. Implants were made in day-6 CAM and read 48 h later

that produced 50% inhibition. The IC_{50} was calculated by the statistical methods described above. Protein levels were determined by a modification of the Lowry procedure in which sodium dodecyl sulfate (SDS) was added to dissolve the proteolipids [14].

Results

The antiangiogenic activities of the 70 suramin analogues were determined in the CAM assay in the presence of 70 nmol of the suramin analogues. The chemical structure, molecular weight and percent inhibition of angiogenesis on treatment with 70 nmol of each suramin analogue tested are shown in Figs. 1–4. Statistical analysis of the results indicated that an inhibition of 50% or higher in the CAM assay with 70 nmol of the analogue had an antiangiogenic activity equal to or greater than suramin. Of the 70 analogues tested, 21 showed an inhibition of 50% or greater in the CAM assay in the presence of 70 nmol of the analogues. The chemical group with the highest activity was the naphthalenesulfonic acid analogues group (6 sulfonic acid groups) with large urea groups (Fig. 1) where 10 of 14 analogues showed an inhibition of 50% or higher at 70 nmol in the CAM assay. Only 1 of 10 naphthalenesulfonic acid analogues with a small urea group (Fig. 2) showed an inhibition $\geq 50\%$ at 70 nmol/disk. Four of 14 naphthalenedisulfonic acid analogues and 3 of 7 benzene sulfonic acid analogues showed $\geq 50\%$ inhibition at 70 nmol/disk (Fig. 2). This includes analogue no. 70 (NF504), which has a different structure (Fig. 3). None of the 14 carboxylic acid analogues (Figs. 1 and 2) showed any significant inhibition in the CAM assay at 70 nmol of the analogue.

Dose response curves for the inhibition of angiogenesis were established for the analogues in the CAM assay for suramin and 19 of the 21 analogues which showed antiangiogenic activity the same or more than suramin at a dose of 70 nmol/disk (see Figs. 1–4). Inadequate amounts of analogues no. 5 (NF157) and no. 9 (NF032) were available for this experiment. The ID_{50} was calculated from the dose response curves (see Table 1). The ID_{50} value of suramin was 75 nmol/disk. All except no. 47 (NF383) of the analogues shown in Table 1 had ID_{50} values similar to or less than suramin, indicating that their ability to inhibit angiogenesis was the same or more than that of suramin.

Statistical analysis of the dose response curves and the calculated ID_{50} values for suramin and the 19 analogues indicated that analogues with ID_{50} values in the range of 56–94 nmol/disk showed antiangiogenic activity equivalent to suramin (75 nmol/disk). An ID_{50} of less than 55 nmol/disk indicated that the analogue was significantly more potent than suramin as an inhibitor of angiogenesis in the CAM assay ($P \geq 0.05$). Of the 19 analogues, 7 had ID_{50} values ≤ 55 nmol/disk (range

