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Inhibition of growth factor binding, Ca^{2+} signaling and cell growth by polysulfonated azo dyes related to the antitumor agent suramin

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Abstract. The ability of the polysulfonated antitumor drug suramin and six related polysulfonated azo dyes to inhibit the cell growth, platelet-derived growth factor (PDGF)-receptor binding, and intracellular Ca^{2+} signaling of Swiss 3T3 fibroblasts was studied. Some of the azo dyes were more potent inhibitors of PDGF binding than was suramin. The concentration giving 50% inhibition (IC_{50}) of PDGF binding was 0.5 μM for the most potent azo dye as compared with 10 μM for suramin. The azo dyes were generally more potent inhibitors of nonmitochondrial Ca^{2+} uptake and of inositol(1,4,5)trisphosphate-mediated Ca^{2+} release in permeabilized Swiss 3T3 cells than was suramin, and they were more potent inhibitors of PDGF-induced Ca^{2+} signaling in intact Swiss 3T3 cells. The azo dyes were only as effective as or less effective than suramin in inhibiting the growth of Swiss 3T3 cells, with IC_{50} values of between 74 and 361 μM being noted for the dyes as compared with 70 μM for suramin. The difference between the growth-inhibitory activity of the azo dyes and that of suramin could not be explained by metabolism of the compounds, which was not detectable in either Swiss 3T3 cells or human liver slice preparations. The results suggest that suramin and some of the azo dyes have actions on cell growth in addition to inhibition of growth factor binding and of Ca^{2+} signaling.

Introduction

Suramin is a polysulfonated naphthylurea that has been used for a number of years to treat African trypanosomiasis and onchocerciasis [27]. Evidence showing that suramin was an inhibitor of retroviral reverse transcriptase [13] and that it could inhibit human immunodeficiency virus (HIV) in vitro [33] led to its clinical trial for the treatment of acquired immunodeficiency syndrome (AIDS) [28]. Although the results of these studies were disappointing, there were anecdotal reports of tumor regression in AIDS patients with tumors [9, 46]. This, combined with evidence that suramin could inhibit the growth of a number of tumor cell lines in vitro [2, 4, 10, 46], led to clinical trials of suramin as an antitumor agent, in which promising results were initially obtained in lymphoma, prostate, renal, and ovarian cancers [1, 14, 21, 26, 34, 47, 51].

The growth-inhibitory activity of suramin is generally ascribed to its ability to inhibit the binding of growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), heparin-binding growth factor 2 (HBGF-2), transforming growth factor β (TGF-β), insulin-like growth factor 1 (IGF-1), and interleukin 2 (IL-2) [4, 11, 32, 36], to their cell-surface receptors. Inhibition of growth factor binding by suramin may lead to the breaking of autocrine loops, which are a common lesion in human cancers [49]. Suramin has inhibitory effects on many isolated intracellular enzymes [22, 23, 30, 40]; however, it is not known whether this action contributes to the drug's growth-inhibitory activity. Suramin causes considerable patient toxicity at the doses employed clinically [27]. Compounds that are more potent than suramin acting by the same antitumor mechanism but with less toxicity may be of clinical utility.

In the present study, we investigated some of the growth factor-related activities, including actions on growth factor-receptor binding and intracellular signaling as well as inhibition of cell growth, of a series of polysulfonated azo dyes related to suramin and compared these activities with those of suramin. Our results suggest that suramin and azo dyes have actions in addition to the inhibition of growth factor-receptor binding that explain their cell growth-inhibitory activity.

