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Eriochrome Black T, structurally related to suramin, inhibits angiogenesis and tumor growth *in vivo*

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The polyanionic species suramin is a potential anti-cancer agent of narrow therapeutic index. Among other pharmacological characteristics, suramin is an inhibitor of angiogenesis. We have targeted its angiostatic properties as part of a program to discover less toxic analogs. From screening a series of commercially available compounds, structurally related to suramin and containing a sulfonic acid substituted naphthylamine moiety, we discovered a new lead, Eriochrome Black T (EBT). EBT is a novel inhibitor of angiogenesis, more potent and less toxic than suramin in the chick chorioallantoic membrane assay. EBT was more active than suramin in inhibiting endothelial cell proliferation in primary culture and in inhibiting proliferation of three tumor cell lines, A431, L1210 and M5076 (IC_{50} 10–100 μM). Cell cycle studies on the A431 line showed that both EBT and suramin caused an accumulation of cells in the S phase, EBT being 10-fold more potent. We suggest that this cell cycle perturbation is linked to inhibition of topoisomerase II catalytic activity. EBT was found to be a moderate but significant inhibitor of matrix metalloproteinases (10 μM range), more efficient than suramin. In a s.c. M5076 sarcoma model in mice, EBT had similar efficacy to suramin both by the i.p. or s.c. route and was moreover better tolerated. Combined pharmacological results show that EBT compared favorably with suramin in all assays, and that *in ovo* and *in vivo*, EBT is an analog of suramin with diminished toxicity.

Key words: Angiogenesis, angiogenesis inhibitors, Eriochrome Black T, suramin.

Introduction

In the early 1920s, the symmetrical polyanionic derivative suramin (Figure 1) was introduced for the treatment of certain protozoal infections (African trypanosomiasis and onchocerciasis) in human beings.[1] In the 1980s, following observations that suramin inhibited reverse transcriptase activity of retroviruses,[2] the compound was studied in AIDS patients. Although the compound was concluded ineffective as an anti-HIV therapy, some circumstantial evidence prompted researchers[3] to investigate its effects in cancer patients. In turn, this led to promising results against hormone refractory prostate carcinoma.[4] However, the use of the compound is hindered by a general toxicity which narrows its therapeutic index.

Many studies have explored the pharmacological properties of suramin.[5] *In vitro*, the compound inhibited cell proliferation at high concentrations[6] and the compound was shown to be antiangiogenic in the chick chorioallantoic membrane (CAM) assay.[7] At the molecular level, the mechanism of action is most probably complex as suramin has been shown, for example, to be an inhibitor of protein kinase C,[8] heparanase[9] and DNA topoisomerase II,[10] an antagonist of the purinergic receptor,[11] an inducer of antiproliferative heparan sulfate production,[12] and a blocker of the activity of interleukin-1[13] and of several growth factors.[14]

In our search for novel antiangiogenic compounds, we have focused on the antiangiogenic and antiproliferative properties of suramin. Angiogenesis, the creation of new blood vessels, occurs during embryogenesis, all stages of growth and development, and wound healing. It is pronounced during the development of primary and secondary tumors, and has been targeted for drug development.[15] Inhibition of blood supply around a tumor was hypothesized by Folkman as a means to hinder tumor growth and to prevent invasion by metastasis.[16] The availability of evaluation tools[17] and the discovery of the endogenous angiogenesis inhibitors angiostatin[18] and endostatin[19] has greatly advanced the possibility of employing angiogenesis inhibitors in tumor dormancy therapy. Regarding exogenous compounds, two major synthetic classes have demonstrated angiostatic potential in the CAM assay; these are the polyanionic naphthalene derivatives such as suramin and the fumagillin analog best represented by TNP-470.[20] Both of these compounds are presently in phase II–III clinical trials.[21,22]

