PLEIOTROPIC ACTIONS OF SURAMIN ON THE PROLIFERATION OF HUMAN BREAST-CANCER CELLS *IN VITRO*

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Suramin, a non-specific growth factor antagonist, is currently under investigation for treatment of cancer patients. We studied its action on 6 different human breast-cancer cell lines *in vitro*. In complete growth medium, pleiotropic effects were observed with respect to cell proliferation, *i.e.* suramin is stimulatory at low concentrations and inhibitory at higher concentrations, for 4 of the 6 cell lines studied. The various cell lines showed marked differences with respect to the antiproliferative action of suramin, the Evsa-T cells being by far the most sensitive ones. A suramin concentration of 100 µg/ml brought about a 100% stimulation of the proliferation of ZR/HERc cells, ZR 75.1 cells ectopically expressing a human epidermal growth factor receptor (EGF-R) cDNA. Although less pronounced (10 to 60% stimulation), a similar response was observed for the parent ZR 75.1 cells, as well as for T-47D and MDA-MB-231 cells. The non-specificity of the action of suramin was established by the observation that suramin-induced inhibition of cell proliferation could be abolished by insulin-like growth factor-1 (IGF-1) or basic fibroblast growth factor (bFGF), and even by estradiol, both in complete growth medium and under defined serum-free conditions. Our data indicate that suramin exerts pleiotropic effects on the proliferation of human breast cancer cells *in vitro*, and confirm the non-specific nature of its action. The stimulatory effect of low concentrations of suramin on the proliferation of breast cancer cells may have important consequences for breast cancer patients treated with suramin.
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Hormones and growth factors play an important role in the growth regulation of several tumors, such as breast, prostate and endometrial cancer. Hormones may exert their action through interference with the production of a variety of growth factors and/or their receptors. Moreover, abnormal growth factor secretion or abnormal growth factor receptor levels may underlie the development of hormone-independence. Amplification of some (onco)genes or overexpression of their protein products in tumor cells, especially of HER2/*neu* and epidermal growth factor receptor (EGF-R), may be of prognostic significance for cancer patients, although no general statement with respect to the prognostic implications for specific subclasses of breast cancer patients can be made as yet (Gullick, 1990; Klijn *et al.*, 1992). In contrast to the availability of many different hormonal therapies, treatment modalities affecting (onco)genes, growth factors, or growth-factor receptors are practically unavailable. One of the potential treatment modalities based upon the interaction of growth factors with their receptors may involve specific growth-factor antagonists. However, such antagonists are not yet available for the growth factors known to be involved in breast-cancer regulation. As a non-specific growth factor antagonist, suramin (a polysulphonated naphthylurea) for use in the treatment of patients with metastatic cancer has attracted much attention in recent years. Promising results have been obtained in patients with adrenal and prostate cancer, and in patients with heavily pre-treated non-Hodgkin's lymphoma (Stein *et al.*, 1989; Vierhapper *et al.*, 1989; LaRocca *et al.*, 1990). Suramin or (functionally) related compounds could, therefore, become of increasing importance in clinical practice.

In preclinical studies, suramin has been shown to exert an antiproliferative effect on a variety of human cancer cell lines grown *in vitro*. These lines include breast, prostate, head and neck, bladder, ovary, lung and colon cancer cells, as well as melanoma, osteosarcoma, glioblastoma and lymphoid cells (Betsholtz *et al.*, 1984; Coffey *et al.*, 1987; Spigelman *et al.*, 1987; Forgue-Lafitte *et al.*, 1989; LaRocca *et al.*, 1990; Klijn *et al.*, 1990; Olivier *et al.*, 1990; Berns *et al.*, 1990). It has been postulated that suramin has mainly extracellular effects (Betsholtz *et al.*, 1984; Coffey *et al.*, 1987), but direct intracellular effects cannot be excluded (Spigelman *et al.*, 1987). The principal mechanism through which suramin acts is probably via interference with the interaction of growth factors and their receptors, and as a result in their signal transduction pathways. The efficacy of suramin in blocking the binding of a variety of growth factors to their specific cell-surface receptors has been shown to vary. These growth factors include platelet-derived growth factor (PDGF) (Betsholtz *et al.*, 1984; Garrett *et al.*, 1984), epidermal growth factor (EGF) (Coffey *et al.*, 1987; Berns *et al.*, 1990), transforming growth factor-β (TGF-β) (Coffey *et al.*, 1987), acidic (Coughlin *et al.*, 1988) and basic (Coffey *et al.*, 1987; Coughlin *et al.*, 1988) fibroblast growth factor (aFGF and bFGF), insulin-like growth factor-1 (IGF-1) (Pollak and Richard, 1990) and interleukin-2 (IL-2) (Mills *et al.*, 1990). Because of the growing interest in suramin and suramin-like compounds as anticancer drugs, and because of the possible biphasic effects suramin can generate on proliferation of cancer cells *in vitro*, (*i.e.* stimulatory at low concentrations and inhibitory at higher concentrations) (Berns *et al.*, 1990; Olivier *et al.*, 1990), we have studied its effects on the proliferation of a variety of human breast-cancer cell lines *in vitro*. To shed some light on the possible mode(s) of action, cell lines with different steroid receptor and growth factor receptor phenotypes were used. We observed a pleiotropic action of suramin with respect to (anti)proliferative effects. Possible unwanted consequences of the treatment of breast-cancer patients with suramin will be discussed.