Material and methods

Compounds and cells. The structures of the compounds used in the study are shown in Fig. 1. Suramin sodium (NSC 34936) and the polysul-

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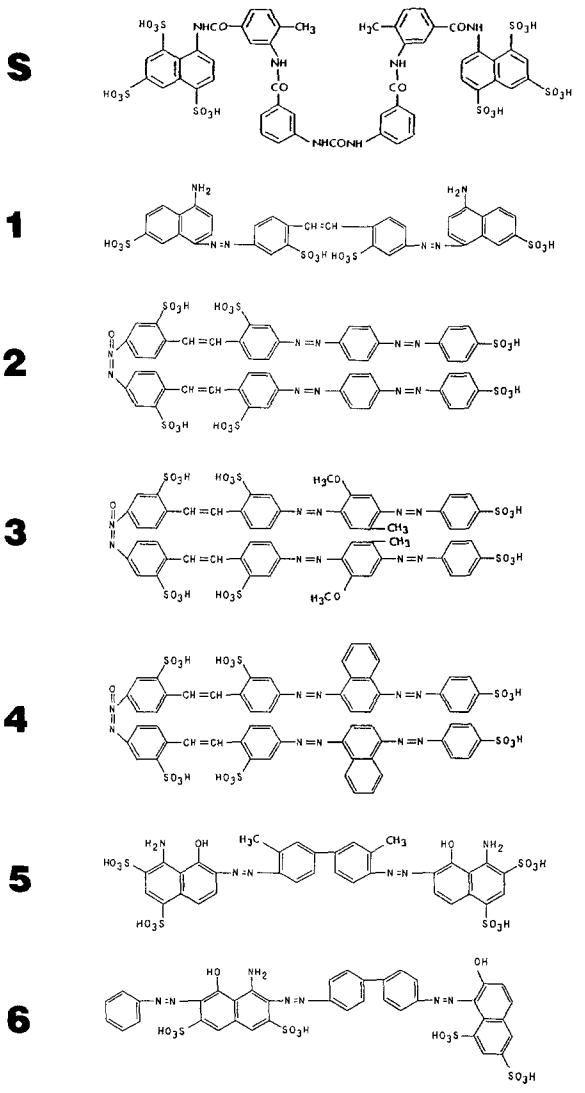

Fig. 1. Structures of suramin (*S*) and the azo dyes (*1–6*) used in the present study

fonated azo dyes 1 (NSC 34933), 2 (NSC 35612), 3 (NSC 25614), 4 (NSC 35616), 5 (NSC 65849), and 6 (NSC 7974) were provided by Dr. R. Schultz, Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health (Bethesda, Md.). $^{45}Ca^{2+}$ (2.5 mCi/mg) and (c-*cis*)-[^{125}I]-PDGF (780 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.). PDGF, human recombinant B-chain dimer, was purchased from Bachem (Torrance, Calif.). Inositol(1,4,5)triphosphate was obtained from Calbiochem (Irvine, Calif.). Aequorin was purchased from Dr. J. Blinks, Friday Harbor Laboratories, University of Washington (Seattle, Wash.). Swiss 3T3 fibroblasts were obtained from Dr. H. R. Herschman, University of California (Los Angeles, Calif.), maintained in bulk culture as previously described [43], and used between passages 24 and 30. Swiss 3T3 fibroblast were chosen for the study because their growth is inhibited by suramin, which inhibits the binding of PDGF to cell-surface receptors and because the signaling cascades activated by PDGF have been extensively characterized in these cells [20, 42, 43].

Growth inhibition. The inhibition of Swiss 3T3 fibroblast proliferation by suramin and the polysulfonated azo dyes was measured according to the colony formation of fibroblast cells growing on plastic culture surfaces over 7 days under continuous exposure to the compounds at concentrations of between 10 and 1,000 μM in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, with no change in the medium, as previously described [37].

PDGF binding assay. The binding of [^{125}I]-PDGF to Swiss 3T3 fibroblasts was measured at 4° C over 4 h by the method of Bowen-Pope and Ross [6].

Ca^{2+} signaling. Changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in intact Swiss 3T3 fibroblasts in response to PDGF were measured using cells loaded with the Ca^{2+}-sensitive photoprotein aequorin as previously described [35]. The cells were exposed to various concentrations of suramin and the azo dyes for 10 min prior to the addition of 3.3×10^{-9} M PDGF. Nonmitochondrial $^{45}Ca^{2+}$ uptake and inositol(1,4,5)trisphosphate-mediated $^{45}Ca^{2+}$ release were measured in saponin-permeabilized Swiss 3T3 fibroblasts using a modification of the method of Gill and Cheuh [17] as described elsewhere [42].