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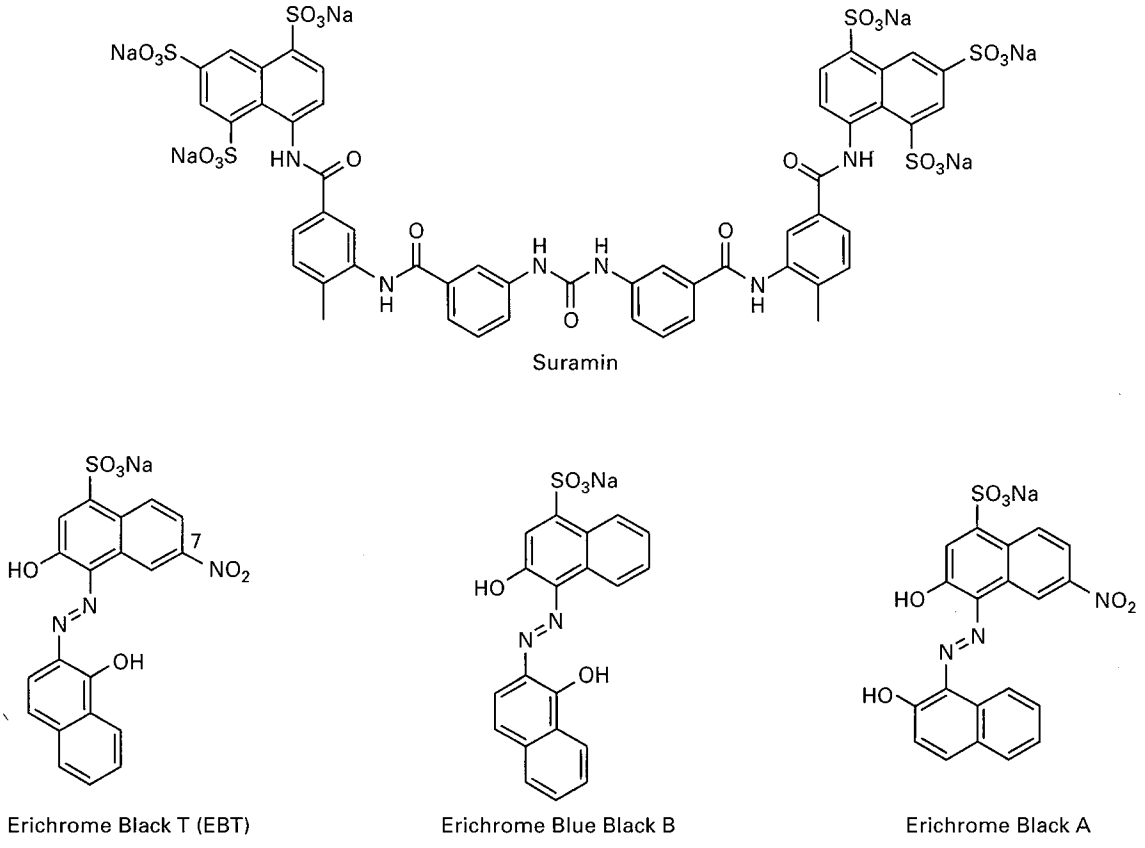

Figure 1. Chemical structure of the lead compound suramin and three compounds selected for screening.

One area of our search for suramin-related structures concentrated solely on the polysulfonated naphthylamine moiety present twice in the molecule (Figure 1). By a structure search of the Available Chemicals Directory (ACD; MDL Information System, San Leandro, CA) over 100 molecules were selected for screening, each bearing from one to three sulfonic acid functions on a naphthylamine residue. As expected, the majority of these compounds were categorized as dyes. It should be noted that we did not consider the symmetrical nature of suramin as a criteria when selecting structures to be screened although other groups have previously favored symmetrical structures in their work.[17,23-25] Similarly, the relative positions of the sulfonic acid functions with regard to the amine was not critical in the choice of structure. Pharmacological screening consisted of dual determination of cytostatic activity on a primary culture of endothelial cells and three tumor cell lines, and antiangiogenic activity in the CAM test. Of the compounds studied, a sole derivative, Eriochrome Black T (EBT) (Figure 1), was more active and potent than suramin in the CAM assay. Therefore the compound was selected for further evaluation. Close analogs of EBT, Eriochrome Blue Black B and Eriochrome Black A (Figure 1), did not fulfill requirements of greater activity than suramin in both screening tests and therefore were not selected for further studies. EBT has previously been identified as an inhibitor of alcohol dehydrogenase,[26] sorbitol dehydrogenase[27] and human skin collagenase.[28] Herein, we report the *in ovo* antiangiogenic activity and *in vivo* anti-tumor activity of EBT, with a preliminary characterization of possible molecular mechanisms.

Materials and methods

Chemicals

The hexasodium salt of suramin was purchased from Research Biochemicals International (Illkrich, France), and used directly. EBT was obtained from Aldrich (St Quentin Fallavier, France) and used directly (cEBT) or after purification (pEBT). Eriochrome Blue Black B was obtained from Aldrich and used as supplied. Erio-

chrome Black A was purchased from TCI America (Asniere, France) and employed without further purification. For all assays, suramin and EBT were dissolved in water, except in the CAM test where compounds were dissolved in 0.5 % methylcellulose.

Reference product Amsacrine (*m*-AMSA), obtained from TopoGEN (Columbus, OH), was dissolved in dimethylsulfoxide at 10^{-2} M and further diluted with water.

