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Suramin Selectively Inhibits Carcinoma Cell Growth that is Dependent on Extracellular Polyamines

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Abstract. *Polyamines are necessary for tumour cell growth. Inhibition of endogenous polyamine biosynthesis results in compensatory up-regulation of polyamine uptake. Here, the combined effect of suramin and the polyamine biosynthesis inhibitor α-difluoromethylornithine (DFMO) on human carcinoma cell proliferation was studied. Suramin selectively inhibited the growth of tumour cells made dependent on extracellular polyamines by DFMO-treatment. In an animal tumour model, low non-toxic doses of suramin resulted in a 2-fold increase in DFMO tumour growth reduction. Moreover, suramin bound strongly to polyamine-agarose and significantly inhibited polyamine uptake in DFMO-treated cells. Our results indicate that non-toxic doses of suramin augment tumour growth inhibition by DFMO, and that a combination of these well-studied anticancer drugs may represent an additional strategy for cancer treatment.*

Polyamines are essential for cellular proliferation and neoplastic conditions are associated with elevated polyamine levels (1-7). Intracellular polyamine content is determined by *de novo* biosynthesis and uptake of extracellular polyamines. Depletion of intracellular polyamines by α-difluoromethylornithine (DFMO), *i.e.* a specific inhibitor of ornithine decarboxylase that catalyses the first step in the synthetic pathway, results in a substantial up-regulation of polyamine transport (1, 3-4, 7). Increased recruitment of extracellular polyamines from the circulation counter-acts the anti-proliferative effect of DFMO on cancer cells *in vivo*. Thus successful antitumour therapy directed at the polyamine system requires efficient inhibition of both *de novo* polyamine synthesis and uptake of polyamines from the extracellular milieu.

Cell-surface proteoglycans (PG), especially those substituted with heparan sulphate (HS), have been implicated in membrane translocation of a number of polybasic compounds (8-11). Previous studies from our group have shown that HS efficiently binds to polyamines and inhibits polyamine uptake competitively (12-13), that cell-surface HSPG facilitates polyamine uptake (13-15) and that combined inhibition of polyamine and HS biosynthesis attenuates tumour growth in a mouse model (14). These and other findings identify cell-surface HSPG as an attractive target in the treatment of malignant diseases (reviewed in 16).

Suramin is a polysulfonated naphtylurea that has been used in the treatment of trypanosomiasis since the 1920's (reviewed in 17). Owing to its polyanionic nature, suramin is often referred to as a heparin/HS mimetic. Accordingly, suramin has been shown to interfere with signalling of HS-binding growth and transcription factors, *e.g.* fibroblast growth factor, platelet-derived growth factor and HIV-Tat (18-22). Another well-established effect of suramin is the inhibition of internalisation and lysosomal degradation of GAG chains (23), which results in substantial cellular accumulation of these polysaccharides. Indeed, a mucopolysaccharidosis-like storage disorder has been demonstrated in suramin-treated animals (24), and increased levels of circulating GAG caused anticoagulation in humans (25). Interestingly, Stein *et al.* suggested increased GAG levels as an alternate mechanism of suramin anticancer activity (26), which was also discussed in a review by Voogd *et al.* (17).

For the purpose of the present investigation, we chose to study a human bladder carcinoma cell line that has been extensively studied in our laboratory with respect to cell-surface HSPG recycling and, more specifically, the effects of suramin on these processes. Suramin intervenes with cell-surface HSPG recycling by arresting internalised PG in endolysosomes, which is reflected by an approximately 3-fold increase in intracellular HS material as compared with

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control cells (27-29). We thus hypothesized that tumour cell growth that is rescued by extracellular polyamines may be efficiently counteracted by suramin *via* inhibition of cell-surface PG turnover and polyamine-GAG interactions.

Materials and Methods

Chemicals and reagents. Polyamines were obtained from Sigma Chemical Co, and ^{14}C-labelled polyamines from Amersham International, UK. DFMO was from Ilex Oncology, USA, and suramin (Germanin) from Bayer, Germany. Cell media and supplements were from Biomedicals Inc., Chemicon, Sweden. Heparin sepharose and Hi-Trap NHS-activated agarose were obtained from Pharmacia-LKB Biotechnology, Sweden. The NHS-activated column was conjugated with spermine according to the manufacturer's instructions.