Phospholipase C assay. The inhibition of phosphatidylinositol phospholipase C (PIPLC) activity in the cytosol of Swiss 3T3 fibroblasts by suramin and the azo dyes was measured as previously described [38].

Statistical analysis. Concentration-response plots were analyzed by a nonlinear least-squares regression-analysis computer program (PC NONLIN; SCI Software, Lexington, Ky.) to find the concentration of agent required to produce a 50% inhibition of the response (IC_{50}) and the standard error. Groups of data were analyzed using Student's *t*-test [45], and a P value of <0.05 was considered to be significant.

Metabolism. The metabolism of suramin and selected polysulfonated azo dyes was studied. Suramin and compounds 1 and 3 were incubated at a concentration of 100 μM with 4×10^6 confluent Swiss 3T3 cells in 2 ml DMEM containing 10% fetal calf serum for up to 24 h or were incubated with 1 g (wet weight) of slices of surgical-waste normal human liver, obtained from a 73-year-old man, in 20 ml William's E medium gassed with 5% CO_2 in air on N_2 [7]. The levels of the compounds in the incubation medium were measured using a modification of the high-performance liquid chromatographic (HPLC) method for suramin of Ruprecht et al. [41]. Briefly, 0.5 ml incubation medium was mixed with 5 μl NaOH and 1 ml methanol at 4° C. After centrifugation at 4,000 *g* for 5 min, the protein pellet was washed twice with 3 ml 67% methanol in 10 mM NaOH and the supernatants were combined and lyophilized. Values were corrected for recovery, which was 85% for suramin and 100% for compounds 1 and 3. The residue was dissolved in 1 ml H_2O and subjected to HPLC on a PRP-1 Hamilton 250 ×4.1-mm reverse-phase column using a mobile phase consisting of 37% 10 mM ammonium acetate, 5 mM tetrabutylammonium phosphate (pH 7.6), and 63% methanol at a flow rate of 1 ml/min. Detection of eluting peaks was carried out by UV absorption at 254 nm for suramin, at 485 nm for compound 1, and at 450 nm for compound 3. The coefficient of variation of the assay was ±4.1%.

Results

Growth inhibition

Suramin and the azo dyes required more than 24 h exposure to the cells to produce significant growth inhibition (results not shown). The IC_{50} values for growth inhibition by the compounds following 7 days continuous exposure are shown in Table 1. Azo dyes 1–5 were 3–5 times less active than suramin in inhibiting cell growth, whereas compound 6 showed similar activity.

Inhibition of growth factor binding

Suramin and the azo dyes inhibited the binding of [^{125}I]-PDGF to Swiss 3T3 fibroblasts (Table 1). Typical concentration-response plots showing the inhibition of [^{125}I]-

Table 1. Some biological activities of suramin and the azo dyes measured in intact Swiss 3T3 fibroblasts

Compound	Growth inhibition (IC_{50}, μM)	Inhibition of PDGF binding (IC_{50}), μM	Inhibition of $[Ca^{2+}]_i$ response (IC_{50}), μM
Suramin	70±10	10.2±3.2	66.3±2.2
1	190±11*	0.5±0.0*	1.1±0.6*
2	361±12*	5.1±3.2	3.6±0.6*
3	340±13*	33.5±7.8*	8.5±2.7*
4	203±31*	1.0±0.3*	ND
5	290±61*	7.6±3.7	4.1±0.8*
6	74±18	0.9±0.3*	ND

Growth inhibition was measured following continuous exposure of cells to the compounds at concentrations of between 10 and 1000 μM over 7 days in culture. Inhibition of [^{125}I]-PDGF binding to confluent cells was measured at a [^{125}I]-PDGF concentration of 3.3×10^{-11} M over 4 h at 4° C. Inhibition of the $[Ca^{2+}]_i$ responses to 3.3×10^{-9} M PDGF was measured after 10 min exposure of cells to the compounds at 37° C. The compound concentrations used for these studies ranged from 0.1 to 80 μM. The peak $[Ca^{2+}]_i$ increase obtained using PDGF in the absence of compound (± SE, $n = 12$) was 872±63 nm. Data are expressed as mean IC_{50} values ± SE calculated from at least 6 determinations. ND, Not determined