MATERIAL AND METHODS

Material

Suramin, the hexasodium salt of 8,8′-{carbonylbis[imino-3,1-phenylenecarbamylimino(4-methyl-2,1-phenylene) carbonylimino]}bis-1,3,5-naphthalenetrisulfonic acid, was purchased from FBA, Bayer, Leverkusen, Germany. Insulin-like growth factor-1 (IGF-1) was a generous gift of Dr. K. Müller (Ciba-Geigy, Basel, Switzerland). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from BTI (Stoughton, MA). Culture media were obtained from Sigma (St. Louis, MO), penicillin/streptomycin and trypsin from Northumbria Biologicals (Cramlington, UK), porcine insulin from Organon (Oss, The Netherlands), gentamicin from Sebak (Aidenbach, Germany), and FCS and iron-supplemented bovine calf serum from HyClone (Logan, UT).

MCF-7 (ER+/PgR+), MDA-MB-231 (ER−PgR−), and T-47D (ER±/PgR+) cells, were obtained from the ATCC (Rockville, MD). ZR 75.1 cells (ER+/PgR+) were a gift from

Dr. R.J.B. King (ICRF, London, UK). Evsa-T cells (ER$^-$/PgR$^-$) were a gift from Dr. N. DeVleeschouwer (Institut Jules Bordet, Brussels, Belgium). ZR/HERc cells (ER$^+$/PgR$^+$) originated from ZR 75.1 cells after introduction of the human EGF-R cDNA utilizing a retroviral factor (Dorssers *et al.,* 1991). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli-umbromide] was supplied by (Sigma).

Cell culture

Cells were routinely grown in their respective complete growth medium. For T-47D cells: RPMI-1640 medium, containing phenol red and 10% heat-inactivated (30 min at 56°C) bovine calf serum, NaHCO$_3$ (10 mM), HEPES (20 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (45 μg/ml) was used. For routine culture of MCF-7 and T-47D cells this medium was supplemented with 10 μg/ml porcine insulin, and for ZR 75.1 and ZR/HERc cells it was supplemented with 1 nM estradiol. Complete growth medium for Evsa-T cells consisted of DMEM/HAM-F12 medium, containing phenol red and 14 mM HEPES, and supplemented with the same additives as for routine MCF-7 cell culture, except that 5% heat-inactivated bovine calf serum was added. MDA-MB-231 cells were routinely cultured in the same medium as MCF-7 cells, except that DMEM/HAM F-12 medium containing 14 mM HEPES was used instead of RPMI-1640 medium.

For experiments in a chemically defined medium, medium consisted of DMEM/HAM F-12 medium (without phenol red), supplemented with NaHCO$_3$ (10 mM), HEPES (15 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (45 μg/ml), 0.2% bovine serum albumin (BSA, purified, Behringwerke, Marburg, Germany), and Na$_2$Se$_2$O$_3$ (50 ng/ml) (=serum-free medium: SFM).

Cell proliferation assay

Cells were harvested by trypsinization, resuspended in fresh medium in 96-well microtiter plates (Greiner, Alphen/Rhine, The Netherlands) and incubated for the time indicated in the Figure legends to allow attachment and flattening of the cells, after which experimental medium containing the respective additives was added. Following incubations (8 wells for each condition studied), and for the time periods indicated in the Figure legends, the MTT assay (Carmichael *et al.,* 1987) was used to evaluate the effects of the various additives on cell proliferation. For the cell lines used we established a linear relation between the MTT-assay and cell number within the range of the experiments shown. Since the presence of suramin in the medium reduced formazan production in the MTT assay in a dose-dependent way, the wells were washed twice with PBS before experimental medium without drugs, but containing MTT (0.65 mg/ml), was added for 4 hr. Possible remaining amounts of suramin attached to the cells after washing did not interfere in the MTT assay, as was established by comparing cell counts and absorbance at 510 nm before and after washing. Subsequently, 100 μl DMSO was added to each aspirated well, the plates were shaken for 10 min, and the optical density at 510 nm was read on an automatic microplate reader (Titertec, Flow, Irvine, UK).

