Effects of Suramin on Human Lung Cancer Cell Lines

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Suramin cytotoxicity was studied in a panel of human lung cancer cell lines by the MTT assay. The concentrations of suramin which induced 50% growth inhibition (IC50) ranged from 130 to 3715 μM for the cell lines growing in medium containing 10% fetal calf serum (FCS). In only one cell line was the IC50 at a concentration that can be reached in plasma of patients treated with suramin. Suramin was 18 and 3.3 times more cytotoxic on NCI-N417 cells growing in 2% FCS and in HITES serum-free medium, respectively, than growing in 10% FCS. No difference in suramin cytotoxicity was observed between small and non-small cell lung cancer cell lines. At the lower concentrations tested, suramin stimulated proliferation of the two small cell lung cancer cell lines, NCI-H187 and NCI-N417. Of several growth factors tested, none induced stimulation of growth in NCI-H187 and NCI-N417 cell lines, nor did they in any way alter the stimulatory effect of suramin. Cell counting, DNA flow cytometric analysis and Ki-67 staining confirmed a higher proliferative state in suramin-exposed NCI-H187 cells as compared with untreated cells. However, topoisomerase II-α gene expression remained unchanged, as assessed by northern blot analysis and immunostaining. Suramin had an inhibitory effect on topoisomerase II activity, as assessed by the kDNA decatenation assay, with an IC50 of approximately 40 μM. In conclusion, suramin has significant cytotoxic activity in a minority of human lung cancer cell lines, and it stimulates proliferation in some instances. The pleiotropic action of suramin observed should caution on the possibility of tumour acceleration in patients being treated with this drug.


INTRODUCTION

Suramin has been synthesised since the beginning of this century as an aminonaphthalene-sulphonate type urea, and has been used in the treatment of trypanosomiasis and onchocerciasis [1]. Recent studies showed that suramin has anti-tumour activity in the treatment of several metastatic cancers such as renal cancer, adrenal carcinoma, lymphoma and prostate cancer [2]. Several mechanisms of suramin anti-tumour action have been described, including inhibition of activity of growth factors [3]. Suramin has been shown to inhibit the receptor binding of platelet-derived growth factor [4], basic fibroblast growth factor, epidermal growth factor [5], transforming growth factors [6], insulin-like growth factors [7, 8] and cytokines [9]. Suramin can also interfere in processes involved in cell adhesion and migration [10, 11], as well as in different signal transduction pathways [12, 13], and it inhibits several nuclear and cytoplasmic enzymes [14, 15] and may induce cell differentiation [12]. Finally, it has recently been shown that topoisomerase II is a target of suramin action in Chinese hamster fibrosarcoma cells and that this may be important for the cytotoxic effect of the drug [16].

Lung cancer is the leading cause of cancer death in people over 35 years of age. Despite advances in cancer treatment, mortality rates due to lung cancer are still nearly identical to incidence rates. Although small cell lung cancer (SCLC) is highly sensitive to chemotherapy at diagnosis, most patients relapse and die of chemorefractory disease. Non-small cell lung cancers (NSCLC) intrinsically respond poorly to chemotherapy. Lung tumours are known to produce a variety of growth factors, some acting in an autocrine loop [17]. New drugs are urgently needed in the treatment of lung cancer, with novel mechanisms of action. Because of its mechanisms of action, and in particular the inhibition of several growth factors important in the biology of lung cancer, suramin could be an interesting new agent to test in this malignancy.

We investigated and characterised the growth inhibitory effect of suramin in a variety of human lung cancer cell lines, and confirmed the inhibitory effect of suramin on human topoisomerase II. We also investigated whether the mechanism of growth inhibition of suramin is due to growth factor pathway interference.

MATERIALS AND METHODS

Cell lines

Cell lines were kindly provided by Dr A. Gazdar (NCI, Bethesda, Maryland, U.S.A.). Four human SCLC cell lines and three NSCLC cell lines were used (Table 1). All cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, U.K.) supplemented with 10% fetal calf serum (FCS) (Sanbio, Uden, The Netherlands) at 37°C in a humid atmosphere with 5% CO2. NCI-N417 cells were also adapted to grow in RPMI 1640...
medium supplemented with 2% FCS and in HITES serum-free medium (which contains hydrocortisone, insulin, transferrin, estradiol and selenium) [18].