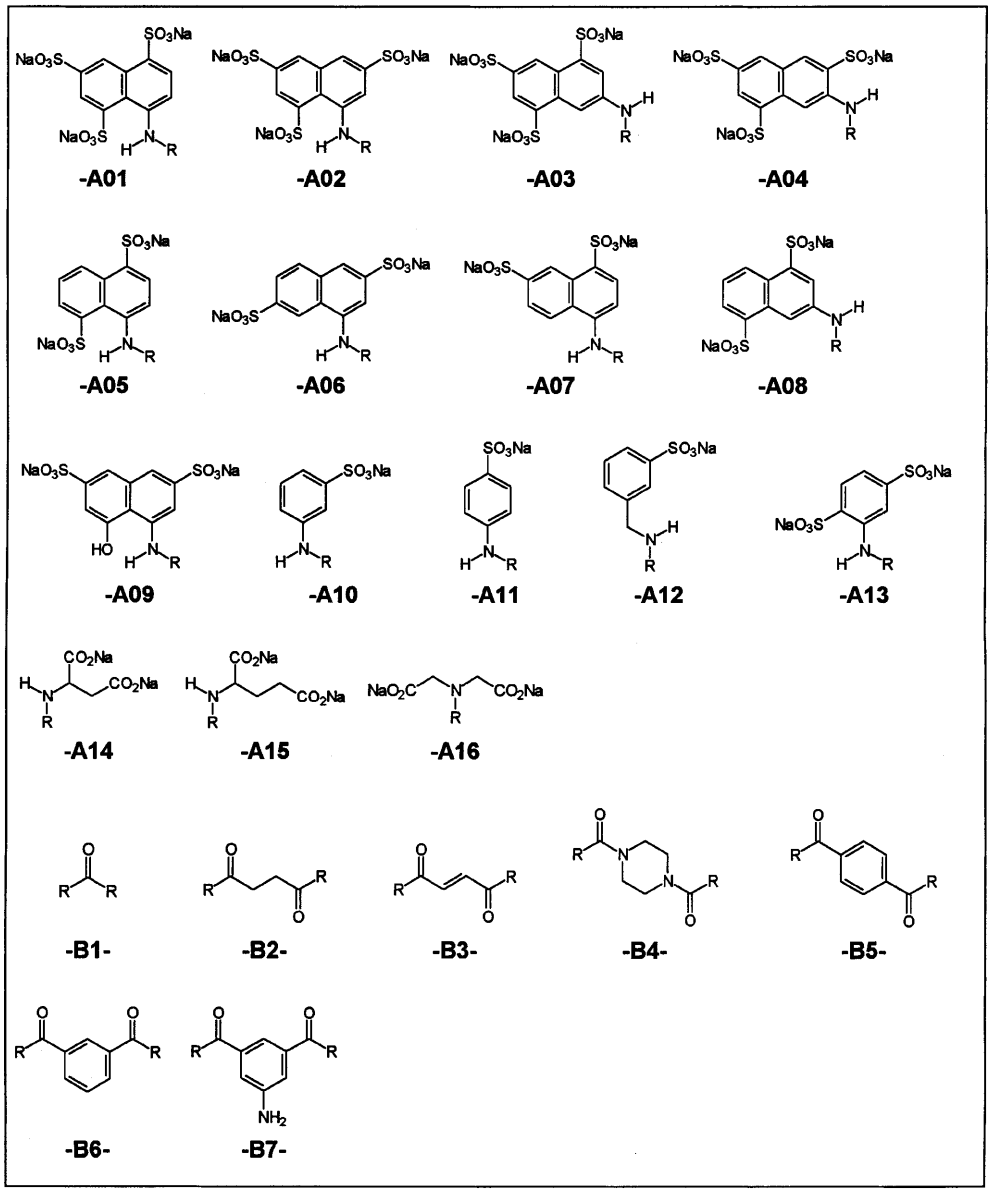


Fig. 4 The structure of acidic groups -R and central bridges -X- in Figs. 1–3

35–55), indicating antiangiogenic activity significantly greater than that of suramin. All seven analogues were naphthalenetrisulfonic acid derivatives of the large urea type (nos. 2, 3, 4, 6, 13, 14, 66; NF127, NF151, NF145, NF171, NF064, NF059, NF279, respectively). All the other analogues shown in Table 1 except no. 47 (NF383) with an ID_{50} of 137 nmol/disk had antiangiogenic activities similar to that of suramin.

Seven analogues and suramin were tested for their ability to inhibit the growth of human microvascular endothelial cells in the presence of 20 ng/ml bFGF. The IC_{50} values for the subconfluent microvascular endothelial cells in the presence of bFGF treated with the analogues are shown in Table 2. Four of the seven aminonaphthalenetrisulfonic acid analogues that showed greater potency than suramin in the CAM assay were tested. Two analogues, no. 4 (NF145) and no. 6 (NF171), were better inhibitors of endothelial growth than suramin as they were in the CAM assay. Another analogue, no. 14 (NF059), which was an excellent inhibitor of angiogenesis in the CAM assay, was not as effective as suramin as an inhibitor of endothelial cell growth. Analogue no. 65 (NF031) was similar to suramin as an inhibitor of both angiogenesis in the CAM assay and endothelial cell growth. The benzene sulfonic acid analogue tested, no. 50 (NF110), was a better inhibitor of human microvascular endothelial cell growth in vitro than suramin (ID_{50} of 280 vs 438 nmol/ml for suramin). A similar pattern was seen for no. 48 (NF293), the only disulfonic acid analogue tested.