IGF-1-R and EGF-R assay

Cells were grown in 75-cm² flasks in complete growth medium until approximately 75% confluence was reached. Cells were rinsed twice with PBS, and further incubated for 2 × 1 hr in SFM before harvesting by a 2-min incubation at 20°C with 0.01% trypsin/0.004% EDTA. This brief trypsin treatment did not affect the amount of measurable growth factor receptor in ligand binding assays.

For the IGF-1-R assay, 500,000 cells per conical 1.5-ml Eppendorf tube were incubated in a final volume of 200 μl SFM with increasing concentrations (range 0.1 to 1.5 nM) of

[125I]IGF-1 (spec.act. 2,000 Ci/mmol: human-Thr^{59}-analog, Amersham, Aylesbury, UK), in the absence or presence of a 200-fold excess of non-radioactive IGF-1 to correct for nonspecific binding. Following incubation for 20 hr at 4°C, cells were washed 3 times with 1 ml PBS at 4°C and centrifuged for 5 min at 800 *g*. Cell-bound radioactivity associated with the pellet fraction was counted in a Cobra 5005 gamma-counter (Canberra Packard, Tilburg, The Netherlands) with 80% efficiency.

For the EGF-R assay, cells were homogenized with a microdismembrator at −196°C according to procedures described by the EORTC for homogenization of human breast tumors (EORTC Breast Cancer Cooperative Group, 1980). The 100,000 *g* pellet fraction was prepared as described (EORTC Breast Cancer Cooperative Group, 1980) and rehomogenized in 1.5 ml buffer A (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 70 μg/ml bacitracin) with 2 × 5-sec bursts of an Omni-1,000 tissue homogenizer (Omni, Waterbury, CT). The homogenate was centrifuged for 10 min at 1,000 *g* and the resulting supernatant fraction was used as membrane preparation. Membrane preparations (100 μl) were incubated for 20 hr at 20°C in a final volume of 140 μl buffer B (buffer A containing 0.1% BSA) with increasing concentrations (0.1 to 2 nM) of [125I]EGF [spec. act. 500 Ci/mmol: mouse-EGF, receptor grade (Sigma), radiolabeled with Protag 125 (J.T. Baker, Philipsburg, NJ), as described by Benraad and Foekens, (1990) in the absence or presence of a 200-fold excess of non-radioactive EGF to correct for nonspecific binding. Separation of bound and free ligand was performed at 20°C by hydroxylapatite (HAP) adsorption of EGF-R and washing away of the non-bound ligand with buffer A (Benraad and Foekens, 1990). HAP-bound radioactivity was counted.

RESULTS

Reversible effects of suramin in complete growth medium

Suramin had a dose-dependent effect on the proliferation of human breast-cancer cell lines. For 4 of the 6 cell lines tested this effect of suramin was biphasic, *i.e.* stimulatory at low concentrations and inhibitory at higher concentrations (Fig. 1). The Figure further shows that the various cell lines display different sensitivities towards suramin, the Evsa-T cell line being by far the most sensitive to the inhibitory effects (IC$_{50}$ = 87 μg/ml of suramin) and the ZR/HERc cells being the most sensitive to the stimulatory effects of suramin.