Cell culture and in vitro growth experiments. Human bladder carcinoma T24 cells (denoted ECV 304 in refs 27-29) were provided by Dr. Inge Olsson, Dept. of Hematology, Lund University, Sweden, and routinely cultured in Dulbecco's MEM supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium). For *in vitro* proliferation experiments, T24 cells were seeded at 3000 cells/well in 96-well micro plates and allowed to adhere for 4 hours in growth medium. Cells were starved in serum-free medium for 24 hours, and then incubated in growth medium with or without substances dissolved in the medium. The growth-rate was measured at different time-intervals, using the crystal violet method: Cells were fixed in 1% glutaraldehyde dissolved in Hanks balanced salt solution (NaCl 80 g/L, KCl 5 g/L, glucose 10 g/L, KH$_2$PO$_4$ 600 mg/L, NaHPO$_4$ 475 mg/L) for 15 minutes, then nucleic acids were stained with the crystal violet dye (0.1%) for 30 minutes. After extensive washing and dissolving in 2% Triton X-100 overnight, the amount of bound dye was determined by A600 measurement in a Multiscan Titertek photometer.

Affinity chromatography on spermine-agarose and heparin sepharose. Suramin and heparin binding to polyamines was analysed on an 1 ml Hi-Trap agarose column coupled with spermine. Samples (1.5 mg) were dissolved in 1 ml PBS (0.137 M NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.5) and applied to the column that was eluted with a guanidine HCl gradient from 0 M (fraction 10) to 2.0 M (fraction 60) in the same buffer at a rate of 0.5 ml/minutes. The effluent was collected and analysed by the dimethylmethylene blue method (30) or UV-absorbance at 280 nm. In the competition experiments on heparin sepharose (1 ml, 10 mg/ml), spermine (2 mg) and 0.1 mCi ^{14}Cspermine were mixed in 1 ml PBS and applied to the heparin column, followed by a 10-minute wash with PBS at a flow rate of 0.5 ml/min. Suramin (10 mg) was then dissolved in 1 ml PBS and applied to the column, followed by another wash. This procedure was repeated three times and finally the column was eluted with 2 M NaCl in PBS for 10 minutes. One minute fractions were collected and analysed for radioactivity by scintillation counting using a LKB Wallac Rackbeta Counter.

Polyamine uptake experiments. T24 cells were plated in 24-well culture plates at 4 x 10^4 cells/well in growth medium supplemented with 5 mM DFMO. Cells were grown for 3 days until subconfluency, the growth medium was aspirated and the cells were rinsed twice with MEM. Then 0.5 ml MEM without serum containing varying concentrations of polyamines and ^{14}Cpolyamines (specific activity, 31 Ci/mol) was added, and the cells were incubated for 20 minutes at 4°C or at 37°C under a 5% CO$_2$ atmosphere. The incubation medium was removed and the cells were washed three times with ice-cold MEM containing 1 mM unlabelled polyamine and then once with MEM. The cells were lysed with 0.5 M NaOH for 1 hour at 37°C, and an aliquot of the homogenate was neutralized with 0.5 M HCl and analysed for radioactivity by scintillation counting. The difference in cell-associated polyamine levels

obtained at 37°C minus 4°C was defined as temperature-dependent cell-associated polyamine uptake. Competition for uptake by suramin was determined by adding varying concentrations of suramin at a fixed concentration of 0.5 μM of the respective polyamine.

Animal experiments and tumour formation in vivo. Female SCIDnodCA mice (7-8 weeks old) were kept under pathogen-free conditions in the animal barrier facility at the Biomedical Centre, Lund University, according to the Swedish guidelines for humane treatment of laboratory animals. The experimental set-up was approved by the ethical committee for animal research in Malmö/Lund, Sweden. For assessment of DFMO and suramin antitumour activity, T24 cells (1 x 10^6 cells in 200 ml PBS) were injected subcutaneously in the dorsal region of 7 to 8-week-old mice. DFMO 1% (w/w) was administered *ad libitum via* the drinking water, and suramin (100 mg/kg in PBS) by intraperitoneal injection once a week. Controls received drinking water with no additives and/or weekly injections with sterile PBS. The animals were killed following 3 weeks of treatment and tumour mass was recorded. Six individuals per group were included in each experiment.

Statistical analysis. All data are presented as means ± S.E.M. Differences from the mean were tested for significance by means of Student's *t* test using Microsoft Excel software.