Purification of EBT

cEBT [500 mg, 79% pure by analytical HPLC: Kromasil C-18, acetonitrile/water/methanesulfonic acid (500/500/1), $\lambda = 210$ nm] was stirred in 30 ml of acetonitrile/water/trifluoroacetic acid (450/550/10). The mixture was filtered and the filtrate injected onto a Merck LiChroprep RP-18 (40-63 μm) glass column (40 × 400 mm). The system was eluted with acetonitrile/water/trifluoroacetic acid (450/550/10) at a flow rate of 10 ml/min. The second burgundy colored band was collected. Acetonitrile was removed from the corresponding fractions by rotary evaporation under reduced pressure and the residual aqueous phase was lyophilized to provide analytically pure pEBT (C, H, N) (200 mg).

Cell and culture proliferation assay

Three tumor cell lines, murine leukemia L1210, murine sarcoma M5076 and human epidermoid carcinoma A431, and a primary culture of endothelial cells from pig aorta (ECPA) were employed. ECPA cells were used during first passage. All cells were cultivated at 37°C under an atmosphere of 5% CO_2 in air and in RPMI 1640 medium (except ECPA in DMEM) supplemented with 10% decomplemented fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES, pH 7.4. All media and supplements were obtained from Gibco (Grand Island, NY).

Inhibition of cell proliferation by test compounds was measured using the microculture tetrazolium assay, as previously described.[29] In brief, cells were seeded in 96-well microplates at the appropriate densities to maintain exponential proliferation in control cells, and linearity between optical density and the number of viable cells. Plates were exposed to graded concentrations of test compounds (nine serial dilutions in triplicate) for approximately four doubling times; 48 h for L1210, 72 h for ECPA, and 96 h for A431 and M5076. After this period, IC_{50} values, corresponding to the concentration of compound required to reduce OD of treated cells by 50% with respect to control cells, were determined.

Cell cycle analysis

A431 cells were incubated for approximately two doubling times (40 h) in the presence of various concentrations of suramin or pEBT. Cells were then fixed with 70% aqueous ethanol (v/v), washed twice with PBS, and incubated for 30 min in PBS containing 100 μg/ml RNase and 50 μg/ml propidium iodide (PI). For each sample, 10 000 cells were analyzed by flow cytometry. Results are expressed as linear histograms of DNA content.

Topoisomerase II assay

Topoisomerase II catalytic activity was determined using the decatenation assay from TopoGEN. The reaction buffer contained 50 mM Tris-HCl, pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/ml BSA and 150 ng of kinetoplast DNA. Various concentrations of test compounds were added and then reactions were initiated by adding 2 U of topoisomerase II and allowed to proceed at 37°C for 30 min. The reaction was stopped by the addition of 1% sarkosyl, 0.025% bromophenol blue and 5% glycerol. Samples were subjected to electrophoresis for 2 h at 180 V in 1% agarose gel containing 0.5 μg/ml ethidium bromide and destained for 30 min in water. DNA bands were visualized by transillumination with UV, photographed and quantified by densitometric scanning of the photographic negatives.

Results are expressed as IC_{50} values, the concentration needed to produce a 50% reduction in the amount of decatenated DNA relative to the control.

CAM assay

Inhibition of angiogenesis was determined using a modification of the CAM assay.[17] Fertilized chick eggs, previously stored at 4°C, were disinfected with 70% aqueous ethanol and then transferred to an egg incubator (Grünbach, Wetzlar, Germany) regulated at 37°C and with 50% humidity for optimal growth conditions. Eggs were continuously rotated. On incubation day 3, an air sac was made by gently aspirating 1 ml albumin with an 18-gauge needle. On incubation day 4 approximately 2 cm^2 of shell was removed just above the air sac using finely tipped

forceps. The window was resealed with adhesive tape and the egg returned to the incubator.

Test compounds were dissolved in 0.5% methylcellulose at specific concentrations. Aliquots of 10 μl were layered on disks which were air-dried and posed on the growing CAM on day 6. A minimum of nine embryos were evaluated at each dose, where dose is defined as the amount of compound expressed in nanomole deposed on one egg. Controls were treated with blank methylcellulose disks. An inhibition of angiogenesis was indicated by an avascular zone around the methylcellulose disk 48 h after implantation. Results are expressed as the percentage of embryos showing inhibition within one experimental group. The surface of the avascular zone was estimated and scored as less than 4, 4-10 and greater than 10 mm. General toxicity was estimated by local necrosis, hemorrhage or marked distortion of large vessels of the embryos.

M5076 *in vivo* tumor model

Specific pathogen-free female C57Bl/6 and BDF1 (C57Bl/6 × DBA2) mice were purchased from Iffa Credo (Lyon, France). Mice were 4-6 weeks old and weighed 20-22 g at the start of the experiments.