To study possible reversible effects of suramin on cell proliferation, cells were cultured in the presence of inhibitory concentrations of 1.0 mg/ml suramin. After 3 days of culture, cells were allowed to further proliferate in the absence or presence of suramin for 6 days. Figure 2 shows that MCF-7, MDA-MB-231 and Evsa-T cells resumed growth after initial inhibition of cell proliferation at day 3, and prolonged culture in the absence of suramin. Again, the various cell lines displayed different sensitivities towards suramin, MDA-MB-231 cells being the least sensitive and Evsa-T cells being the most sensitive. Cultures lacking suramin during the last 6 days of culture after an initial exposure to suramin showed rapid regrowth of MDA-MB-321 cells, while growth of MCF-7 and Evsa-T cells did not recover after a 3-day exposure to 1 mg/ml suramin (Fig. 2). Experiments with exposure to lower concentrations of suramin (0.1, 0.25 and 0.5 mg/ml) also showed for MCF-7 and for Evsa-T cells a regrowth of the cells (not shown). After pre-exposure of the cells to these lower concentrations of suramin, restoration of proliferation was more pronounced since the cell number, *i.e.* the absorbance at 510 nm measured at day 9, was higher than that of control cultures at day 3 (data not shown). This was not the case for MCF-7 and Evsa-T cells after a 3-day exposure to the high concentration of 1.0 mg/ml suramin (Fig. 2). Reversibility of the effects of
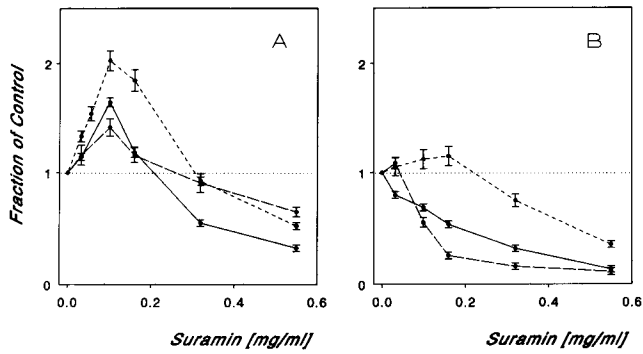
Figure 1 – Dose-response effect of suramin on proliferation of human breast cancer cells *in vitro*. Cells were plated at a density of 5,000 cells/200 μl (for T-47D cells, 7,500 cells/200 μl) in their respective complete growth medium. After 24 hr, medium was replaced by complete growth medium with and without the addition of various concentrations of suramin (ranging from 0.016 to 0.55 mg/ml). Medium was renewed after 3 days. After 5 days of continuous drug exposure, the MTT assay was performed. *(a)* ZR/HERc cells (----), ZR 75.1 cells (--), and T-47D cells (—). *(b)* MDA-MB-231 cells (----), MCF-7 cells (--), and Evsa-T cells (—). The results are depicted as the fraction of control-cell proliferation ± standard error of the quotient *vs.* suramin concentration.
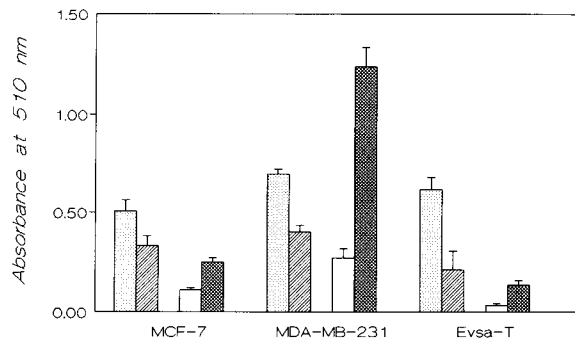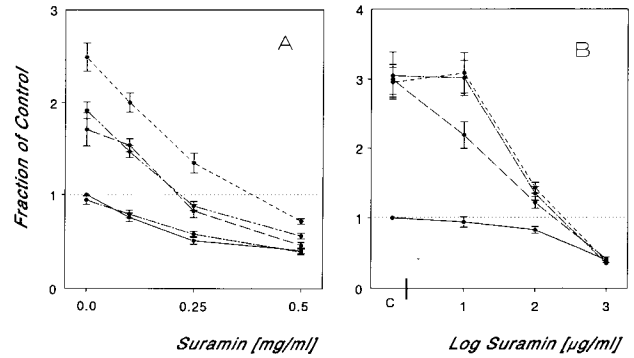

Figure 2 – Reversible effect of suramin on proliferation of human breast-cancer cells *in vitro*. Cells were plated at a density of 5,000 cells/200 μl and cultured for 3 days in their respective complete growth medium with or without (control) the addition of 1.0 mg/ml suramin. After 3 days the MTT assay was performed on control and suramin-containing cultures. In parallel cultures, suramin was washed away—after 3 days' preincubation with suramin—with 2 washes of complete growth medium, and the cells were allowed to proliferate for 6 more days in the presence or absence of suramin before the MTT assay was performed. Results are expressed as the mean absorbance at 510 nm ± SD of suramin-lacking control cultures at day 3 (▦), suramin-containing cultures at day 3 (▨), suramin-containing cultures at day 9 (□), and cultures lacking suramin during the last 6 days of culture after an initial 3-day exposure to suramin, *i.e.* at day 9 (▩).


Figure 3 – Effect of suramin on proliferation of stimulated and non-stimulated MCF-7 cell cultures in serum-free medium (SFM). MCF-7 cells were plated at a density of 10,000 cells/200 μl *(a)* or 5,000 cells/200 μl *(b)* in complete growth medium, and allowed to attach and flatten for 4 hr. Following 2 washings with PBS, cells were cultured for 6 days in SFM in the absence or presence of increasing concentrations of suramin and/or estradiol, IGF-1, and bFGF. *(a)* Control (—), estradiol (0.01 nM, --; 1.0 nM, ----), IGF-1 (1 ng/ml, --; 100 ng/ml, --·-). *(b)* bFGF (0 ng/ml, —; 1 ng/ml, --; 10 ng/ml, ----; 100 ng/ml, -·-). The MTT assay was performed after 6 days of culture, and results are depicted as the fraction of control-cell proliferation ± standard error of the quotient in the absence of suramin, hormone and growth factors (the horizontal dotted lines) *vs.* suramin concentration (*a*, linear scale; *b*, logarithmic scale).

suramin was also observed for the other 3 breast-cancer cell lines tested (ZR 75.1, ZR/HERc and T-47D).