Results

Effects of DFMO, polyamines and suramin on cell-growth in vitro. The polyamine synthesis inhibitor DFMO had a maximal antiproliferative effect on human bladder carcinoma T24 cells at a concentration of ≥ 5 mM (approx 55% growth-inhibition as compared with control), and extracellular addition of the polyamine spermine restored growth of DFMO-treated cells in a dose-dependent manner (Figure 1a). These results indicate that T24 cells exhibit a functional transport system for the utilization of extracellular polyamines. This was confirmed by polyamine uptake experiments (Figure 1b), showing Michaelis-Menten kinetics up to 50 μM with a Km of approx 1.5 μM, as determined by using the Lineweaver-Burke formulation (not shown). In the following experiments, the effects of suramin on either untreated cells or cells treated with DFMO and various polyamines were investigated (Figure 2). Putrescine, spermidine and spermine, *i.e.* the polyamines found in mammalian cells, all restored growth of DFMO-treated cells by 90-100% at a concentration of 0.5 μM. In untreated T24 cells, suramin exhibited no significant effect on growth up to 0.2 mM, and only limited growth-inhibition (approx 18% growth-inhibition as compared with control) was observed at 0.5 mM (Figure 2, filled diamonds). However, T24 cells made dependent on extracellular polyamines by DFMO-treatment, were significantly growth-inhibited at 0.1-0.2 mM suramin. At 0.5 mM suramin, cells treated with DFMO and restored by either putrescine, spermidine, or spermine were growth-inhibited by approx 57 (84) %, 58 (86) % and 54 (80) %, respectively. The figures within brackets represent the inhibitory activity of suramin on growth restored by extracellular polyamines. These results indicate that tumour cells were preferentially susceptible to the antiproliferative activity of suramin when made dependent on extracellular
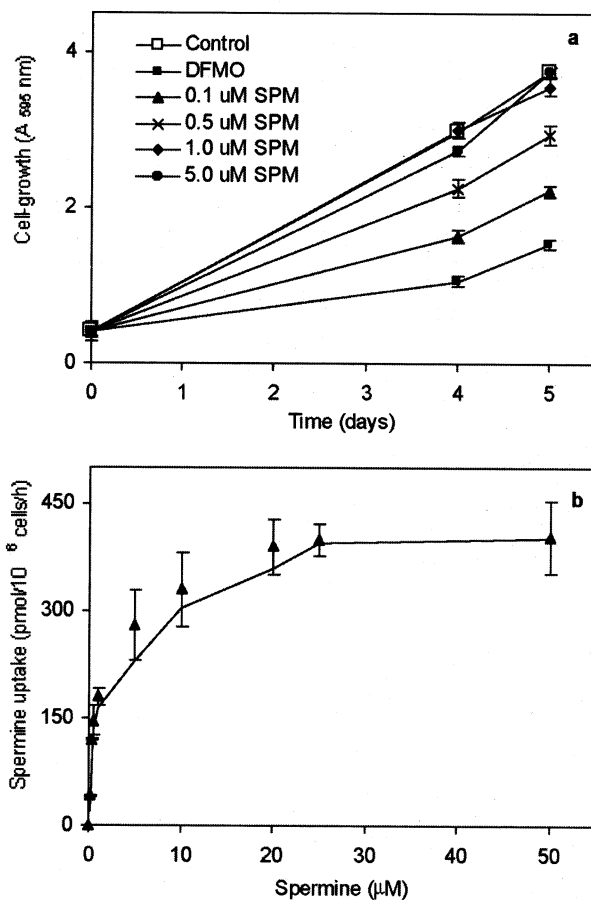
Figure 1. *Uptake of and growth-restoration by extracellular polyamines in T24 carcinoma cells. a) T24 cells were incubated in growth medium with no additions (control), or 5 mM DFMO (DFMO), or 5 mM DFMO and spermine at the various concentrations indicated in the Figure. Cell number was determined at days 4 and 5 by the crystal violet method. Values are means ± S.E.M. (n=6). In some cases error bars (S.E.M.) were smaller than the symbols. b) Subconfluent T24 cells were incubated with varying concentrations of ^{14}Cspermine (specific activity, 31 Ci/mol) for 20 minutes at either 4°C or 37°C under a 5% CO_2 atmosphere, and temperature-dependent polyamine uptake was determined as described in "Materials and Methods". The results are expressed as pmol/10^6 cells/h and represent the mean ± S.E.M. (n=6).*