M5076 reticulosarcoma was obtained from the Division of Cancer Treatment, Tumor Repository, NCI. The tumor was regularly propagated in C57Bl/6 strain mice in ascitic form by i.p. inoculation. For experiments, ascitic fluid was withdrawn and a cell suspension prepared on day 0. Cells were counted in the presence of Trypan blue and then diluted in RPMI 1640 medium. Two million M5076 cells in 0.2 ml were s.c. injected into the mid-dorsal pelvic region of each BDF1 test mouse. The implanted mice were randomly distributed in treated (eight animals per dose) or in control group (32 animals). One day after tumor implantation, compounds were administered i.p. or s.c., once daily for 12 days at the indicated doses. On day 13, tumor volume was estimated by two-dimensional measurements performed with a slide caliper and following the formula [length (mm) × width^2 (mm^2)]/2. The median tumor volume (MTV) of each treated group was compared with that of the control group and the results expressed as percentage T/C:

$$\text{median \%T/C (tumor volume)} = \frac{\text{MTV of treated group}}{\text{MTV of control group}} \times 100$$

As the M5076 model is particularly responsive to alkylating agents,[30] cyclophosphamide was employed as a reference molecule.

Matrix metalloproteinase (MMP) inhibition assay

MMP inhibition was determined using the fluorogenic substrate Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH_2 purchased from Bachem (Budendorf, Switzerland). The substrate is cleaved by the MMPs between amino acids Gly and Cys(Me) to yield a quantifiable, fluorescent fragment.[31] Recombinant human MMP-1 (interstitial collagenase), MMP-2 (72 kDa gelatinase A), MMP-3 (stromelysin 1) and MMP-9 (92 kDa gelatinase B), supplied by G Murphy (Strangeways Laboratory, Cambridge, UK), were employed at a final concentration of 3.4, 0.48, 4.8 and 0.36 μg/ml, respectively, after (4-aminophenyl) mercuric acetate activation. Assays were performed in a total volume of 100 μl of 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$ and 0.1% Brij 35 at pH 7.7, and were initiated by addition of 2 μM of substrate. Mixtures were incubated at 37°C for 6 h before measuring fluorescence with a fluorometer (Cytofluor 2350, Millipore-Perseptive Biosystems (Voisins le Bretonneux, France) equipped with filters for excitation and emission at 365 and 450 nm, respectively.

Results

Inhibition of cellular proliferation

Antiproliferative effects of suramin, pEBT and cEBT were evaluated on murine leukemia L1210, human epidermoid carcinoma A431 and murine sarcoma M5076 cell lines, and on a primary culture of endothelial cells from pig aorta (ECPA).

Results are expressed as IC_{50} values and are presented in Figure 2. On all cell types, suramin appeared mildly cytostatic with IC_{50} values ranging from 224 to 513 μM. pEBT was significantly more cytostatic than suramin with IC_{50} values ranging from 10 to 55 μM, and cEBT provided values ranging from 27 to 97 μM. EBT appears less cytostatic towards ECPA cells than the tumor cells.

During screening, Eriochrome Black A and Eriochrome Blue Black B showed IC_{50} values ranging from 115 to 226 and 50 to 98 μM, respectively (data not shown).

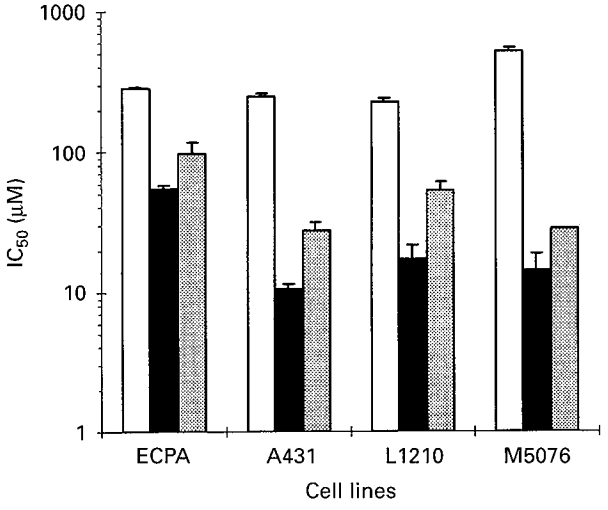

Figure 2. Inhibition of cell proliferation by suramin (□), pEBT (■) and cEBT (▒) on ECPA and three tumor cell lines A431, L1210 and M5076. Results are expressed as IC_{50} (μM) and SEM are obtained in at least three independent experiments.