Effects of suramin in serum-free medium

Under serum-free conditions both estradiol and insulin-like growth factor-1 (IGF-1) were potent mitogens for MCF-7 cells (Fig. 3*a*). The growth of stimulated and non-stimulated cultures was inhibited by suramin in a dose-dependent way. Moreover, in serum-free medium (SFM) the net inhibitory effect on cell proliferation, at concentrations of suramin as high as 0.5 mg/ml, could be partly overruled by the simultaneous addition of estradiol or IGF-1 to the cultures (Fig. 3*a*). At lower concentrations of suramin (0.1 and 0.25 mg/ml)

IGF-1 and/or estradiol were still able to stimulate cell proliferation, even when compared to cultures without suramin (up to 100% stimulation by 1 nM estradiol in the presence of 0.1 mg/ml suramin; Fig. 3*a*). A similar occurrence was observed in subsequent experiments in which MCF-7 cells were cultured in SFM in the absence or presence of increasing concentrations of basic-fibroblast growth factor (bFGF). Concentrations of bFGF as low as 1 ng/ml stimulated cell proliferation by 200% when compared to control cultures at day 6 (Fig. 3*b*). Higher concentrations of 10 and 100 ng/ml bFGF were unable to further stimulate cell proliferation. Suramin inhibited both the b-FGF-stimulated and the basal MCF-7 cell proliferation in a dose-dependent manner. The 200% stimulation caused by 1 ng/ml bFGF was reduced to only 120% by concentrations of suramin as low as 0.01 mg/ml. However, suramin at a concentration of 0.01 mg/ml was not able to inhibit the bFGF-stimulated proliferation caused by higher concentrations (10 and 100 ng/ml) of bFGF (Fig. 3*b*). At the intermediate suramin concentration of 0.1 mg/ml net stimulatory effects caused by bFGF (up to 43% stimulation) were still observed. In the presence of 1.0 mg/ml suramin, bFGF was no longer able to stimulate cell proliferation or abolish the suramin-induced inhibition, and a net inhibitory effect (±60% inhibition) was observed in both the absence and presence of bFGF up to 100 ng/ml (Fig. 3*b*).

Effects of hormones and growth factors on suramin action

To gain more insight into the specificity of suramin action, MCF-7 cells (IGF-1-R positive: Fig. 4, left), ZR 75.1 cells (EGF-R negative: data not shown), and ZR/HERc cells (ZR 75.1 cells ectopically expressing the human EGF-R cDNA; EGF-R-positive: Fig. 4, right), were cultured in complete growth medium in the absence or presence of increasing concentrations of suramin and/or IGF-1 (MCF-7; Fig. 5*a*) and/or estradiol (MCF-7; Fig. 5*b*), and/or EGF (ZR 75.1; Fig. 5*c*, and ZR/HERc; Fig. 5*d*). In contrast to what was observed in serum-free medium with slowly proliferating cells (Fig. 3*a*), in the absence of suramin, neither IGF-1 at concentrations up to 10 μg/ml nor estradiol affected the already maximally

Bound/Free   Bound/Free
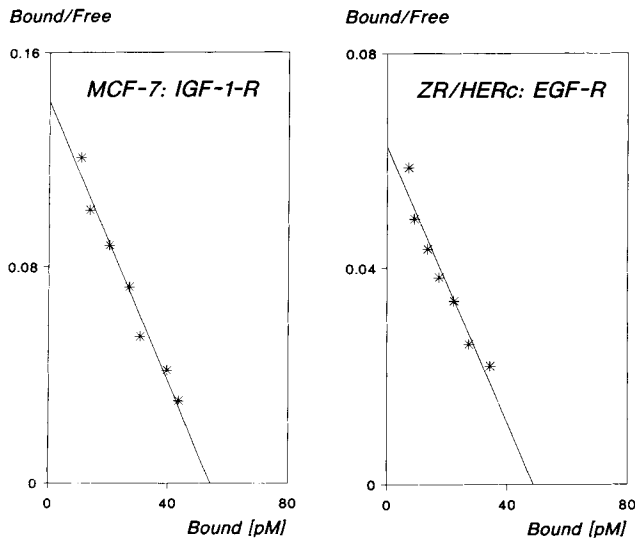
FIGURE 4 – Scatchard analysis of IGF-1 and EGF binding. MCF-7 and ZR/HERc cells were cultured 75-cm² flasks in complete growth medium to approximately 75% confluence. ^{125}I-IGF-1 (MCF-7 cells; left panel) and ^{125}I-EGF (ZR/HERc cells; right panel) binding studies were performed as described in the text. Specific binding is plotted. The number of specific ^{125}I-IGF-1 binding sites was 2.8×10^4 molecules/cell (K_D: 0.4 nM), and the amount of ^{125}I-EGF binding sites was 207 fmol/mg membrane protein (K_D: 0.9 nM).

