Nature of the Interaction of Growth Factors with Suramin

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ABSTRACT: Suramin inhibits the binding of a variety of growth factors to their cell surface receptors. The direct interaction of suramin with acidic fibroblast growth factor has been detected by the enhancement of the drug's fluorescence in the presence of the protein with the maximum effect occurring at a molar ratio of suramin to aFGF of 2:1. This interaction stabilizes aFGF to thermal denaturation and partially protects a free thiol in its polyanion binding site from oxidation. The binding of suramin to aFGF also induces aggregation of the growth factor to at least a hexameric state as detected by static and dynamic light scattering as well as by gel filtration studies. Both CD and amide I′ FTIR spectra of aFGF in the presence and absence of suramin suggest that the drug may also be causing a small conformational change in the growth factor. Suramin produces an even greater aggregation of bFGF and PDGF but not of EGF or IGF-1. Evidence for a suramin-induced conformational change in IGF-1 but not EGF is found by CD, however. It is concluded that suramin binds to many growth factors and that this induces microaggregation and, in some cases, conformational changes. In the case of aFGF, suramin interacts at or near its heparin binding site. The relationship between these phenomena and the anti-growth factor activity of suramin remains to be clearly elucidated.

Suramin is a symmetrical polysulfonated naphthylurea which has been extensively used to treat trypanosomiasis and onchocerciasis (Webster, 1991; Olenick, 1975). It has also been employed therapeutically in a variety of other disorders, including AIDS, with limited success (Cheson et al., 1987). Most recently, it has been evaluated as an anti-tumor agent (La Rocca et al., 1990a). These latter investigations are partially based on the ability of suramin to disrupt the interaction of many growth factors with their membrane receptors [e.g., see Coffey et al. (1987) and Coughlin et al. (1988)]. The mechanism by which suramin disrupts the activity of growth factors is incompletely understood but is presumed to involve direct binding of the drug to the growth factor itself rather than its complementary receptor (Hosang, 1985). Since the growth factors inhibited by suramin are primarily heparin binding proteins and suramin itself is a polyanion (La Rocca et al., 1990a), it seems likely that the interaction of suramin with these proteins involves their polyanion binding site. On the basis of the crystal structure of one of these growth factors, human recombinant basic fibroblast growth factor (bFGF),[1] it has been suggested that suramin inhibits receptor binding by either sterically occluding the receptor binding region or inducing a conformational change in the growth factor (Eriksson et al., 1991). This proposal is consistent with the hypothesized mechanism of action of suramin in a variety of other situations in which this polysulfonated compound binds to specific enzymes, noncompetitively inhibiting their activity [e.g., see La Rocca et al. (1990a) and references cited therein].

Acidic fibroblast growth factor provides a unique system with which to explore the molecular basis of suramin's actions (Coughlin et al., 1988; Huang et al., 1986). This growth factor is much less stable in the absence of polyanions and requires the presence of a molecule like heparin for significant biological activity (Burgess & Maciag, 1989; Copeland et al., 1991). We have therefore conducted a comprehensive analysis of the interaction of suramin with aFGF. In contrast to previous suspicions (Eriksson et al., 1991; La Rocca et al., 1990b), it is found that a major effect of suramin is to cause microaggregation of the growth factor. Less detailed studies suggest that this result can be extended to other growth factors as well, although induced conformational changes do seem to occur in some cases.

EXPERIMENTAL PROCEDURES

Materials. Suramin was obtained from Mobay Chemical Corp. Recombinant human aFGF was isolated from transformed *Escherichia coli* cell as described previously (Copeland et al., 1991; Linemeyer et al., 1987). Human EGF, PDGF (the BB isomer with Ala_1Val_{12} substitutions), bFGF (with the six N-terminal amino acids truncated), and IGF-1 (Thr_{59} substitution) were provided by Creative BioMolecules. Rat EGF and murine EGF were donated by R. C. Cavalli (Temple University). All of the proteins of this study were judged to be homogeneous by the criteria of the formation of single bands upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Heparin was purchased from Hepar, and sulfated β-cyclodextrin was provided by American Maize. Evans Blue and 1,3,(6 or 7)-naphthalenetrisulfonic acid (trisodium salt hydrate) were obtained from Aldrich Chemical Co. All other reagents were from Sigma.

Mitogenic Activity. The biological activity of aFGF in the presence and absence of various polyanions was monitored as the mitogenic response of Balbc/3T3 cell lines and followed by the uptake of tritiated thymidine as described previously (Linemeyer et al., 1987).

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[1] Abbreviations: aFGF, human recombinant acidic fibroblast growth factor; bFGF, human recombinant basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, human recombinant platelet-derived growth factor; rEGF, rat EGF; hEGF, human EGF; IGF-1, human recombinant insulin-like growth factor 1; BSA, bovine serum albumin; GluOx, glucose oxidase; RibA, ribonuclease A.

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In some experiments (Table I), aFGF (50 μg/mL) was preincubated with a 10× molar excess of the indicated polyanion. These solutions were then introduced into the tissue culture media (0.25–500 ng/mL) containing 0.5 g/L of the same polyanion. The specific activity of aFGF in these experiments was defined as the midpoint of a plot of mitogenic response versus aFGF concentration (IC_{50}) which was normalized to an aFGF reference standard containing heparin. In studies to explore the effect of suramin concentration on aFGF mitogenic activity, aFGF (500 μg/mL) was preincubated with increasing molar ratios of suramin to growth factor. These samples were then diltued into cell-containing media in the absence of any extrinsically added polyanion. The mitogenic response (percent) was then defined as the degree of [^{3}H]thymidine incorporation after background correction at the indicated concentrations of aFGF. These values were then normalized to the mitogenic response in the absence of suramin. Standard errors of these values ranged from 5 to 10%.