Effect on A431 cell cycle

Perturbations induced by test compounds on the cell cycle of A431 cells were studied by flow cytometry. Representative results are shown in Figure 3. Both compounds caused a significant increase in the percentage of cells in the early S phase of the cycle, approximately 55% of cells were accumulated in the early S phase for suramin and pEBT compared to 31% for untreated cells. pEBT is clearly more potent than suramin, the compounds giving similar results at 50 and 500 μM, respectively.

Inhibition of topoisomerase II catalytic activity

In the presence of eukaryotic topoisomerase II, kinetoplast DNA (KDNA, covalently linked DNA circles) is monomerized into nicked and relaxed DNA monomers. The reference compound, *m*-AMSA, completely inhibited decatenation of KDNA at a concentration of 100 μM. Suramin and pEBT induced a 50% inhibition of decatenation at 6.4 and 2.35 μM, respectively (Figure 4).

Antiangiogenic activity in the CAM assay

The antiangiogenic activity of pEBT and suramin was compared in the CAM assay at equimolar doses on

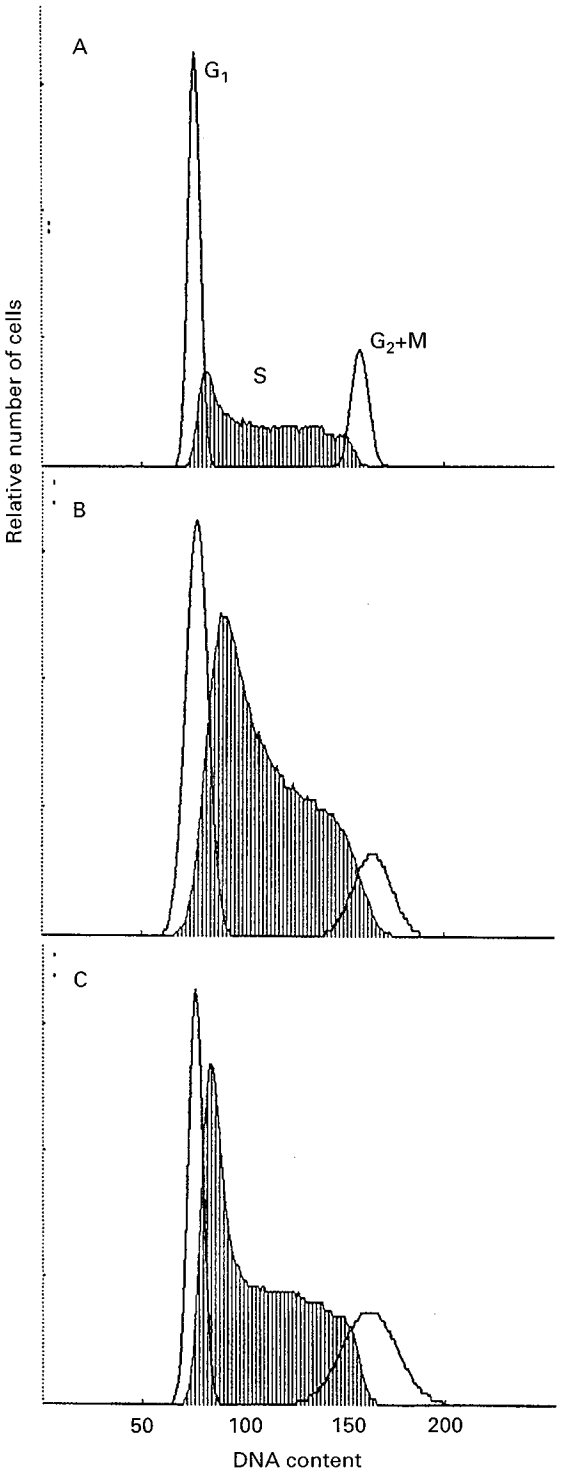

Figure 3. Effect of suramin and pEBT on the cell cycle of A431 cells. (A) Typical flow cytometric histogram of untreated A431 cells stained with PI. (B) Effect of 500 μM suramin on A431 cells treated for 40 h. (C) Effect of 50 μM pEBT on A431 cells treated for 40 h.