Figure 2. *Suramin selectively inhibits T24 cell-growth that is dependent on extracellular polyamines. Effects of suramin alone (◆), DFMO alone (dotted line), or DFMO and various concentrations of suramin (□), or DFMO and either putrescine (■), spermidine (▲), or spermine (●) and various concentrations of suramin on growth of T24 cells was determined at day 4. In all cases, 5 mM DFMO and 0.5 μM of the respective polyamine were used. Values represent the mean +± S.E.M. (n=6-10).*

polyamines by DFMO-treatment. Support for this notion comes from the finding that suramin was unable to further suppress growth below the level of DFMO alone (Figure 2, dotted line) even in the absence of extracellular polyamines (Figure 2, open squares).

Suramin binds strongly to polyamines and inhibits polyamine uptake in DFMO-treated tumour cells. To gain further insight into the mechanism of the inhibitory activity of suramin on growth of DFMO-treated tumour cells, we next studied binding of suramin to polyamines by affinity chromatography.
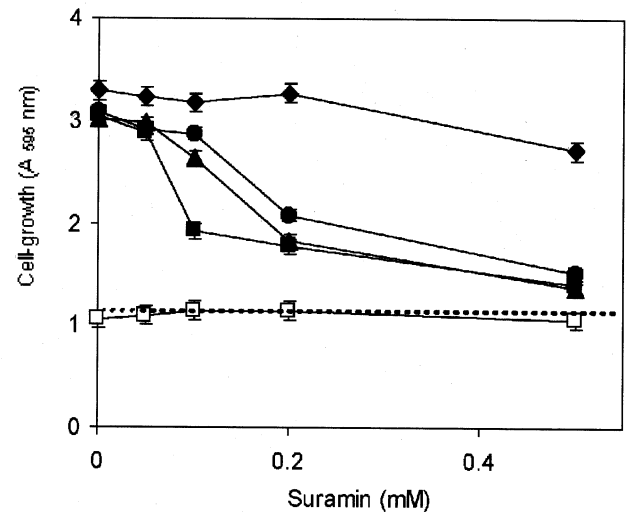
Suramin bound avidly to spermine-substituted agarose, and eluted as a narrow peak at approx 1.5 M guanidine. As a comparison heparin, a highly sulphated GAG, eluted as a more heterogeneous peak at a salt concentration of approximately 0.5-1.4 M (Figure 3a). Moreover, suramin efficiently eluted putrescine and spermidine (results not shown), as well as spermine from a heparin sepharose column (Figure 3b), indicating that suramin is a strong competitor of polyamine-GAG interactions. These results and the fact that HSPG previously has been shown to facilitate polyamine transport (13-14) prompted us to investigate the effect of suramin on polyamine uptake in bladder carcinoma cells. DFMO-treated cells were incubated for 20 minutes with ^{14}C-labelled spermidine or spermine at 0.5 μM, *i.e.* at a concentration that will restore growth in DFMO-treated cells (see Figures 1 and 2), and increasing concentrations of suramin. As shown in Figure 4, suramin inhibited polyamine uptake in a dose-dependent manner. At 0.5 mM, uptake of spermidine and spermine was inhibited by approx 45-50%.