Fluorescence Measurements. Fluorescence spectra were measured with either an SLM 8000, a Hitachi F-2000, or a Spex Fluorolog fluorometer at 4–10-nm resolutions. Suramin was excited at 315 nm and its emission spectrum recorded from 350 to 550 nm in a 2 × 10 mm cuvette. All spectra were recorded at 10 °C in 20 mM sodium phosphate/0.15 M NaCl, pH 7.2. Suramin titrations were conducted by adding aliquots of either a stock solution of protein (1 g/L) into a 10 μM suramin solution or a stock 1 mM suramin solution into 6–10 μM protein. Suramin manifests a significant inner filter effect at higher levels of the compound in the latter experiments, and therefore titration of protein into solutions of suramin of fixed (low) concentrations was normally employed. When necessary, inner filter effects were corrected as described by Lakowicz (1983). Thermal melting experiments were performed with the Spex instrument using a computer-controlled circulating water bath and a protocol which varied the temperature from 10 to 80 °C in 2 °C intervals with a 2-min incubation period (sufficient to reach equilibrium) between each measurement. The thermal denaturation of aFGF was monitored by the shift in emission maximum from 306 to 350 nm which occurs when the protein unfolds (Copeland et al., 1991).

Calorimetry. Differential scanning calorimetry was performed with a Hart 7708 calorimeter at a scan rate of 60 °C/h. A 0.6-mL solution of 1 g/L aFGF was employed. To minimize interference from exotherms due to protein aggregation at higher temperatures, the solutions contained small sections of glass tubes (Steadman et al., 1989). All thermograms were background-corrected.

Copper-Catalyzed Oxidation. aFGF (80 μg/mL) was incubated in a 20 mM Tris/0.15 M KCl, pH 8, solution containing 20 μM copper chloride for varying periods of time. The reaction was terminated by the addition of 100 μM EDTA in the same buffer. A solution of 0.7 mL of 0.25 M Tris (pH 8) containing 2 mM EDTA and 7 M GuHCl was then added followed by 35 μL of 4 mg/mL DTNB reagent (Sigma). Samples were incubated for 30 min and filtered through a 0.22-μm filter, and the optical density was measured at 412 nm to determine the sulfhydryl content (Riddles et al., 1983).

Light Scattering. Static and dynamic light-scattering measurements were performed with a Malvern 4700 multiangle apparatus equipped with a 256-channel autocorrelator and a 5W Spectra Physics argon ion laser. In general, studies were conducted at 8 °C, 90° scattering angle, with 250–mW laser power at 488 nm and 50–100-s data acquisition times. Protein concentrations of 1 g/L were used in 20 mM sodium phosphate/0.15 M NaCl, pH 7.2, which also contained 5 mM Na_2SO_4 for stabilization purposes (Copeland et al., 1991). The presence of Na_2SO_4 did not affect any of the reported results. All samples were filtered through 0.1-μm filters prior to examination. A cumulant analysis (Koppel, 1972) was used to derive diffusion coefficients (D) from autocorrelation functions. These values were then used to calculate the z-average mean equivalent hydrodynamic diameter (d) from the Stokes–Einstein equation ($d = kT/3\pi D\eta$). Over the range of protein sizes and concentrations employed in these studies, it was found that scattering intensity was independent of angle and linearly dependent on concentration. Thus, protein aggregation within a solution of constant concentration (weight/volume) should at least initially result in an increased scattering intensity which is proportional to the weight average molecular weight of the solute components. We have therefore defined the ratio of scattering intensity in the presence of ligand to that in its absence (I_+/I_-) as a measure of the extent of aggregation induced by ligand. This ratio actually also depends on both the shape of any aggregates and the relative amount of free protein in solution. Thus, the experimental values are lower limits of the estimate of relative aggregate size that approach true values as long as their dimensions are significantly less than the wavelength of the scattered light.

Size-Exclusion Chromatography. Gel filtration studies were performed at 25 °C with a Toso-Haas G3000 SW_{XL} column (0.78 × 30 cm) and a Rainin Rabbit HPLC system equipped with a Rheodyne 7161 injector (20-μL loop). Peaks were detected at 280 nm with a Spectra Physics forward optical scanning detector. Data acquisition and integration employed software from Dyanamax. A mobile phase of 25 mM sodium phosphate/0.15 M NaCl, pH 7.2, was used at a flow rate of 1 mL/min. The column was calibrated with five standards consisting of vitamin B_{12} (M_r ~1350), equine myoglobin (17 000), chicken ovalbumin (44 000), bovine γ-globulin (158 000), and thyroglobulin (670 000) (Bio-Rad Laboratories) with molecular weights of unknowns estimated from extrapolation of a plot of log M_r versus elution time of the standards. Protein was applied in 20-μL volumes at concentrations from 6.3 to 0.3 μM in the mobile phase buffer.

Circular Dichroism. Circular dichroic spectra were obtained with either a JASCO J720 or an AVIV 62DS spectropolarimeter. The instruments were calibrated with camphorsulfonic acid, and all spectra were measured at 1-nm resolution and 10 °C. Path lengths of 0.1 and 0.01 cm were employed, and reported spectra are the average of two runs. Protein concentrations of 5–50 μM were used in 20 mM sodium phosphate, 0.15 M NaCl, and 5 mM Na_2SO_4, pH 7.2. Similar results were obtained in the absence of Na_2SO_4.

Fourier Transform Infrared Spectroscopy. Infrared spectra were measured with a Digilab FTS60 FTIR spectrometer at 2-cm^{-1} resolution. A protein concentration of 113 μM in D_2O containing 20 mM sodium phosphate/0.15 M NaCl, pD ~7.2, was used in 0.1-mm cells. All spectra were obtained at room temperature, and suramin was added as a solid to protein solutions. Spectra were partially deconvoluted by the method of Kauppinen et al. (1981) employing parameters of $K = 2$ and $\sigma = 12$. The partially deconvoluted spectrum was fit to a series of mixed Gaussian/Lorentzian peaks (Copeland et al., 1991), and assignments were made on the basis of the studies of Byler and Susi (1986). The results of this analysis differ slightly from those reported previously (Copeland et al., 1991) because of the lower protein concentration and consequently decreased signal-to-noise ratio of this study.

Table I: Effect of Polyanions on the Mitogenic Activity of aFGF[a]

polyanion (0.5 g/L)	sp act. (units/mg × 10^{-6})
none	<0.18
heparin	4.26
sulfated β-cyclodextrin	3.70
inositol hexasulfate	2.30
suramin[b]	<0.14
Evan's Blue[b]	<0.13
naphthalenetrisulfonate[b]	<0.13

[a] aFGF (50 μg/mL) was preincubated with 10× molar excess of polyanion. Experiments were performed (0.25–500 ng/mL aFGF) with 0.5 g/L polyanion in the culture media. [b] The addition of 0.5 g/L heparin into the culture medium partially restored mitogenic activity in the range 1.11–2.44 (×10^{-6} units/mg).