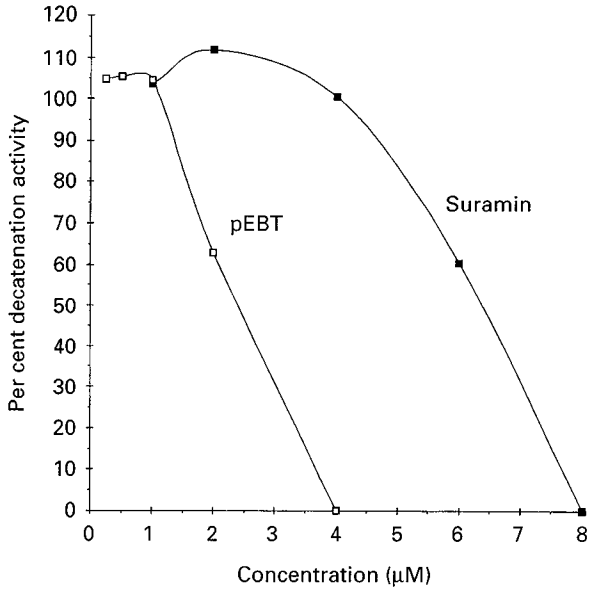

Figure 4. Inhibition of topoisomerase II by suramin and pEBT: percentage of decatenation activity versus compound concentration.

methylcellulose disks. The percentage of eggs showing an avascular zone and the distribution of zone diameter, as observed in a typical, representative experiment, are given in Figure 5. In this assay, suramin showed no angiostatic effect at 31.25 nmol but induced avascular zones in 33% of treated eggs at 62.5 nmol. Inhibition, as previously noted,[32] was dose dependent with 50 and 75% of treated eggs scoring as positive at 125 and 250 nmol, respectively. At 250 nmol, the antiangiogenic effect was associated with toxicity as vessel damage and local hemorrhage was observed in all of the positively scored eggs.

In comparison to suramin, pEBT started to induce avascular zones in 50% of eggs at the lower dose of 31.25 nmol and had a maximum effect at 125 nmol where 100% of the eggs scored positive. At 250 nmol, pEBT was still highly active, avascular zones were observed in 78% of eggs, with no observable signs of toxicity. The size of the avascular zone around the disks is significantly greater in the case of pEBT (and cEBT, results not presented) when compared to

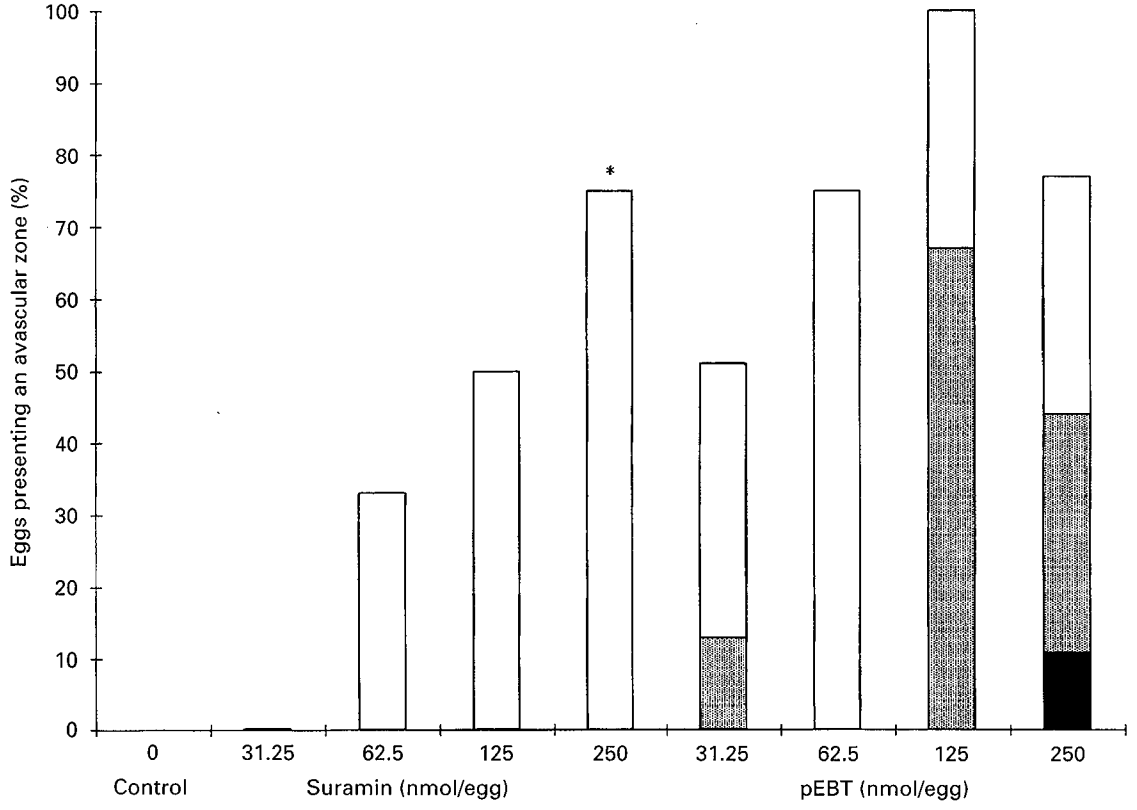

Figure 5. Inhibition of angiogenesis in the CAM assay by suramin and pEBT: percentage of eggs presenting an avascular zone and distribution of zone diameter (□, avascular zone <4 mm; ▩, avascular zone 4–10 mm; ■, avascular zone > 10 mm). * Toxic dose.