Effects of DFMO and suramin on tumour growth in mice. To translate these findings to *in vivo* conditions, we next investigated the tumour-forming properties of bladder carcinoma T24 cells. T24 cells formed solid tumours in a subcutaneous model in which the cells (1×10^6 cells/animal) were injected dorsally in immunodeficient mice (female SCIDnodCA). After 21 days of incubation, the average tumour load was approximately 142 mg in untreated animals
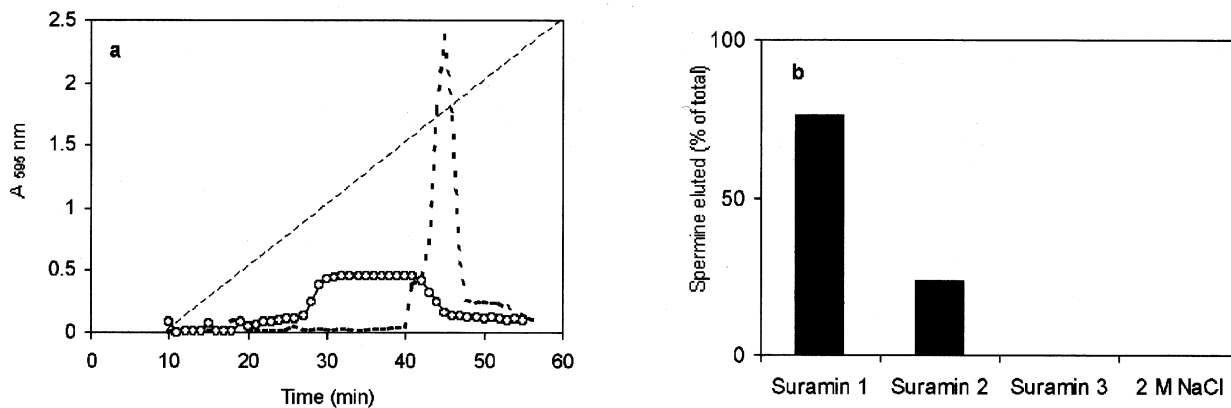
Figure 3. *Suramin binds strongly to polyamine agarose and efficiently competes with polyamine-heparin binding. a) 1.5 mg of either suramin (---) or heparin (-O-) were dissolved in PBS and chromatographed on a spermine-substituted Hi-Trap affinity column that was eluted with a guanidine gradient (dashed line) going from 0 M (fraction 10) to 2 M (fraction 60). The effluent was analysed by the dimethylmethylene blue method (heparin, A595 nm) or UV-absorbance (suramin, A280 nm). The results are representative of three independent experiments. b) Spermine (2 mg) and 0.1 μCi ^{14}Cspermine were mixed in 1 ml PBS and applied to the heparin column, followed by a 10-minute wash with PBS at a flow rate of 0.5 ml/minute. All of the spermine applied bound to the column under these conditions. Suramin (10 mg in 1 ml PBS) was then added to the column by three consecutive injections (Suramin 1, 2 and 3 in the Figure). Finally, the column was eluted with 2 M NaCl in PBS for 10 min. Collected fractions were analysed for radioactivity and results were expressed as % of total ^{14}Cspermine applied to the column.*

(Figure 5). At 1% (w/w) DFMO, tumour formation was reduced by approx 65% (p=0.003). Mice receiving intraperitoneal injections of suramin, 100 mg/kg/week, which is a low, non-toxic suramin dosage, formed tumours that were not significantly different from controls (p>0.4), which is consistent with the results from other mouse studies (31, and refs. therein). The combined effect of DFMO (1% w/w *ad libitum*) and suramin (100 mg/kg/week *i.p.*) was then assessed. DFMO and suramin markedly decreased tumour formation relative to control (approx 88% growth-inhibition, p=0.0005), which corresponds to an additional 2-fold reduction of tumour growth as compared with DFMO alone (p=0.02). Moreover, no adverse symptoms or significant changes in body weight as compared with controls were observed in mice receiving both DFMO and suramin under these conditions.

Discussion

Suramin and DFMO represent well-established, registered drugs that have been used in the treatment of protozoan infections and tested for antitumour activity in animal models and cancer patients. The major limitations of suramin and DFMO as anticancer agents *in vivo* are dose-limiting toxicity and reversal by circulating polyamines, respectively. We have recently demonstrated a role for cell-surface HSPG in polyamine uptake (13, 14). In this study we show that I) suramin binds strongly to polyamines, II) suramin efficiently competes with binding of polyamines to the highly sulphated GAG heparin, III) suramin inhibits uptake of polyamines in DFMO-treated human bladder carcinoma cells, IV) suramin inhibits the biological activity of extracellular polyamines in DFMO-treated cells, and V) that non-toxic doses of suramin significantly enhance the antitumour activity of DFMO in a
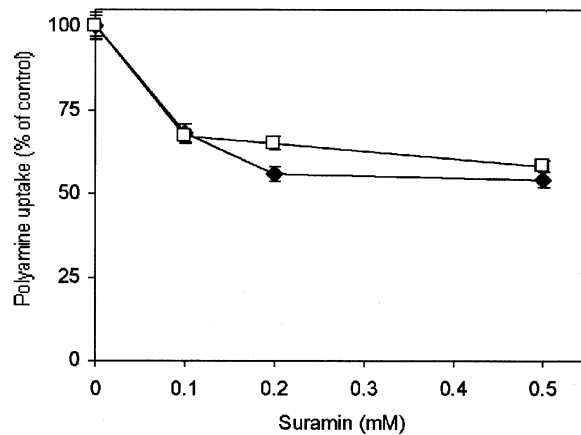