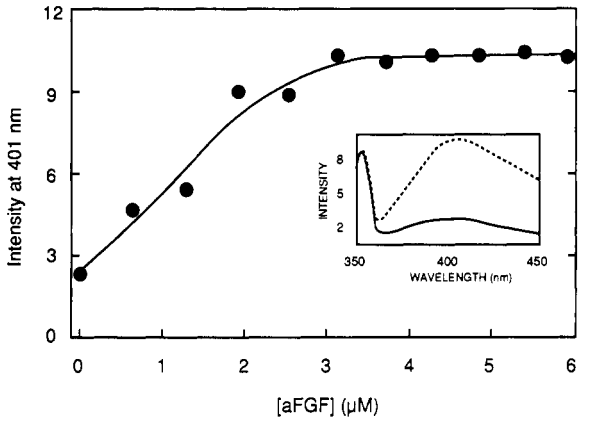

FIGURE 1: Effect of aFGF concentration on the fluorescence of suramin. The increase in suramin fluorescence as aFGF is added to a 10 μM suramin solution containing 20 mM sodium phosphate/0.15 M NaCl, pH 7.2, was monitored as the magnitude of the fluorescence intensity at 401 nm. Suramin was excited at 315 nm with 2-nm resolution. The spectrum of suramin (10 μM) in the absence (—) and presence (- - -) of aFGF (5 μM) is shown in the inset.

RESULTS

To confirm the inhibitory influence of suramin upon the activity of aFGF as well as several other growth factors, the effects of suramin and two compounds related to suramin (Evan's Blue and trisulfated naphthalene) as well as heparin and two lower molecular weight sulfated polyanions were examined for their perturbation of the aFGF stimulation of the mitogenesis of 3T3 cells (Table I). As previously shown (Gimenez-Gallego et al., 1986; Gospodarowicz & Cheng, 1986), aFGF possesses only low activity in the absence of polyanions. Addition of heparin stimulates this residual activity over 20-fold. Lower molecular weight sulfated polyanions such as inositol hexasulfate and sulfated β-cyclodextrin (Folkman et al., 1989) were also effective at stimulating aFGF activity. In contrast, suramin as well as the two related compounds was ineffective. This lack of ability of suramin, Evan's Blue, and trisulfated naphthalene to support aFGF-stimulated mitogenesis could be at least partially overridden by the presence of heparin in the incubation medium.

To test whether suramin binds to aFGF, we took advantage of the fact that the compound has weak fluorescence emission near 400 nm. Upon addition of aFGF to a solution of suramin, this fluorescence is significantly enhanced (Figure 1, inset). Using the intensity of suramin fluorescence at 401 nm as a measure of the interaction of the compound with aFGF, titration of a solution of suramin with aFGF finds this fluorescence enhancement complete at a molar ratio of suramin to aFGF of approximately (2–3):1 (Figure 1). Similar values were obtained by titration of aFGF with suramin. Fluorescence enhancement was reversed by the addition of an equal molar amount of heparin or a 50-fold molar excess of inositol hexasulfate (not illustrated).

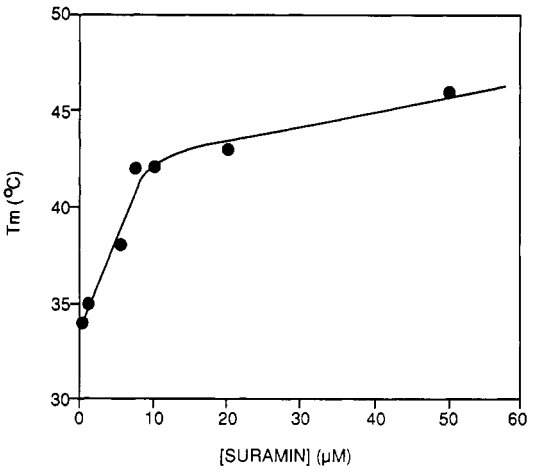

FIGURE 2: Effect of suramin concentration on the thermal denaturation of aFGF. The T_m is defined as the midpoint of the temperature transition in plots of fluorescence wavelength emission maximum (280-nm excitation) versus temperature. Experiments were performed with 6.2 μM aFGF in 20 mM sodium phosphate/0.15 M NaCl, pH 7.2. The precision of the T_m estimates is ±2 °C.

Table II: Effect of Ligands on the Thermal Denaturation Temperature (T_m) of aFGF As Measured by Differential Scanning Calorimetry[a]

ligand (ligand:aFGF molar ratio)	T_m (°C)
buffer	43
naphthalenetrisulfonate (10:1)	45
suramin (3:1)	50
suramin (10:1)	49
Evan's Blue (10:1)	52
sulfated β-cyclodextrin (10:1)	67
heparin (3:1)	67

[a] Samples contained 1 mg/mL (62 μM) protein in PBS buffer, pH 7.2.

The binding of polyanions to aFGF is also known to stabilize aFGF against thermal unfolding (Copeland et al., 1991; Gospodarowicz & Cheng, 1986). This can be simply followed by the relaxation of the quenching of the protein's single tryptophan residue upon thermal perturbation of the protein's native structure (Copeland et al., 1991). Defining the midpoint of this fluorescence change as the thermal melting temperature (T_m) of aFGF, the effect of suramin upon the stability of aFGF was examined. Addition of suramin shifted the T_m of aFGF from 34 °C to ca. 45 °C, with the effect saturating at a molar ratio of suramin to aFGF of approximately 2:1 (Figure 2). Similar results were obtained when thermal unfolding was monitored by circular dichroism (not illustrated) and by differential scanning calorimetry in which a 7 °C increase in the thermal unfolding temperature of the suramin-complexed protein was observed. For comparison purposes, the effect of a number of other sulfated compounds upon the thermal stability of aFGF as detected by differential scanning calorimetry is reported in Table II. The effect of the suramin analog Evan's Blue is similar to that seen with suramin but greater than that found with the trisulfated naphthalene. In contrast, both heparin and sulfated β-cyclodextrin have a significantly greater stabilizing effect upon aFGF. Suramin, however, is the only compound that produces a biphasic transition in the aFGF unfolding endotherm (not illustrated). It should be noted that both CD and DSC find a higher unfolding temperature than intrinsic protein fluorescence experiments, presumably due to the greater thermal