Cytotoxicity assay

Growth inhibition was determined using the MTT assay with slight modifications [19], after 4 days of continuous exposure to different concentrations of suramin. Doubling times of all tested cell lines were also determined with the MTT assay, as described previously [20]. Briefly, cells were harvested from exponentially growing cultures, counted and plated in 96-well plates (Greiner Labortechnik, Solingen, Germany) (100 μl cell suspension per well) at seeding densities which sustained exponential growth during the 4-day drug exposition of the assay. Only the number of cells was selected based on preliminary experiments, in order to have the best conditions supporting cell growth for the duration of the experiment. Twenty-four hours after plating, 100 μl culture medium alone (control wells) or drug-containing medium was added to the wells, and 96 h after drug addition, 20 μl MTT (5 mg/ml) was added. After 4 h incubation, the medium was removed, the formazan crystals were dissolved with 150 μl dimethylsulphoxide containing 0.5% FCS, and the optical density (OD) was measured at 540 nm using a Titertek microplate reader (Multiskan MCC/3-40, Flow, Urvine, U.K.).

Experiments were performed in triplicate. Suramin was purchased from Bayer (Leverkusen, Germany) and stored at 25 mM concentration of 0.35 M. Nuclear proteins were extracted for 30 min at 4°C, and then the mixture was centrifuged at 16 000 g for 20 min at 4°C. Protein concentrations were determined by the method of Bradford. The enzyme solution was diluted with an equal volume of 87% glycerol. The reaction mixture contained 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM PMSF, pH 6.4 and an equal volume of nuclear buffer containing NaCl was added to obtain a final NaCl concentration of 0.35 M. Nuclear proteins were extracted for 30 min at 4°C and then the mixture was centrifuged at 16 000 g for 20 min at 4°C. Protein concentrations were determined by the method of Bradford. The enzyme solution was diluted with an equal volume of 87% glycerol. The reaction mixture contained 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM 2-mercaptoethanol.
165 mM KCl, 1 mM ATP and 200 ng of kinetoplast DNA (TopoGen, Ohio, U.S.A.). The reaction was initiated with the addition of the nuclear extracts and allowed to proceed at 37°C for 30 min. Reactions were terminated with SDS, bromophenol blue and glycerol (3%, 0.3% and 30% v/v, respectively). The samples were electrophoresed in 1% agarose gels at 100 V for 2 h in Tris/borate/EDTA buffer at pH 8. The gels were then stained with ethidium bromide for 20 min, destained and photographed by transillumination with UV light.

**Immunohistochemistry**

Cytospins of NCI-H187 cells after 4 days in culture (3 days of drug exposure) were dried overnight, fixed in acetone (paraformaldehyde plus acetic acid for topoisomerase II-α antibody) for 10 min at room temperature, washed in PBS and pre-incubated with normal rabbit serum at 1:50 in 1% PBS RSA for 10 min. The indirect immunoperoxidase method was used. Blocking serum was drained off and the slides were incubated with the antibodies for 1 h. Slides were then washed three times for 10 min with PBS and incubated for 15 min with undiluted affinity-purified rabbit anti-mouse biotinylated immunoglobulin (Zymed, California, U.S.A.). They were then conjugated to horseradish peroxidase streptavidin (Zymed) at 1:500 in 1% PBS BSA for 30 min. All incubations were performed at room temperature in humidity chambers. Diaminobenzidine (Sigma Chemical Co., St Louis, Missouri, U.S.A.) was used as chromogen (5 mg of DAB tetrahydrochloride in 100 ml of PBS with 70 μl of 30% hydrogen peroxide). Slides were rinsed in water, counterstained with haematoxylin, dehydrated, cleared and mounted. In negative control slides all steps were repeated, substituting the primary antibody with an irrelevant mouse monoclonal antibody (IgG). Frozen tonsil tissue sections were used as positive controls for Ki-67 staining, while NCI-H322 cells were used as positive controls when topoisomerase antibodies were used. The Ki-67 monoclonal antibody (Dako, Glostrup, Denmark) was used at 1:10 dilution; the monoclonal antibodies to human DNA topoisomerases I, IIα, and IIβ [23, 24] were generously provided by Dr G. Astaldi Ricorti (Pavia, Italy), and used at dilutions of 1:25, 1:50, 1:25, respectively.