Figure 4. *Suramin inhibits polyamine uptake in DFMO-treated T24 cells. T24 cells were grown for 72 hours in the presence of 5 mM DFMO. Cells were then rinsed and uptake of either ^{14}Cspermidine ($\square$) or ^{14}Cspermine ($\blacklozenge$) at 0.5 μM (specific activity, 31 Ci/mol) for 20 minutes in the presence of increasing concentrations of suramin was determined. The results are expressed as % of control (no suramin added) $\pm$ S.E.M. (n=6).*

mouse tumour model. Suramin is known to inhibit lysosomal degradation of GAG chains, which results in intracellular accumulation and leakage of polysaccharides into the extracellular compartment. In a suramin anticancer trial, Stein *et al.* (26) observed a close temporal correlation between the development of GAG-mediated anticoagulation and tumour shrinkage, suggesting that the antitumour activity was, at least in part, mediated by accumulated GAG chains. The effect of suramin on HSPG turn-over in T24 cells has been extensively studied. Suramin treatment resulted in

intracellular accumulation of HS chains (approx 3-fold as compared with untreated cells), conceivably due to inhibition of lysosomal heparanase activity (23, 27). Suramin may thus inhibit bioavailability of extracellular polyamines by a dual mechanism, involving: I) Direct binding to polyamines (see Figures 3 and 4), and II) Disturbance of recycling HSPG that facilitate polyamine uptake.

To our knowledge, this is the first study that reports the combined effect of suramin and DFMO on *in vivo* tumour growth. Previous studies investigated the effects of suramin on polyamine metabolism in B16 melanoma cells (32-33) However, the effect of suramin on the biological activity of polyamines in the growth medium or in the circulation of animals was not studied, which, from a pathophysiological point of view, should be more relevant. Moreover, the direct effect of suramin on polyamine uptake in DFMO-treated cells was not explored in the previous studies.

In general, the antiproliferative activity of suramin has been attributed to the propensity of suramin to interact with HSPG-binding growth factors, *e.g.* fibroblast growth factors and platelet-derived growth factor (18-21). Our results indicate that suramin inhibits the bioavailability of yet another group of growth-promoting, polybasic compounds with HS-binding properties, *i.e.* the polyamines, and that a combination of the polyamine biosynthesis inhibitor DFMO and suramin may provide an additional strategy for cancer therapy.

Acknowledgements

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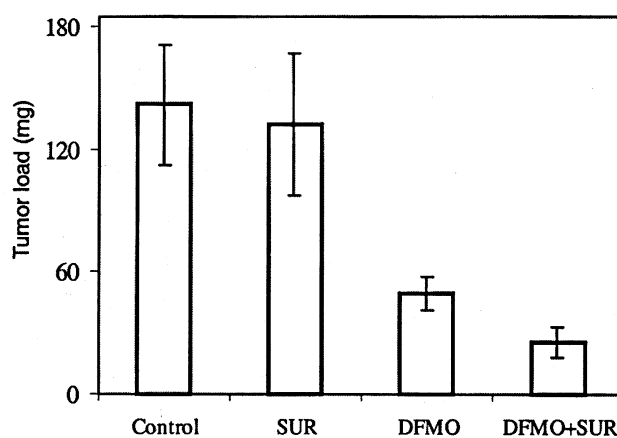20 Huang SS and Huang JS: Inhibition of angiogenesis by suramin. Cancer Res *52:* 5073-5075, 1992.

Figure 5. *Combined antitumour activity of DFMO and suramin in immunodeficient mice. Female ScidnodCA mice (7-8 weeks old) were injected s.c. with 1×10^6 T24 cells in 100 ml sterile PBS, and then received no treatment (control), or 100 mg/kg suramin via intraperitoneal injections once a week in 200 µl sterile PBS (SUR), or 1% (w/w) DFMO ad libitum via the drinking water (DFMO), or combined treatment with DFMO and suramin (DFMO + SUR). After 3 weeks of treatment, the tumour mass was determined and expressed as the mean ± S.E.M. (n=12). The results were obtained by two separate experiments.*

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