Table III: Effect of Polyanions on the Oxidation of aFGF Thiols

sample	% thiols remaining (10 min)
buffer	39
naphthalenetrisulfonate	49
inositol hexasulfate	74
suramin	80
heparin	99
sulfated β-cyclodextrin	110

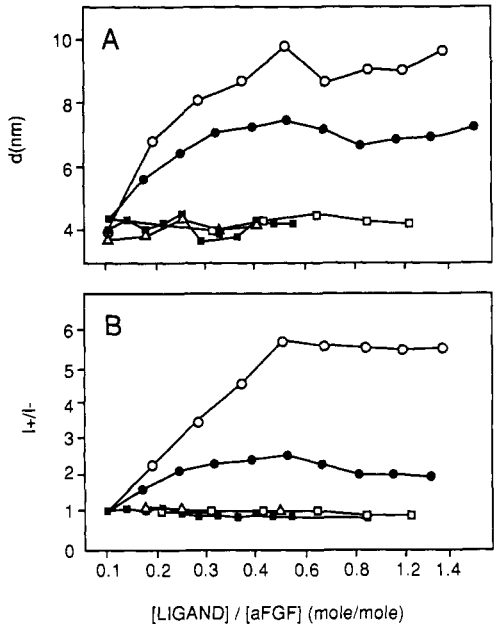

FIGURE 3: Effect of polyanions on the size of aFGF. Size was estimated by determination of the equivalent hydrodynamic diameter (d) from dynamic light-scattering experiments (A) or from the ratio of the steady-state intensity of light scattered in the presence (I_+) or absence (I_-) of the indicated agent (B). Measurements were made at a protein concentration of 62 μM in 20 mM sodium phosphate, 0.15 M NaCl, and 5 mM Na_2SO_4 (pH 7.2) at 8 °C and 90° scattering angle. Data are expressed in terms of the molar ratio of ligand to aFGF, and the ligands are (○) suramin, (●) sulfated β-cyclodextrin, (■) inositol hexasulfate, (△) Evan's Blue, and (□) naphthalenetrisulfonic acid.

lability of the local tertiary structure near aFGF's single tryptophan residue (Copeland et al., 1991).

To confirm that the presumptive suramin binding site on aFGF is at or near the previously described polyanion binding site, the ability of suramin to protect the exposed sulfhydryl group in this vicinity from copper-induced oxidation was examined.[2] In the absence of polyanion, approximately 40% of the thiols of aFGF remain after 10 min of copper-induced oxidation (Table III). Addition of heparin or sulfated β-cyclodextrin completely protects aFGF from this reaction over this time period. In contrast, both suramin and inositol hexasulfate provide partial but significant protection while trisulfated naphthalene has a much smaller effect.

The potentially bifunctional nature of suramin caused us to examine its ability to alter the aggregation state of the normally monomeric M_r 15 900 aFGF molecule. The 4-nm hydrodynamic diameter of aFGF as measured by dynamic light scattering increases upon the addition of suramin to approximately 9 nm (Figure 3A). This growth in size is initially complete at a ratio of suramin to aFGF of 0.5–0.7. The intensity of scattering (at right angles) also increases over this same range of ligand concentration to about 6 times

[2] D. B. Volkin, P. K. Tsai, J. M. Dabora, J. O. Gress, C. J. Burke, R. J. Linhardt, and C. R. Middaugh, submitted for publication.

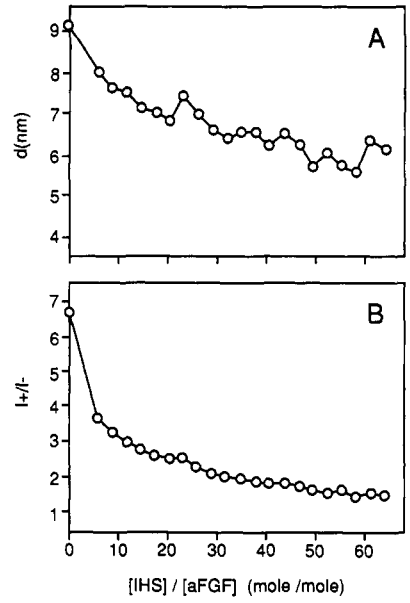

FIGURE 4: Effect of high concentrations of a representative polyanion (inositol hexasulfate) on the size of aFGF/suramin complexes. Size was estimated from the equivalent hydrodynamic diameter (d) (A) and the intensity of scattering in the presence (I_+) and absence (I_-) of ligand (B) as described in Figure 3.

the level observed with monomeric aFGF (Figure 3B). Sulfated β-cyclodextrin also appears to induce a similar aggregation to a diameter of about 7 nm and a scattering intensity double that seen with unliganded aFGF. In contrast, the presence of inositol hexasulfate, trisulfated naphthalene, and Evan's Blue at the same molar ratios of polyanion to aFGF has no significant effect on the size of the growth factor. As shown in Figure 4A,B, this effect is reversible since the size of the suramin/aFGF complex can be reduced substantially back toward that of the monomeric protein by the addition of an excess of inositol hexasulfate.

If the growth in size of aFGF is produced by suramin-mediated cross-linking, high concentrations of suramin (above those illustrated in Figure 4) should decrease the size of the complex as all of the polyanion binding sites become occupied. As shown in Figure 5, this is indeed the case. Expansion of the range of suramin concentrations examined now reveals a peak in complex size at suramin:aFGF ratios of 2–5 and a gradual decrease in diameter above this value. A complete return of aFGF to a monomeric state is observed at ratios above 150.