Table 1 The ID_{50} of angiogenesis in the CAM assay for suramin and a series of analogues (ID_{50} dose in nmol/disk that induced 50% inhibition of angiogenesis)

Suramin analogue[a]		ID_{50} (nmol/disk)
No.	Code	
Naphthalenetrisulfonic acid derivatives		
1	NF060 (suramin)	75
2	NF127	54
3	NF151	45
4	NF145	36
6	NF171	35
8	NF280	68
13	NF064	55
14	NF059	40
31	NF201	71
65	NF031	75
66	NF279	55
68	NF506	70
69	NF507	65
Naphthalenodisulfonic acid derivatives		
36	NF289	72
47	NF383	137
48	NF293	60
49	NF324	92
Benzenesulfonic acid derivatives		
50	NF110	70
54	NF440	70
70	NF504	60

[a] The corresponding chemical structures are shown in Figs. 1–4

Table 2 ID_{50} for inhibition of cell growth in basic FGF-stimulated human microvascular endothelial cells by suramin and a series of analogues (ID_{50} dose in nmol/disk that induced 50% inhibition of angiogenesis)

Suramin analogue[a]		IC_{50} (nmol/ml)
No.	Code	
Naphthalenetrisulfonic acid derivatives		
1	NF060 (suramin)	438
4	NF145	143
6	NF171	170
14	NF059	800
26	NF023	750
65	NF031	440
Naphthalenedisulfonic acid derivatives		
48	NF293	150
Benzenesulfonic acid derivatives		
50	NF110	280

[a] The corresponding chemical structures are shown in Figs. 1–4

Discussion

The chemical structure of suramin was systematically varied in the suramin analogues used in this study. The naphthalenetrisulfonic acid residues -A01 were replaced by the acidic groups -A02 to -A16 shown in Fig. 4. The central urea bridge -B1- of suramin was replaced by the dicarboxylic acid diamide bridges -B2- to -B7- shown in Fig. 4. The size of the molecules was varied by modifying the number of benzoyl residues from zero (Fig. 3, nos. 62–64) via two (small urea type, Fig. 2) to four (large urea type, Figs. 1 and 3). Further, the rigidity of the molecules was modified. Thus, two aminobenzoyl residues of suramin were replaced by the 2-phenylbenzimidazole residue (Fig. 3, nos. 68–70). These suramin analogues have a similar size to suramin but a reduced flexibility.

The following molecular features seemed to be important for high antiangiogenic activity. A structure with two agglomerations of highly acidic groups in a certain distance was essential. Asymmetric molecules with only one highly acidic group (e.g. only one naphthalenetrisulfonic acid residue -A1) were inactive (data not shown in this report). The number of anionic groups was also important. The most active compounds were found among the naphthalenetrisulfonic acid derivatives. Naphthalenedisulfonic acid derivatives were, in general, less active. Among the benzene sulfonic acid derivatives, only those with four sulfonic acid residues (Figs. 2 and 3; nos. 50, 54 and 70; NF110, NF440 and NF504, respectively) showed significant antiangiogenic activity. None of the 13 carboxylic acid derivatives having two or four carboxylate residues (Fig.1, 18–25; Fig. 2, nos. 56–61) had significant antiangiogenic activity. The distance between the acidic groups was also important. Analogues with small bridges without benzoyl groups between the naphthalenesulfonic acid residues had no antiangiogenic activity (Fig. 3, nos. 62–64) and analogues of the small urea type with only two benzoyl residues (Fig. 2) were, in general, less active than those of the large urea type with four benzoyl residues (Fig. 1). It seems that steric factors and the rigidity of the molecule also had an important influence on the antiangiogenic activity. In the suramin analogues 10–14 (Fig. 1), the central urea bridge of suramin is replaced by dicarboxylic acid diamides. In the case of no. 13 (NF064) and no. 14 (NF059), the bridges are diamides of terephthalic (-B5-) and isophthalic acid (-B6-), respectively. The resulting analogues are, like suramin (no. 1, NF060), rigid and flat molecules. Both showed an inhibitory activity (76% and 87%, respectively) superior to that of suramin. Analogue no. 10 (NF061) had a much lower inhibitory activity (15%) than suramin. The central bridge of no. 10 is formed by succinic acid diamide. This is a very flexible bridge which allows many conformations of the molecule. Analogue no. 12 (NF299) has a dicarbamic acid diamide (-B4-) as the central bridge. This bridge contains two urea groups. The two-dimensional formula for this bridge -B4- shown in Table 2 seems to be very similar to the terephthalic diamide bridge -B5-, but the three-dimensional structure of -B4- differs greatly from that of -B5-. Two preferred conformations of analogue no. 12 can be expected. In neither of these conformations can the aromatic ring systems of the molecules be arranged in the same plane.