suramin. At the optimal dose of 125 nmol of pEBT, 30% of eggs show an avascular zone of less than 4 mm; however, the remaining 70% have avascular zones in the region of 4–10 mm. At the highest used dose of 250 nmol, avascular zones of greater than 10 mm became visible.

Over a series of CAM assays, pEBT averaged at $80 \pm 3\%$ ($n=45$), cEBT averaged at $68 \pm 8\%$ ($n=4$) and suramin averaged at 30 ± 5 % ($n=12$) for a deposit of 125 nmol of test compound. The two structurally related derivatives, Eriochrome Black A and Eriochrome Blue Black B, induced avascular zones in 0 and 18% ($n=2$) of eggs, respectively.

Antitumor activity

The antitumor activity of suramin and pEBT were compared in the same experiment against s.c. grafted M5076 sarcoma (Table 1). In this model, daily i.p. administration of suramin over 12 days provoked a weak inhibition of tumor growth, providing a T/C of 65% at the maximum tolerated dose (MTD) of 30 mg/kg. At 60 mg/kg i.p., suramin caused death in three out of eight mice. Subcutaneous administration of suramin provoked stronger antitumor activity (T/C = 21% at 60 mg/kg) and less toxicity, the MTD being 2-fold higher at 60 mg/kg. At 120 mg/kg s.c. and i.p., suramin provoked mortality in all animals.

Under the same conditions, pEBT was clearly less toxic than suramin. There were no cases of toxic death from pEBT with the schedules used up to the optimal dose of 120 mg/kg. pEBT was active by either the i.p. or s.c. route and induced T/C values of 24 and 34% at 120 mg/kg, respectively. At 240 mg/kg i.p., pEBT provoked mortality. Up to 120 mg/kg, there were no major signs of toxicity regarding body weight change, although slight weight differences between i.p. treated animals and the control indicate a better tolerance with s.c. over i.p. administration of pEBT.

Similar to pEBT (experiment not shown), cEBT was well tolerated with no toxic deaths being observed up to 120 mg/kg and induced a T/C value of 33% at 120 mg/kg on day 13 after daily i.p. administration over 12 days.

Inhibition of MMP activity

As EBT had previously been described as an inhibitor of skin collagenase,[26] its inhibitory effect on four representative MMPs was determined. The IC_{50} values for interstitial collagenase, 72 kDa gelatinase A, stromelysin 1 and 92 kDa gelatinase B (MMP-1, MMP-2, MMP-3 and MMP-9, respectively) are given in Table 2. Suramin had an IC_{50} range from 54.7 to greater than 1000 μM. pEBT was more active than suramin with 50% inhibition being observed in the 10 μM range.

Table 1. Antitumor activity of purified EBT (pEBT) and suramin against s.c. M5076 sarcoma

Experimental group	Schedule and route	Dose (mg/kg)	Body weight change day 12 – day 1 (g)	Total of toxic deaths by day 13	Median tumor volume on day 13 (mm^3)	Median T/C on day 13 (%)
Suramin	days 1–12 i.p.	15	+0.0	0/8	166	59
		30	+0.8	0/8	182	65
		60	+0.1	3/8	28	10
		120	toxic	8/8	–	–
	days 1–12 s.c.	15	+0.7	0/8	198	71
		30	+1.3	0/8	157	56
		60	+1.5	0/8	60	21
		120	toxic	8/8	–	–
pEBT	days 1–12 i.p.	15	+0.6	0/8	211	75
		30	+1.3	0/8	237	85
		60	+0.8	0/8	159	57
		120	−0.2	0/8	66	24
	days 1–12 s.c.	15	+1.0	0/8	225	80
		30	+0.9	0/8	255	91
		60	+1.9	0/8	235	84
		120	+1.8	0/8	95	34
Cyclophosphamide	days 1,5,9 i.p.	60	+1.1	0/8	0	0
Control	–	–	+1.7	0/32	280	100

A suspension of 2×10^6 M5076 cells was implanted s.c. on BDF1 mice on day 0. Drugs were administered once daily on days 1–12 by i.p. or s.c. route. Eight mice for each treated group and 32 mice for the control group were employed.