Size exclusion chromatography was used to obtain additional evidence in support of the suramin-induced aggregation of aFGF (Table IV). A molecular sieve column was used with exclusion limits of 10–500 kDa for globular proteins and calibrated with protein standards. In this system, aFGF elutes as a single sharp peak of apparent M_r 7500. This low value probably reflects the migration of the growth factor near the extreme limit of the calibration curve. Addition of inositol hexasulfate has no effect on the position of the monomeric aFGF peak as does a 1:1 molar ratio of sulfated β-cyclodextrin to aFGF. At a much higher ratio (10:1) of the cyclodextrin to aFGF, however, two additional higher molecular weight peaks appear of approximately 2 and 3 times the size of native aFGF. As increasing concentrations of suramin are added to aFGF solutions and then applied to the column, a peak corresponding to a species of M_r ~90 000 appears. At the highest amounts of suramin examined (2:1 molar ratio of suramin to aFGF), the proportion of lower molecular weight material (overlapping peaks of ca. 7.5K and 11K) begins to

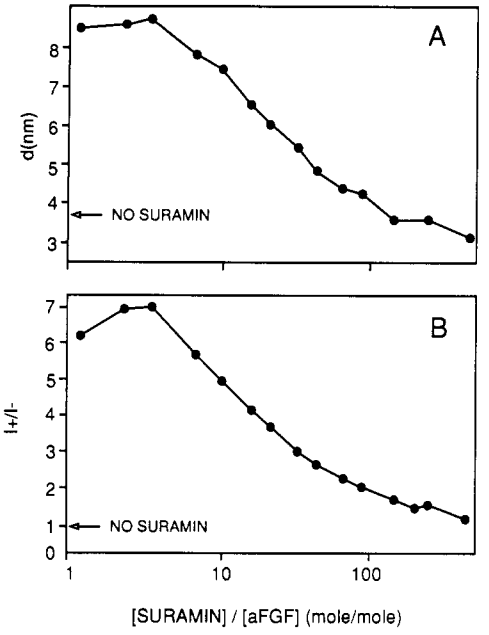

FIGURE 5: Effect of high concentrations of suramin on the size of suramin/aFGF complexes. The conditions of the experiment are the same as those described in Figures 5 and 6.

Table IV: Gel Filtration Analysis of the Size of aFGF/Polyanion Complexes

ligand:aFGF ratio (mol/mol)	app mol wt from size-exclusion chromatography
aFGF	7500
inositol hexasulfate/aFGF (1:1)	7500
sulfated β-cyclodextrin/aFGF (1:1)	7500
sulfated β-cyclodextrin/aFGF (10:1)	7500; 16000; 27000
suramin/aFGF (1:4)	7500
suramin/aFGF (1:2)	7500; 90000 (13:1)[a]
suramin/aFGF (1:1)	7500; 90000 (1.4:1)[a]
suramin/aFGF (2:1)	7500; 11000; 90000 (1.2:1:1.9)[a]

[a] Two or three peaks are resolved at the indicated ratio of peak areas.

increase relative to the large 90K aggregate. To examine the concentration dependence of the formation of the M_r 90 000 aggregate material, aFGF and suramin at a 1:1 molar ratio were applied at decreasing concentrations to the column. Both the M_r 7500 and the M_r 90 000 species were detected at unchanging relative peak areas down to the lowest concentrations which could be detected at (50 nM).

To further explore the question of whether the suramin-induced aggregation of aFGF might occur under biological conditions, we took advantage of the fact that aFGF is known to bind to heparin and heparin-like molecules in vivo [e.g., see Vlodavsky et al. (1991)]. As illustrated above with suramin, light scattering can be used to estimate the size of polyanion/aFGF complexes. Using this approach, approximately 9–10 aFGF molecules are found to bind strongly to heparin of M_r ~16K as suggested by I_+/I_- values in this range (Figure 6; Mach et al., 1992). Heparin contributes little to the total scattering observed under the chosen experimental conditions. When suramin is added to a solution in which heparin is saturated with aFGF molecules and no free aFGF is present, the size of the complexes decreases to a size (Figure 6) similar to that observed with suramin/aFGF complexes (Figure 3). This decrease in size is essentially complete at suramin to aFGF molar ratios of approximately 1, arguing that the affinities of suramin and heparin for aFGF are of the same order of magnitude. The known interaction between heparin

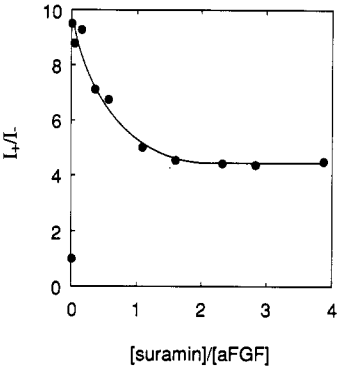

FIGURE 6: Dissociation of aFGF/heparin complexes by suramin. A mixture of heparin (6.3 μM) and aFGF (63 μM) was prepared to produce heparin chains saturated with growth factor at a molar ratio of 10:1 (Mach et al., 1992). Under these conditions, little free aFGF is present in solution, and relative scattering intensities (I_+/I_-) of 9–10 are observed. This solution was then titrated with aliquots of a 15 mM suramin solution to produce the indicated molar ratios of suramin to aFGF and the reduction in scattering intensity recorded. Further experimental details are described in the legend to Figure 4 and the text.

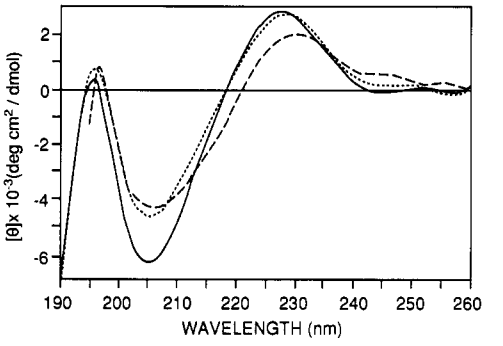

FIGURE 7: Effect of suramin on the far-UV CD spectrum of aFGF. The solid line is the spectrum of aFGF in the absence of ligand. Spectra recorded using optical path lengths of 0.1 and 0.001 cm are identical. The spectra of aFGF in the presence of a 3.6× molar excess of suramin are represented by the dashed and dotted lines for 0.1- and 0.01-cm path-length cells, respectively. All spectra were obtained at 10 °C at protein concentrations of 5.5 (0.1) and 42 μM (0.01 cm) in 20 mM sodium phosphate, 0.15 M NaCl, and 5 mM Na_2SO_4, pH 7.2.

and aFGF in vivo thus suggests that the suramin-induced aggregation of aFGF would probably occur under similar conditions.