**Northern blotting**

NCI-H187 cells of day 3 were trypsinised, resuspended in ice-cold culture medium, washed twice in PBS at 4°C and pelleted. Total RNA was extracted after lysis with guanidine isothiocyanate and centrifugation in a caesium chloride gradient [25]. Ten micrograms of total RNA were electrophoresed on a denaturing 1% agarose/formaldehyde gel and transferred to a nylon membrane (GeneScreenFlus; NEN Research Products, Boston, Massachusetts, U.S.A.). Prehybridisation, hybridisation with [α-32P]dCTP-labelled cDNA probes (Random Primer Labelling System; Bethesda Research Laboratory, Rockville, Maryland, U.S.A.), and washing of the membrane were performed according to the vendor's instructions. A 1.8-kb human p170 topoisomerase II-α cDNA fragment was kindly provided by Dr L.F. Liu (Baltimore, Maryland, U.S.A.) and a human cDNA GRP fragment was kindly provided by Dr B. Johnson (NCI-Navy, Bethesda, Maryland, U.S.A.). Differences in RNA loading were corrected by dividing the expression of the topoisomerase IIα gene by that of the GAPDH gene (gift from C. Thiele, NCI, Bethesda, Maryland, U.S.A.) on the same northern blot, as described previously [20].

**RESULTS**

**Suramin cytotoxicity in human lung cancer cell lines**

A maximum of 28-fold difference in cytotoxicity induced by suramin was observed, with NCI-H209 and NCI-H187, both SCLC cell lines, being the most sensitive and the most resistant cell lines, respectively. There was no differential sensitivity based on histological type or doubling time (Table 1). By observing the shape of the growth inhibitory curves, it appears that suramin has a more cytostatic than cytotoxic effect (Figures 1, 2, Table 1).

In NCI-N417 cells, adapted to grow in media containing different serum concentrations, there was a striking influence of serum concentration on the cytotoxicity that suramin induced in this cell line: IC50 values varied from 97 μM in RPMI + 2% FCS medium to 1713 μM in RPMI + 10% FCS medium. Interestingly, cells grown in HITES medium (serum-free) had...
exposed to suramin 0.1 mM. The cells were plated on day -1 and suramin was added on day 0. Experiments were repeated three times; error bars are S.D.

Figure 2. Growth curves of NCI-H187 cell line: control (---) and exposed to suramin 0.1 mM (--). The cells were plated on day -1 and suramin was added on day 0. Experiments were repeated three times; error bars are S.D.

Although the cytotoxicity curves produced by suramin were concentration-dependent for most of the cell lines assessed, relatively low concentrations of suramin induced marked growth stimulation in two instances. The growth of the NCI-H187 cell line was stimulated to a maximum of 2.4 times over the control cells at drug concentrations between 5 and 250 μM (Figure 1a). A less marked stimulatory effect was also observed in the NCI-N417 cell line growing in HITES serum-free medium, where suramin concentrations ranging from 5 up to 100 μM caused up to a 1.7 increase over the control cells (Figure 1b). Cells growing in 10% FCS were also stimulated (up to 1.5 times the control) at concentrations between 50 and 150 μM, while no stimulation of growth was observed in NCI-N417 cells grown in 2% FCS-containing medium. The maximum stimulatory effect of suramin was therefore observed at lower suramin concentrations in NCI-N417 cells grown in HITES medium than that observed in cells grown in 10% FCS. Although the addition of BSA to NCI-N417 cells growing in HITES did not influence the growth stimulatory pattern induced by suramin, it did cause a reduction of the steepness of the growth inhibitory curve, with a 5-fold increase in IC50 value, from 500 μM to 1 mM (data not shown).