* $P < 0.05$ as compared with the value obtained using suramin

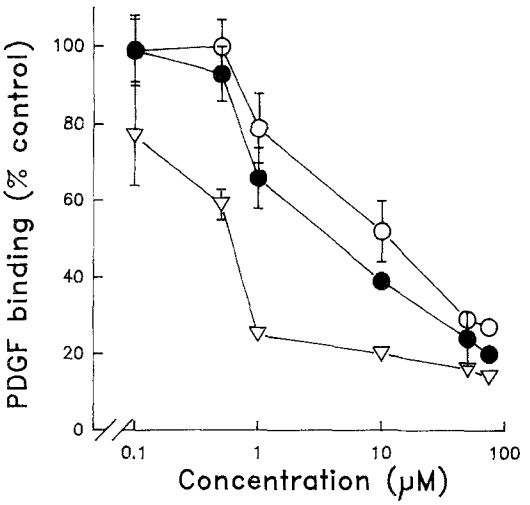

Fig. 2. Inhibition of PDGF binding to Swiss 3T3 fibroblasts by suramin and azo dyes. Cells were grown to a density of 10^5 cells/cm^2 and were exposed to 3.3×10^{-11} M [^{125}I]-PDGF and various concentrations of suramin (○), compound 2 (●), or compound 6 (▽) at 4° C for 4 h; 50% of the maximal binding of [^{125}I]-PDGF to Swiss 3T3 cells occurred at 3.6×10^{-11} M [^{125}I]-PDGF. Data represent mean values for 4 determinations and are expressed as a percent of binding in the absence of added compound. Bars indicate the SEM

PDGF binding to Swiss 3T3 fibroblasts by suramin and azo dyes 2 and 6 are illustrated in Fig. 2. The IC_{50} values obtained for the inhibition of [^{125}I]-PDGF binding by all of the compounds are shown in Table 1. Compound 3 was less active than suramin in inhibiting [^{125}I]-PDGF binding, compounds 2 and 5 showed similar activity, and compounds 1, 4, and 6 were at least 10 times more active than suramin.

Inhibition of $[Ca^{2+}]_i$ responses

We have previously reported that suramin inhibits the increase in $[Ca^{2+}]_i$ in response to PDGF in Swiss 3T3 fibroblasts [42]. The azo dyes were also found to block the increase in $[Ca^{2+}]_i$ in response to PDGF in Swiss 3T3 fibroblasts. The compounds tested (1, 2, 3, and 5) were up to 60 times more effective than suramin in inhibiting $[Ca^{2+}]_i$ responses (Table 1).

Inhibition of the uptake and release of $^{45}Ca^{2+}$

Suramin is an inhibitor of the ATP-dependent uptake of $^{45}Ca^{2+}$ and of the $^{45}Ca^{2+}$ release caused by 10 μM inositol(1,4,5)triphosphate in saponin-permeabilized Swiss 3T3 fibroblasts [42]. The azo dyes also inhibited $^{45}Ca^{2+}$ uptake and inositol (1,4,5)triphosphate-mediated $^{45}Ca^{2+}$ release in saponin-permeabilized Swiss 3T3 fibroblast (Table 2). Only compounds 2 and 3 were less active than suramin in inhibiting Ca^{2+} uptake. Compounds 2 and 3 were as active as suramin and compounds 1, 4, 5, and 6 were more active than suramin in inhibiting inositol(1,4,5)triphosphate-mediated Ca^{2+} release.