The possibility also exists that suramin may induce some type of conformational change in aFGF. We therefore examined the far-UV CD spectrum of aFGF in the presence and absence of suramin (Figure 7). All spectra display positive ellipticities near 228 and 195 nm as well as a negative peak at 205 nm consistent with the β-sheet-rich secondary structure of aFGF (Copeland et al., 1991). Decreasing the optical path length and increasing the concentration of unliganded aFGF have no effect on the spectrum. Addition of a 3.6 molar excess of suramin causes a marked change in the CD spectrum of aFGF. At lower concentrations (5.5 μM) of aFGF and longer path length (0.1 cm), this alteration involves a substantial reduction in intensity and small shifts in wavelength position of both the 228- and 205-nm peaks as well as the addition of some small positive ellipticity near 247 nm. At shorter path length (0.01 cm) and higher concentration (42 μM), the change is primarily limited to the 205-nm negative ellipticity. Other polyanions examined (inositol hexasulfate, sulfated β-cyclodextrin, heparin, Evan's Blue, naphthalenetrisulfonate) had no effect on the CD spectrum of aFGF.

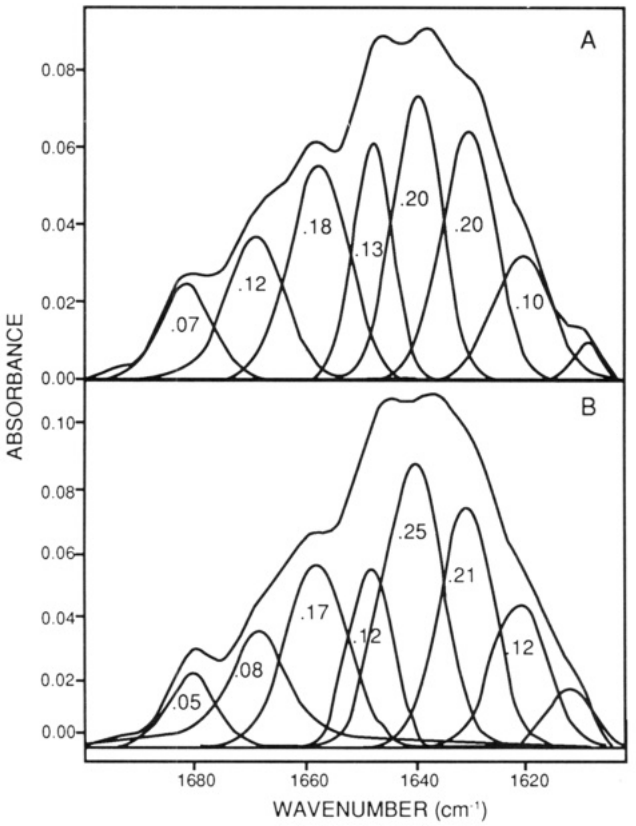

FIGURE 8: Partially deconvoluted amide I′ infrared spectrum of aFGF in the presence (B) and absence (A) of suramin. Spectra were recorded at 25 °C employing a protein concentration of 113 μM in D_2O containing 20 mM sodium phosphate/0.15 M NaCl, pD 7.2. A 3-fold molar excess of suramin was used in (B). The original spectra were deconvoluted employing values of $K = 2$ and $\sigma = 12$ (Kauppinen et al., 1981), and curve fitting was performed with a mixed Gaussian/Lorentzian function (Copeland et al., 1991). The positions of the peaks (± 1 cm^{-1}) are 1610, 1621, 1631, 1640, 1648, 1657, 1668, and 1681 cm^{-1}, and the numbers under each peak represent the relative contribution of each band to the total partially deconvoluted spectral area with the 1610-cm^{-1} band eliminated from the analysis. On the basis of the assignments of Byler and Susi (1986), the peaks are due to α-helix or random structure (1657 cm^{-1}), β-sheet (1621, 1631, 1640, and 1681 cm^{-1}), turns (1681 cm^{-1}), and less ordered structure (1648 cm^{-1}) (see the text).

To further explore the effect of suramin on the conformation of aFGF, the deconvoluted amide I′ spectrum of 1.8 g/L aFGF in buffered D_2O was examined in its presence and absence. As shown previously (Copeland et al., 1991), the amide I region is dominated by peaks due to β-structure and turns at 1621, 1631, 1668, and 1681 cm^{-1} (Figure 8A). The peak at 1657 cm^{-1} would usually be assigned to α-helix (Byler & Susi, 1986), but the absence of this type of secondary structure in aFGF suggests that it, as well as the more usual 1648-cm^{-1} feature, is due to less ordered structure (Byler & Susi, 1986). The 1640-cm^{-1} peak could be due to either β-structure or less ordered structure. Addition of a 3-fold molar excess of suramin produces only slight changes involving primarily a 25% increase in area of the 1640-cm^{-1} peak and a 30% decrease in the 1668-cm^{-1} absorption band (Figure 8B).

To test the generality of the preceding observations, we also examined the interaction of suramin with several other growth factors that have been reported to be inhibited by the drug. For this purpose, bFGF (Coughlin et al., 1988; Huang et al., 1986), PDGF (Coffey et al., 1987), EGF (Coffey et al., 1987), and IGF-1 (Pollak & Richard, 1990) were investigated. Because only limited quantities of each of these proteins were available, we performed only those analyses that had shown

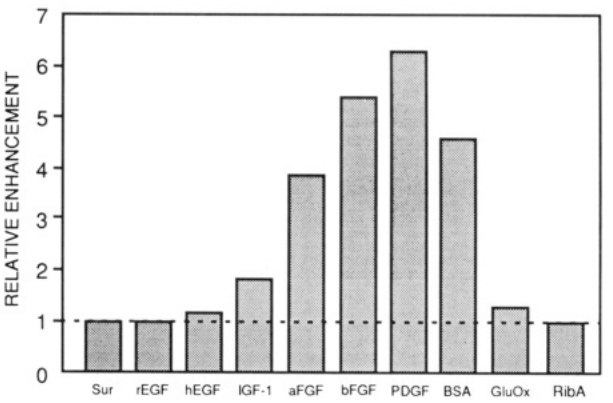

FIGURE 9: Effect of growth factors and other proteins on the fluorescence of suramin. Results are shown at a 1:1 molar ratio of suramin to protein and are expressed relative to the fluorescence intensity at 402 nm of suramin itself. Protein concentration is approximately 9 μM in 20 mM phosphate buffer containing 0.15 M NaCl, pH 7.2.

distinctive changes in aFGF in the presence of suramin. These included fluorescence, light scattering, and CD studies.