Table 2. Inhibition of MMP activity by suramin and pEBT

Compound	MMP-1	MMP-2	MMP-3	MMP-9
Suramin	167.1±22.8	54.7±21.7	>1000	281.1±130.7
pEBT	10.7±6.3	12.7±3.5	39.9±4.4	22.3±6.3

Mean IC_{50} (μM) values determined from two experiments carried out with triplicate samples.

Discussion

Suramin has been employed as an antiparasitic drug in man for over 70 years, and more recently in trials in HIV and cancer patients. It has, however, broad spectrum toxicity and necessitates patient monitoring irrespective of actual therapeutic use. The compound, among other properties, is a known inhibitor of angiogenesis and it is this specific property that prompted us to look at structurally similar compounds in our search for tumor angiogenesis modulators. From these studies, we have identified the comparatively low molecular weight, non-symmetrical angiostatic agent EBT. Some structure-activity relationships in this screening were stringent. For example, close EBT analogs Eriochrome Blue Black B, where the 7-nitro group is replaced by hydrogen, and Eriochrome Black A, an isomer of position, were more cytostatic than suramin on the cell types studied, but were devoid of significant antiangiogenic activity in the CAM assay and therefore not selected for *in vivo* evaluation.

In cell proliferation assays, suramin was a mild and non-selective cytostatic on the four cell types tested. EBT was predominantly more cytostatic than suramin but similarly not selective between tumor lines. EBT was less cytostatic towards endothelial cell culture in comparison to those tumor cell lines employed. These results demonstrate that neither suramin nor EBT are selective inhibitors of endothelial cell proliferation as seen with certain tumor angiogenesis regulators.[18,19] Analysis of cell cycle was performed on human epidermoid carcinoma A431 cells, a line over-expressing epidermal growth factor receptor.[33] Cell cycle was perturbed in the same manner by suramin and EBT, both compounds enhancing the percentage of cells in the early S phase. However, EBT was 10-fold more potent.

At the molecular level, suramin is known to inhibit the interaction of DNA with topoisomerase II without stabilizing the transient cleavable complex.[10] Topoisomerase II levels vary during the cycle with an increase in the S and G_2/M phases. As a possible explanation for the effect of EBT on the cell cycle, we looked at its effect on the catalytic activity of topoisomerase II, measured by the decatenation of KDNA, in comparison to suramin. EBT was significantly more potent than suramin in this assay. As previously proposed for suramin,[10] it may be hypothesized that accumulation of cells in the S phase of the cell cycle by EBT is a consequence of the inhibition of the topoisomerase II activity and that this ultimately inhibits cell proliferation.

Inhibition of angiogenesis in the CAM assay found EBT to be both more potent and less toxic than suramin. The mechanism of this antiangiogenic activity could be due, in part, to a direct inhibition of endothelial cell proliferation. Compared to the tumor cell lines, pig aorta endothelial cell cultures were less sensitive to EBT. However, as the endothelial cells employed *in vitro* and those *in ovo* are of different origin and proliferate in two and three dimensions, respectively, a direct inhibition of cell proliferation in the CAM assay may only be tentatively disfavored. Similar to suramin, a study of the molecular mechanisms of action of EBT is rendered complex as suramin, for example, is also a non-selective inhibitor of growth factor binding to cell surface receptors[14]—a property which could be involved in the inhibition of cell proliferation by these compounds.

The MMP family of enzymes is known to be involved in the remodeling of tissues preceding angiogenesis.[34] EBT, previously reported to inhibit skin collagenase,[26] was found to have a moderate and non-selective inhibitory activity against MMP-1, MMP-2, MMP-3 and MMP-9, and EBT was again more active than suramin. It is thus feasible that inhibition of MMP activity may be involved in the antiangiogenic activity observed in the CAM assay.

In vivo, on the murine reticulum sarcoma M5076 model, EBT was active at inhibiting tumor growth and was substantially less toxic than suramin. This enlarges the possible therapeutic window compared to the parent compound. Regarding possible mechanisms of action, antiangiogenic activity may contribute to the antitumor activity observed, although a direct inhibition of M5076 tumor cell proliferation may also be implicated.

In cell proliferation studies, antiangiogenic activity in the CAM assay and tumor growth inhibition *in*

vivo, the pharmacological response to purified EBT was slightly more pronounced than to commercial EBT. However, the difference in biological effects shows that purification of commercial EBT is not necessary.

Conclusion

EBT compares favorably to suramin regarding inhibition of cell proliferation, antiangiogenic activity in the CAM test and tumor growth inhibition *in vivo*. The compound is less toxic than suramin *in ovo* and better tolerated in mice. EBT may be considered as a chemical lead and a potential candidate for further exploratory studies and chemical optimization.

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