proliferating MCF-7 cells in complete growth medium (Fig. 5a and b, respectively). However, the net inhibition of cell proliferation caused by 0.1 and 0.25 mg/ml of suramin (15% and 45%, respectively) was completely abolished by IGF-1 in a dose-dependent manner (Fig. 5a), or by estradiol which however displayed a less dose-dependent relationship (Fig. 5b). Inhibition of cell proliferation caused by 0.5 mg/ml of suramin (70% inhibition) could only partially be overcome by the concentrations of IGF-1 or estradiol used (Fig. 5a, b). The intermediate concentration of suramin used (0.1 mg/ml) caused a 40% stimulation of ZR 75.1 cells over basal proliferation in complete growth medium (Fig. 5c), an occurrence which was also shown in Figure 1b where twice the amount of cells were originally plated. Surprisingly, however, in the presence of the lowest concentration of suramin used (0.03 mg/ml), EGF was able to stimulate the proliferation of the EGF-R-negative ZR 75.1 cells (up to 40% stimulation; Fig. 5c). EGF had no effect on the suramin-induced inhibition (at 0.3 mg/ml) or stimulation (at 0.1 mg/ml) of proliferation of the EGF-R-negative ZR 75.1 cells, nor on the basal proliferation. The ZR 75.1 cells which were virally infected with EGF-R cDNA, the ZR/HERc cells, showed a marked stimulation of proliferation (30% and 55%) by the 2 lowest concentrations of suramin used (0.03 and 0.1 mg/ml; Fig. 5d). The basal proliferation of the EGF-R-positive ZR/HERc cells in complete growth medium was stimulated by EGF (up to 40% stimulation; Fig. 5d), and furthermore a slightly additive growth-stimulatory effect of 0.03 mg/ml suramin and EGF was observed, reaching the level caused by 0.1 mg/ml suramin alone (Fig. 5d). This level could not be attained by the addition of EGF alone. Moreover, inhibition of cell proliferation by the highest concentration of suramin used (0.3 mg/ml) was unaffected or only partly abolished by EGF (Fig. 5d).

DISCUSSION

In the past decade, it has become clear that growth factors are involved in the negative or positive growth regulation of a

variety of cancer types. Until very recently, treatment modalities based upon growth-factor-receptor interactions, or interference with their signal transduction pathways, were not available. Evidence that suramin not only inhibits several enzyme systems, including reverse transcriptase (De Clerq, 1987), some other nucleic-acid-related enzymes (Ono *et al.*, 1979; Spigelman *et al.*, 1987), ATPase (Fortes *et al.*, 1988), and protein kinase C (Hensey *et al.*, 1973), but also the binding of several growth factors and peptide hormones to their specific cell-surface receptors of tumor cells (Betsholtz *et al.*, 1984; Garrett *et al.*, 1984; Coffey *et al.*, 1987; Spigelman *et al.*, 1987; Coughlin *et al.*, 1988; Pollak and Richard, 1990), has made suramin the first available growth-factor "antagonist". Moreover, pleiotropic actions of suramin on the proliferation of various tumor-cell lines *in vitro* have been described. Suramin was stimulatory at low concentrations (50–125 µg/ml) and inhibitory at higher concentrations for some osteosarcoma, melanoma, ovarian-carcinoma, bladder-cancer, head-and-neck cancer, and breast-cancer cell lines tested (Olivier *et al.*, 1990). Similar effects were observed for neuroblastoma cells (Guo *et al.*, 1990) and for androgen-responsive DDT-1 hamster ductus deferens tumor cells (Berns *et al.*, 1990) (however, for these latter cells only in the presence of testosterone) (Berns *et al.*, 1990). In the absence of testosterone, suramin exclusively caused inhibitory effects on DDT-1 cell proliferation (Berns *et al.*, 1990). The studies described here show that, provided the concentration used was high enough (> 0.3 mg/ml), suramin could inhibit the proliferation of 6 different human breast cancer cell lines *in vitro*. The various cell lines showed different sensitivities with respect to reversibility of suramin action, *e.g.* for MDA-MB-231 cells a rapid regrowth of the cells occurred. This suggests that, for some breast-cancer cell lines, suramin is cytostatic rather than cytotoxic, an effect which was also observed earlier for androgen-responsive LNCaP human prostate-cancer cells (Berns *et al.*, 1990) and AKR-2B mouse embryo cells (Coffey *et al.*, 1987). The efficacy of the effect of suramin differed markedly for the various breast-cancer cell lines studied. Whereas a concentration of, *e.g.*, 0.1 to 0.15 mg/ml of suramin in complete growth medium containing serum was inhibitory for some cell lines, it was stimulatory for others (Fig. 1). From data presented in the literature and from our experiments, it is clear that suramin is far from being specific to a certain type of growth-factor receptor pathway. Moreover, the efficacy with which suramin can block the binding of different growth factors to their specific receptors and influence cell proliferation varies widely, and is also dependent on the density of the cell cultures (Olivier *et al.*, 1990). Concentrations of suramin causing 50% inhibition of growth factor binding ranged from ±0.03 mg/ml for bFGF binding, through ±0.04 mg/ml for TGF-β binding, to ±0.5 mg/ml for EGF binding (Coffey *et al.*, 1987). The inhibition of growth-factor-stimulated mitogenicity by suramin appeared to be directly correlated to the concentration of suramin required to inhibit the binding of these growth factors (Coffey *et al.*, 1987). Other studies also showed a wide concentration range in which suramin interfered in the binding of growth factors (PDGF, FGF, IGF-1, IL-2) to their receptors (Betsholtz *et al.*, 1984; Garrett *et al.*, 1984; Coughlin *et al.*, 1988; Pollak and Richard, 1990; Mills *et al.*, 1990).