As shown in Figure 9, both bFGF and PDGF produce an even greater enhancement of suramin fluorescence than that observed with aFGF. A marked increase in suramin fluorescence is also seen with IGF-1, although this increase is only half that seen with aFGF. Bovine serum albumin has also been reported to bind suramin, which is confirmed by its significant effect on suramin fluorescence (Figure 9). In contrast, EGF, which has been reported to be only weakly inhibited by suramin (Coffey et al., 1987; La Rocca et al., 1990b), had no effect on the compound's emission properties. Two proteins selected for their extremes of pI [glucose oxidase (pI = 4.3) and ribonuclease (pI = 10)] also had little effect.

The effect of suramin on the size of these other growth factors was also analyzed by light scattering. Suramin induced extensive aggregation of both bFGF and PDGF (Figure 10), with the maximum size (1200–1600 nm) occurring at a ratio of suramin to growth factor of approximately 1. At the very highest concentrations of suramin examined, some decrease in size of the protein aggregates is also apparent. In contrast, suramin had no effect on the z-average hydrodynamic diameter or scattering intensity of EGF, IGF-1, or BSA over the range of suramin concentrations shown in Figure 10.

To search for suramin-induced conformational changes, the far-UV CD spectrum of each of the growth factors in the presence and absence of suramin was examined. The spectra of bFGF and PDGF were clearly distorted by the extensive aggregation induced by suramin, manifesting large decreases in their 200–230-nm ellipticity minima (not illustrated). Significant changes in the CD spectrum of IGF-1 were, however, induced in monomeric IGF-1. As shown in Figure 11, suramin produced a 14% decrease in the 207-nm minimum and a 43% ellipticity decrease and 3-nm red shift in the 192-nm positive peak. As reported previously by others (Müller & Wollert, 1976), a small 2% decrease in the magnitude of the negative double ellipticity minimum of BSA was found to be induced by suramin (not illustrated), but this is very close to the resolution limit of this technique. No change in the CD spectrum of either rat, murine, or human EGF was detected in the presence of suramin.

DISCUSSION

Suramin is currently widely used to inhibit the activity of a number of growth factors (La Rocca et al., 1990a,b). The

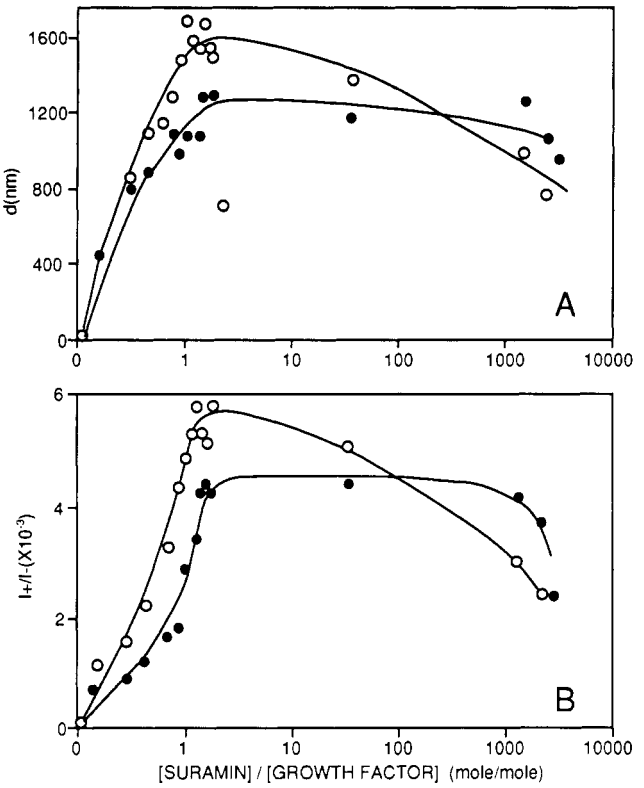

FIGURE 10: Effect of suramin on the size of bFGF and PDGF. Experiments were performed as described in Figures 3–5. PDGF is indicated by the open symbols while bFGF is designated by the closed ones. The lines have been hand-drawn through the data to illustrate general trends and do not reflect any particular model.

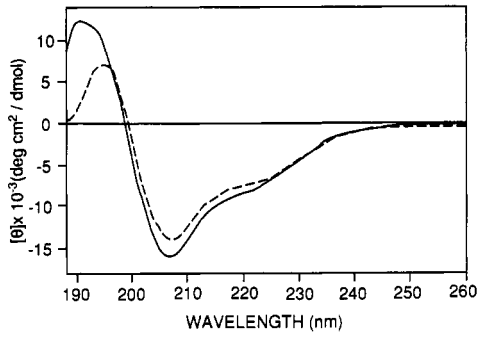

FIGURE 11: Far-UV CD spectra of IGF-1 in the presence (- - -) and absence (—) of suramin. Spectra were recorded at a protein concentration of 270 μM (0.01-cm path length), and suramin was present at a 1.9× molar excess.

drug has been shown to act primarily by blocking the binding of these growth factors to their cell surface receptors (Coffey et al., 1987). Indirect evidence suggests that at least in the case of PDGF, suramin interacts directly with the growth factor itself to produce this inhibition (Hosang, 1985). In fact, this polysulfated naphthylurea is known to interact with a surprisingly wide variety of proteins and enzymes in addition to the postulated interaction with growth factors (La Rocca et al., 1990a,b).

We now provide the first direct evidence that suramin does bind to many growth factors. Using the effect of suramin on aFGF as a prototype of this interaction, it is found that the fluorescence of suramin is significantly enhanced in its presence, indicating a direct interaction between suramin and aFGF. Like many of the growth factors that have been found to be inhibited by suramin, aFGF is a heparin binding protein. It therefore seems likely that the binding site of the polysulfated drug is located at or near the relatively nonspecific polyanion binding site of aFGF. This is supported by the observations (a) that the presence of suramin stabilizes aFGF against thermal denaturation like other polyanions (Copeland et al., 1991) and (b) that suramin protects the highly reactive sulfhydryl group of aFGF (known to be near the heparin binding site) against oxidation.[2] Furthermore, polyanions including heparin effectively compete the binding of suramin to aFGF. Changes in the fluorescence of suramin seen in the presence of aFGF saturate at a ratio of suramin to aFGF of approximately 2, suggesting the presence of two binding sites for the drug on the aFGF molecule. This is consistent with either the significant extent of the growth factor's polyanion binding site or the presence of two distinct polycationic interaction regions.[2] This stoichiometry is confirmed by thermal stabilization experiments in which maximum induction of the stability of aFGF occurs in the presence of approximately two molecules of suramin.