Table 2. Inhibition of Ca^{2+} signaling targets by suramin and the azo dyes

Compound	Inhibition of PIPLC (IC_{50}, μM)	Ca^{2+} uptake		Ca^{2+} release	
		% Control	IC_{50} (μM)	% Control	IC_{50} (μM)
Suramin	63.3±2.6	59.6±3.0	119.1±3.2	35.9±6.9	31.8±8.7
1	1.6±0.1*	31.0±2.9*	24.1±0.9*	7.1±2.1*	10.8±3.1*
2	7.9±7.1*	87.4±7.6	ND	32.1±9.3	ND
3	6.7±0.4*	69.3±1.8*	ND	30.6±1.1*	ND
4	2.6±0.3*	39.7±4.7*	ND	19.1±6.2*	ND
5	2.5±0.2*	32.9±2.9*	26.9±1.6*	0.0*	19.0±4.2*
6	1.2±0.1*	43.5±6.1*	ND	14.5±1.4*	ND

PIPLC activity in the cytosol of Swiss 3T3 fibroblasts was measured by the hydrolysis of [^{3}H]-phosphatidylinositol(4,5)bisphosphate. Data are expressed as IC_{50} values obtained using compound concentrations of between 0.1 and 100 μM. Control PIPLC activity was 42.2±2.6 nmol mg^{-1} 10 min^{-1}. Inhibition of $^{45}Ca^{2+}$ uptake and of release of $^{45}Ca^{2+}$ by 10 μM inositol(1,4,5)triphosphate from nonmitochrondrial stores was measured in saponin-permeabilized Swiss 3T3 cells using compounds at a concentration of 75 μM. The control value for $^{45}Ca^{2+}$ uptake was 0.39±0.01 nmol 10^{-6} cells/6 min^{-1}, and that for $^{45}Ca^{2+}$ release was 0.15±0.01 pmol/10^6 cells over 1 min. Data are expressed as a percentage of control values. For suramin and compounds 1 and 5, IC_{50} values were also obtained in studies using compound concentrations of between 1 and 100 μM. All data represent mean values ± SE. ND, Not determined

* $P < 0.05$ as compared with the value obtained using suramin

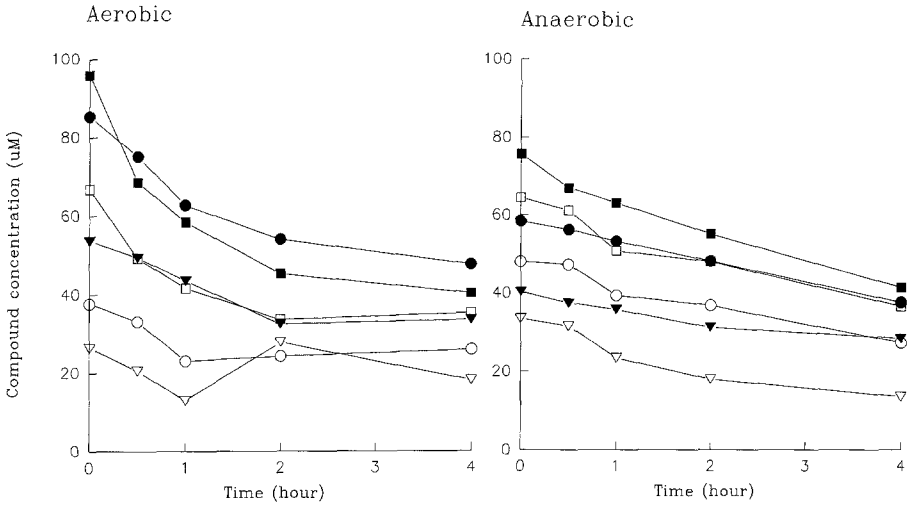

Fig. 3. Loss of suramin and polysulfonated azo dyes incubated with human liver slices. The compounds were incubated at an initial concentration of 100 μM with 1 g of fresh human liver slices (*open symbols*) or human liver slices treated for 10 min at 100° C in 20 ml Williams' E medium at 37° C (*closed symbols*). (□, ■), Suramin; (△, ▼), compound 1; (○, ●), compound 3. *Left panel*, incubations in 95% air: 5% CO_2 (*aerobic*); *right panel*, incubations in 95% N_2: 5% CO_2 (*anaerobic*)

PIPLC inhibition

Suramin and the azo dyes were found to inhibit PIPLC activity in the cytosol of Swiss 3T3 fibroblasts (Table 2). All of the azo dyes were considerably more potent inhibitors of PIPLC (IC_{50} values, 1–8 μM) than was suramin (IC_{50}, 63 μM).