In the series of naphthalenedisulfonic acids, only one analogue, no. 48 (NF324), showed an inhibitory activity superior to that of suramin (75% versus 64%).

Interestingly, this compound is a 2-aminonaphthalenedisulfonic acid derivative, whereas the 2-aminonaphthalenetrisulfonic acid derivatives, no. 34 (NF249) and no. 35 (NF252), had very low activity (12% and 14%, respectively).

When the ID_{50} values were calculated from the 21 analogues shown to inhibit angiogenesis in the CAM assay by 50% or more, 17 were found to have antiangiogenic activity the same or greater than suramin, as indicated by ID_{50} values $\leq$ 75 nmol/disk (see Table 1). The ID_{50} value of 92 nmol/disk for no. 49 (NF324) was not significantly different from suramin, whereas the ID_{50} of 137 nmol/disk for no. 47 (NF383) indicates that no. 47 was significantly less antiangiogenic than suramin. The ID_{50} values were not calculated for no. 5 (NF157) and no. 9 (NF032) because inadequate amounts were available. The distribution of the most active suramin analogues was not uniform in different groups, showing that there was a clear relationship between the chemical structure and the inhibition of angiogenesis. When the partial structure of suramin was altered, 7 of the 18 analogues showed ID_{50} values that were significantly lower ($\leq$55 nmol/disk), indicating that their antiangiogenic activity was significantly greater than that of suramin. All of these analogues were from the naphthalenetrisulfonic acid group and contained large urea groups. In general, the naphthalenedisulfonic acid and benzene sulfonic analogues were less active than the naphthalenetrisulfonic acid analogues. Replacement of the trisulfonic acid groups by carboxylic acids resulted in analogues with very low antiangiogenic activity in the CAM assay.

Our results show that the suramin analogues were effective inhibitors of bFGF-stimulated growth of human microvascular endothelial cells. The polyanionic structure of the analogues seems to be an important factor for the interaction with various growth factors, such as bFGF, VEGF, epidermal growth factor, PDGFβ and IGF-1 [13, 15, 18, 19, 21]. These results are supported by results from other polyanions such as polysulfonated distamycin-A derivatives, which inhibit PDGFβ [4], and pentosan polysulfate, which inhibits bFGF-stimulated growth of SW13 cells [24] and blocks tumor growth in nude mice [25].

In studying the naphthalenetrisulfonic acids, great differences in antiangiogenic activity were noted, suggesting that other structural properties also influence this activity. Firsching et al. [7] indicated a similar pattern for antiproliferative activity with nine similar analogues synthesized by Dr. Peter Nickel. Structural modifications of suramin have been reported to markedly influence trypanosomal and antifilarial activity. Alterations of methyl groups leads to a significant decrease in trypanosomal activity [16]. The antifilarial activity of suramin analogues is also sensitive to structural changes and symmetry is essential for antifilarial activity [16]. For HIV-1 reverse transcriptase activity, no clear relationship between chemical structure and inhibitory activity has been demonstrated [11]. Two of the most active inhibitors of HIV-1 reverse transcriptase, analogues no. 65 (NF031) and no. 32 (NF013), have no trypanocidal or antifilarial activity. Firsching et al. [7] found that no. 65 (NF031) was the most potent antiproliferative compound against five tumor cell lines and had antiangiogenic activity similar to suramin in the CAM assay. We found that no. 65 (NF031) was similar to suramin with regard to antiangiogenic activity in the CAM assay and the inhibition of bFGF-induced growth of human microvascular endothelial cells in vitro. However, we found seven analogues that showed significantly greater antiangiogenic activity than suramin and no. 65 (NF031). Analogue no. 32 (NF031) did not significantly inhibit angiogenesis in our CAM assay.

Our results, in general, agree with the findings of Braddock et al. [3], who studied the effects of ten different suramin analogues on endothelial cells in vitro and in the CAM assay using analogues different from those used in our study. In conclusion, we identified seven analogues which have significantly greater antiangiogenic activity than suramin in the CAM assay. These analogues also appear to have significantly greater inhibitory activity against bFGF-stimulated growth in human microvascular endothelial cells in vitro. These results suggest that these more potent analogues may be developed into therapeutic agents for angiogenesis-dependent and proliferative diseases.

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