Our study shows that, under serum-free conditions, suramin could inhibit both the basal and IGF-1-, bFGF- and estradiol-stimulated proliferation of breast cancer cells. In addition, increasing concentrations of these mitogens (partly) abolished the growth inhibition caused by lower concentrations of suramin (Fig. 3a, b). Regarding the effects of IGF-1 and bFGF, we suggest that suramin bound, at least in part, to these proteins, thus preventing their mitogenic action. With respect to the nullifying effect of estradiol on the inhibition of basal-cell proliferation caused by suramin, we suggest that
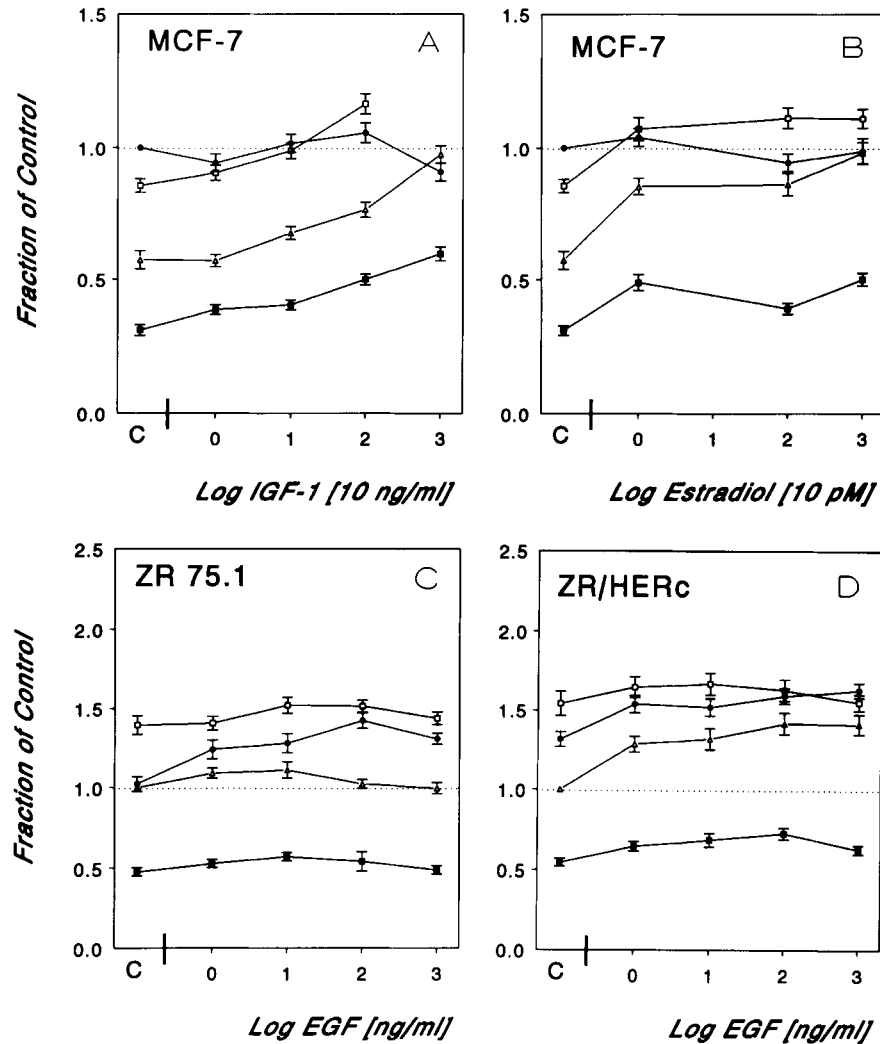
FIGURE 5 – Effects of IGF-1, EGF or estradiol on suramin-affected proliferation of breast cancer cells. MCF-7 cells were plated at a density of 5,000 cells/200 μl and ZR 75.1 and ZR/HERc cells at a density of 2,500 cells/200 μl in their respective complete growth medium. After 24 hr, fresh complete growth medium with and without 0.01, 0.1, 1.0, and 10 μg/ml IGF-1 (MCF-7; a), with and without 0.01, 1, and 10 nM estradiol (MCF-7; b), or with and without 0.001, 0.01, 0.1, and 1 μg/ml EGF (ZR 75.1 and ZR/HERc; c and d, respectively) was added. MCF-7 cells were cultured in the absence (●) and presence of 0.1 (□), 0.25 (△), or 0.5 mg/ml suramin (■). ZR 75.1 and ZR/HERc cells were cultured in the absence (△) and presence of 0.03 (●), 0.1 (□), or 0.3 mg/ml suramin (■). Medium was refreshed at day 3, and the MTT assay was performed on day 5. The results are depicted as the fraction of control-cell proliferation ± standard error of the quotient vs. the log IGF-1 [10 ng/ml] (a), log estradiol [10 pM] (b), or log EGF [ng/ml] (c and d) added. "C" indicates cell proliferation in the absence of added respective hormone or growth factors, and the horizontal dotted line in each panel represents basal-cell proliferation in complete growth medium.