Metabolism

When suramin and azo dyes 1 and 3 were incubated with Swiss 3T3 fibroblasts, no significant decrease was observed in the concentration of any of the compounds in the incubation medium over 24 h (data not shown). To determine whether differences in metabolism might lead to different in vivo antitumor activities for suramin and the azo dyes, we examined the metabolism of suramin and compounds 1 and 3 by human liver slices (Fig. 3). The recovery of the compounds from incubations using heat-inactivated liver slices were considerably greater at all times than from incubations employing fresh liver slices. Although a progressive loss of all of the compounds from the medium during the 4-h period of incubation occurred under both aerobic and anaerobic conditions, this loss did not appear to be attributable to metabolism since it was observed in both the fresh and the heat-treated liver slices. The loss may represent binding of the compounds to the liver slices that were not extracted for assay in this study. No metabolite peak was detected on the chromatograms for any of the compounds.

Discussion

The cell growth inhibition caused by suramin is generally ascribed to the drug's ability to interfere with the binding of growth factors to cell-surface receptors [2, 12, 20]. In the present study we found that 70 μM suramin produced a 50% inhibition of the growth of Swiss 3T3 fibroblasts. This is similar to the growth-inhibitory concentrations of suramin reported for tumor cell lines [3, 10, 12, 15] and is generally lower than the serum concentration of suramin seen in clinically responding cancer patients [47]. We found the IC_{50} value for the inhibition of PDGF binding to Swiss 3T3 fibroblasts by suramin to be 10 μM, which is similar to the values reported for suramin's inhibition of PDGF binding in other cells [20, 43, 44].

Polysulfonated azo dyes related to suramin have been reported to inhibit the growth of tumor cells in vitro [40, 49]. Polysulfonated azo dyes have also been reported to localize in tumors when given to animals, but to lack significant antitumor activity (reviewed by Regelson [39].) Isamine blue, a precursor of suramin, has undergone clinical trial as an antitumor agent, with negative results being obtained [39]. It was of interest for us to compare some biological activities thought to be important for the growth-factor action of a series of polysulfonated azo dyes related to suramin with those of suramin in an attempt to explain the basis for the differences in antitumor activity. All of the azo dyes studied inhibited Swiss 3T3 cell growth; however, none was more effective than suramin and only one dye (compound 6) showed growth-inhibitory activity equivalent to that of suramin. The activity of the other compounds was from 3 (compounds 1 and 4) to 5 times (compounds 2 and 3) lower than that of suramin. All of the azo dyes were found to be inhibitors of the binding of PDGF to Swiss 3T3 cells and were up to 20 times more potent than suramin. However, no correlation was found between the ability of the azo dyes to block PDGF binding and their inhibition of cell proliferation. Although PDGF is a major growth factor in serum for fibroblast proliferation [18], other growth factors are also required [8, 19]. The receptor binding of growth factors other than PDGF is also inhibited by suramin [4, 11, 32, 36] and may be inhibited by the azo dyes. However, the potencies of the azo dyes for inhibition of the binding of other growth factors are unknown.

The azo dyes inhibited the acute response of Swiss 3T3 fibroblasts to PDGF as measured by an increase in $[Ca^{2+}]_i$. The block of $[Ca^{2+}]_i$ signaling appeared to be related to the ability of the azo dyes to block PDGF receptor binding, and all of the azo dyes were more effective than suramin in blocking the $[Ca^{2+}]_i$ response to PDGF. The compounds were also found to have inhibitory effects on other aspects

of $[Ca^{2+}]_i$ signaling. Suramin and the azo dyes inhibited cytosolic PIPLC, which is responsible for the PDGF-mediated hydrolysis of phosphatidylinositol(4,5)bisphosphate to inositol(1,4,5)trisphosphate that leads to a release of intracellular Ca^{2+} [25]. The azo dyes were more potent inhibitors of PIPLC than was suramin. We have previously reported that suramin blocks Ca^{2+} uptake and inositol(1,4,5)trisphosphate-mediated Ca^{2+} release from the intracellular stores of permeabilized Swiss 3T3 fibroblasts [42]. The azo dyes were also found to block these processes and were at least as effective as suramin in doing so. Bootman et al. [5] have reported that polysulfonated azo dyes compete with inositol(1,4,5)trisphosphate for binding to its receptor on the endoplasmic reticulum. If the azo dyes can penetrate intact cells as does suramin [24, 48], they may have direct effects on PIPLC and Ca^{2+} uptake and release, which might explain their greater inhibition of $[Ca^{2+}]_i$ signaling as compared with that induced by suramin. It should be noted that the biological actions of the polysulfonated azo dyes were originally investigated because of their ability to localize in cells, including tumor cells [39].