This raises the crucial question as to why suramin inhibits the activity of aFGF. Other polyanions which stabilize aFGF and protect its free thiols from oxidation actually stimulate the growth factor's activity (Table I). In contrast, suramin has no stimulating effect upon aFGF and will inhibit the growth factor's activity in the presence of stabilizing ligands. It is therefore apparent that suramin must have additional effects upon aFGF other than those produced by a wide variety of other polyanions.

The symmetrical nature of suramin immediately suggests one possibility in this regard. The combination of potential bifunctionality and two suramin binding sites on aFGF implies that suramin could produce aggregation of the growth factor. Light-scattering and gel filtration studies argue that this is indeed the case. The addition of less than one suramin molecule per aFGF produces an oligomeric species with approximately 6 times the scattering intensity of the monomer (Figure 3) and therefore at least 6 times its molecular weight. This aggregation appears to occur in the concentration range over which suramin inhibits growth factor activity in vitro as well as the levels at which suramin exhibits clinical effects (La Rocca et al., 1990a,b). Molecular sieve chromatography demonstrates the existence of a suramin-induced species with a mass also approximately 6 times greater than monomer (using the 15 900 molecular weight of monomeric aFGF rather than the apparent value of 7500 measured at the extreme end of the column calibration curve). Furthermore, the size of this entity as measured by dynamic light scattering is in general agreement with the estimated diameter of a compact hexameric aggregate. Could suramin actually be causing the aggregation of aFGF at the low growth factor concentrations present under biological conditions? In the presence of equimolar amounts of suramin and aFGF, there is no evidence for decreases in aggregate formation at 50 nM protein, and thus at least some aggregation must be present in the low nanomolar range. Furthermore, partial inhibition of aFGF-stimulated mitogenesis of 3T3 cells with relief of this effect at higher suramin:aFGF ratios can be detected at growth factor concentrations as low as 3 pM (not illustrated). This pattern of inhibition is entirely consistent with the cross-linking mechanism as proposed above since monovalent interactions leading to loss of the inhibitory effect should begin to predominant at higher suramin concentrations. Additional support for the ability of suramin to induce aFGF aggregation under biological conditions comes from the observation that suramin can complete with the binding of aFGF to heparin (Figure 6), a more natural ligand of the growth factor (Vlodavsky, 1991), with unimolar stoichiometry. This suggests

similar binding affinities of aFGF for suramin and heparin and the potential appearance of suramin-stimulated aFGF association in physiological environments.

The noncovalent nature of the suramin-induced aFGF association is also confirmed by the ability of other polyanions to reduce the size of the suramin-induced aggregate. It is interesting to note, however, that the formation of multiple aFGF-containing complexes is not itself sufficient to inactivate the growth factor since sulfated β-cyclodextrin (which binds ~2–3 aFGF molecules; Figure 3 and Table IV) and heparin (M_r ~16 000 binds ~10 aFGF molecules; Sommer & Rifkin, 1989; Mach et al., 1992) stimulate growth factor activity. In addition, Evan's Blue, which appears to bind to aFGF and is also symmetrical, has no effect on the overall size of aFGF. Possible explanations for these differences are that the nature of the ligand/aFGF complexes is fundamentally different (e.g., direct contact between aFGF molecules in suramin-induced complexes as opposed to a lack of protein/protein interactions in the sulfated β-cyclodextrin and heparin-mediated aggregates) or conformationally altered aFGF molecules are found in the presence of suramin. Obviously, these two different mechanisms are not mutually exclusive. To further explore this question, we examined the conformation of aFGF in the presence and absence of suramin using CD and FTIR. Although interpretation of the CD spectra appears to be complicated by differential light scattering and/or absorption flattening (Schneider, 1973), changes in the 195–240-nm region which are independent of optical path length suggest some type of conformational alteration may be occurring. Such changes need not involve significant changes in secondary structure since aromatic residues may also make a large contribution in this region (Woody, 1978). Furthermore, although free suramin does not contribute to the circular dichroism at the sensitivity of these experiments, induction of optical activity in chromophoric suramin itself upon binding to growth factors could also occur. A simple interpretation of corresponding amide I′ FTIR spectra would suggest that suramin is causing a small (~5%) increase in either the protein β-structure or the protein's random structure content (1640 cm^{-1}) and a decrease in turn content (1668 cm^{-1}) (Byler & Susi, 1986) but changes of this magnitude are difficult to quantitate unambiguously. Nevertheless, the cumulative data suggest that suramin may also be producing a small conformational change in aFGF as well as causing the much more marked aggregation process. The relationship, if any, between these two phenomena is unknown.

To what extent can we generalize these findings with aFGF to the action of suramin on other growth factors? Both bFGF and PDGF, which are also inhibited by suramin, are even more dramatically aggregated by the compound than aFGF. In contrast, IGF-1 and EGF, which are only weakly inhibited by suramin (Coffey et al., 1987; La Rocca et al., 1990b; Pollak & Richard, 1990), do not appear to undergo drug-induced aggregation. In contrast, IGF-1, but not EGF, does show evidence of a small suramin-induced conformational change. In this regard, it has recently been reported that suramin is a more potent inhibitor of IGF-1 binding to its receptor than previously recognized (Baghdiguian et al., 1992). We thus suggest that one effect of suramin upon its target growth factor is probably due to induced microaggregation of the proteins which effectively prevents their interaction with cell surface receptors. Although this aggregation is primarily due to the potentially bifunctional nature of both suramin and the polyanion binding site of the growth factors themselves, a small conformational change may also play a role in the process. The latter possibility may reflect the presence of a significant apolar region and/or the urea-like linkage in suramin, structural features not apparent in other polyanions examined.

It is possible that these results are relevant to a variety of other activities of suramin. For example, it has been proposed that suramin may inhibit phosphoglycerate kinase by cross-linking the active site of the enzyme (Hart et al., 1989). Suramin inhibition of a wide variety of enzymes is noncompetitive (La Rocca et al., 1990a; Nakajima et al., 1991; Constantopoulos et al., 1980; Spigelman et al., 1987), consistent with intermolecular cross-linking as an inactivating event. Indeed, several proteins have been reported to be precipitated by high concentrations of suramin (Town et al., 1950). Cross-linking could also potentially explain the immunological adjuvant activity of suramin (Van Der Meer et al., 1977; Brandely et al., 1985).

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