estradiol stimulated the secretion of growth-stimulatory growth factors which subsequently abrogated the effect of suramin.

In serum-containing medium, IGF-1 and estradiol were able to abolish the inhibition of proliferation caused by suramin (Fig. 5a, b). In complete growth medium, IGF-1 did not affect the proliferation of the already maximally proliferating MCF-7 cells. The inhibition of cell proliferation caused by suramin was prevented by the simultaneous addition of IGF-1 in a dose-dependent manner, suggesting that IGF-1 was able to replace suramin-blocked mitogens (IGF-1, insulin, or unidentified growth factors) present in the serum. Regarding the nullifying effect of estradiol on inhibition of cell proliferation caused by suramin, a dose-dependent relationship was less clear and maximal restoration was already obtained at a concentration of 10 pM estradiol. This may be explained by the production of a secretory growth stimulating factor(s) whose synthesis is estro-

gen-dependent and which is already produced at maximal levels once the estradiol-receptor pathway has been activated.

Due to the fact that suramin interferes with different efficacies in the binding of various growth-stimulatory and growth-inhibitory growth factors to their cell-surface receptors, the observed differences in sensitivity of the various breast-cancer cell lines towards suramin probably depend on the phenotype of specific known and as yet unknown growth-factor receptors of the various cell lines. The growth-stimulatory effects of low concentrations of suramin (below 0.25 mg/ml) on the proliferation of several of the breast-cancer cell lines studied herein (Fig. 1) may have important consequences in the treatment of patients with breast cancer. In the treatment of patients, and using a loading dose of 350 mg/m²/day (Stein et al., 1989; LaRocca et al., 1990; Klijn et al., 1990) to reach a plasma level of ±280 μg/ml of suramin after

10 days (Klijn *et al.*, 1990), a growth stimulation of some specific breast tumors may initially occur. The stimulation observed *in vitro,* and which may also occur in patients, may result from nullifying effects of growth-inhibitory factors, *e.g.* of TGF-β, the binding of which is very effectively blocked by very low concentrations of suramin (Coffey *et al.,* 1987). In addition, growth stimulation may be caused by potentiating the growth-stimulatory effects of specific growth factors, *e.g.* of EGF at low concentrations, as may be inferred from the experiment described in Figure 5. The reason for this slightly potentiating effect of EGF and low concentrations of suramin on the EGF-receptor-containing ZR/HERc cells in particular remains at present unclear, but may have resulted from effective removal of an unknown molecule from the EGF receptor which prevented EGF from fully exerting its mitogenic action, or by blockade of the action of a growth-inhibitory factor.

In conclusion, suramin acts as a non-specific growth-factor antagonist for breast-cancer cells. At concentrations approaching the critical plasma concentration in patients (±300 μg/ml) suramin is effective in inhibiting breast-cancer cell proliferation *in vitro.* However, in the treatment of breast-cancer patients, unwanted effects may arise and growth stimulation of some breast cancers may occur as a result of low suramin concentrations during the loading phase with suramin, or when plasma levels decrease below certain concentrations for weeks after one course of treatment (Klijn *et al.,* 1990).

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