No correlation was found between the inhibition of $[Ca^{2+}]_i$ signaling and the inhibition of cell proliferation by suramin and the polysulfonated azo dyes. Activation of PIPLC by PDGF, leading to an increase in $[Ca^{2+}]_i$, is a necessary but not sufficient step for cell proliferation [31]. Suramin is a more potent inhibitor of cell proliferation than are most of the azo dyes, and it may have effects on other pathways important for cell proliferation that are not shared with the azo dyes. Suramin has been reported to be an inhibitor of protein kinase C [30], DNA polymerases [23], and DNA topoisomerase II [22]. Inhibition of these enzymes by suramin might contribute to its inhibition of cell growth. The effects of polysulfonated azo dyes on these enzymes is not known.

We studied the possibility that differences in the biological activity of the polysulfonated azo dyes and that of suramin could be due to metabolism of the azo bond of the dyes. The azo bond in many compounds is susceptible to metabolism by cellular reductases, particularly under anaerobic conditions [29]. Suramin, on the other and, does not undergo appreciable metabolism by cells in culture [15] or in vivo [16]. Metabolism of the polysulfonated azo dyes in vivo could limit their potential usefulness as therapeutic agents. However, metabolism alone was insufficient to explain the differences in cell growth inhibition observed between suramin and the polysulfonated dyes, since no loss of suramin or of compounds 1 or 3 was detected over a 24-h period of incubation with cells in serum-containing medium. Moreover, no metabolism of suramin or of compounds 1 or 3 occurred during a 4-h period of incubation with human liver slices under aerobic or anaerobic conditions. We did, however, observe extensive tissue binding of suramin and, particularly, of the azo dyes to both fresh and heat-treated liver tissue. The lack of metabolism of the polysulfonated azo dyes is, perhaps, surprising and may mean that the dyes no not penetrate cells in sufficient concentrations to be metabolized.

The question remains as to whether the polysulfonated azo dyes might be effective antitumor agents in vivo. We found that at least one of the azo dyes (compound 6) was more effective than suramin in inhibiting PDGF receptor binding and as effective as suramin in inhibiting the growth of Swiss 3T3 cells. Neither suramin nor the azo dyes have shown significant antitumor activity against murine or human transplantable tumors in animals (Dr. V. Naryanan, National Cancer Institute, unpublished data) despite the demonstrable clinical activity of suramin. Therefore, the likely relative antitumor activity of the azo dyes as compared with suramin in humans cannot be predicted on the basis of animal studies. This presents a dilemma for the selection of suramin-related for clinical development. Further work in tumor cell lines and additional animal toxicology studies will be required to establish whether the polysulfonated azo dyes might offer any therapeutic advantage over suramin for human use.

In summary, we found that suramin and a series of polysulfonated azo dyes are inhibitors of PDGF binding and of cell growth in Swiss 3T3 fibroblasts. Nearly all of the azo dyes were more effective inhibitiors of PDGF-receptor binding than was suramin, but none was more effective than suramin in inhibiting cell growth, and some were considerably less effective. The azo dyes and suramin inhibited PIPLC and blocked PDGF-dependent $[Ca^{2+}]_i$ signaling in intact cells, with suramin being the least potent of the compounds tested. There was no detectable metabolism of suramin or the azo dyes by Swiss 3T3 cells or human liver slices. The present results suggest that although inhibition of growth factor-receptor binding may contribute to the inhibition of cell growth by suramin and some of the polysulfonated azo dyes, other actions also appear to be important.

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