We performed additional experiments in NCI-H187 cells, where the stimulatory effect was greatest. In a 7-day growth curve, a stimulation of cell proliferation was confirmed with a specific topoisomerase II-α antibody to which most of the cells stained positive. In addition, most cells also displayed strong staining with antibodies against topoisomerase II-β and topoisomerase I. While staining was homogenously nuclear for topoisomerase I and II-α, staining for topoisomerase II-β was mainly nucleolar, in agreement with prior reports [23, 24] (data not shown).

As increased proliferation after suramin exposure had been reported to be mediated by EGF in a cell line [27], we assessed whether EGF or other growth factors influenced growth of NCI-H187 cells. None of the growth factors tested (EGF, GM-CSF, TGF-β, IGF-II) alone or in combination with suramin showed any effect on growth of NCI-H187, the cell line with the highest growth stimulation in the presence of suramin after 4 days of incubation (Figure 4, and data not shown). In addition, NCI-H417 cells, known to express the receptor for EGF [28], growing in 10% FCS or in HITES, also did not display any changes in proliferation after exposure to EGF or IGF-II alone or in combination with suramin (data not shown).

Finally, we assessed whether suramin exposure could result in expression of the gastrin-releasing peptide (GRP) gene in NCI-H187 cells, which it was known to be negative for under normal conditions [29]. The product of the GRP gene is a member of the bombesin family of peptides which is implicated in autocrine growth in some SCLC cells [30]. By using, as positive control, RNA extracted from the SCLC cell line NCI-H209, no GRP expression was seen in NCI-H187 cells either in the presence or absence of 0.1 mM suramin (Figure 5).

**Suramin inhibits the catalytic activity of topoisomerase II in vitro**

Although suramin has been shown to have an inhibitory action on topoisomerase II of Chinese hamster cells [16], no data on human cells are available so far. We assessed the inhibitory effect of suramin on the decatenating activity of two human lung cancer cell lines, NCI-N417 and NCI-H460, which displayed a 6-fold difference in cytotoxicity. The topoisomerase II catalytic activity was assayed by decatenation of kDNA networks. kDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb) that form extremely large networks that fail to enter an agarose gel. Upon incubation with topoisomerase II, which engages DNA in a double-stranded breaking and reunion cycle, minicircular DNAs are effectively released and are able to move rapidly into the gel. Three different suramin concentrations (10, 40 and 80 μM) were assayed in the inhibition of the decatenation activity. The decatenating activity induced by nuclear extracts of NCI-H460 cells was approximately comparable to that observed in NCI-N417 (Figure 6). The IC50 concentration of suramin inhibiting the decatenating activity of nuclear extracts of both cell lines was around 40 μM. The decatenating activity of the nuclear extracts of NCI-N417 cells was largely inhibited by 40 and 80 μM suramin, as shown by the disappearance of the 2.5-kb minicircles, although a faint band was still visible at the highest protein concentrations used. A weak inhibition by 10 μM suramin could only be seen at the lowest protein concentrations employed (Figure 6a). The topoisomerase II inhibitory effect of suramin was dose-dependent at concentrations in the range of 10–40 μM in NCI-H460 cells (Figure 6b).
DISCUSSION

Two principal mechanisms of action have been proposed for suramin activity: growth factor blockade and inhibition of cellular enzymes implicated in signal transduction pathways [3, 8, 13]. In vitro studies have shown a wide range of suramin concentrations that are required to produce 50% inhibition of growth (IC50) [5, 31].

In the lung cancer cell lines tested, the effect of suramin, although dose-dependent over the range of concentrations employed in most cell lines, was more cytostatic than cytotoxic, with no notable differences between SCLC and NSCLC cell lines. Because suramin causes dose-dependent inhibition of MTT conversion by mitochondrial dehydrogenases, beginning approximately at $10^{-4}$ M, the activity shown in our assay could have been overestimated, and consequently the stimulatory effect underestimated [32]. In two SCLC cell lines, remarkable growth stimulation was observed at relatively low suramin concentrations (5–250 μM). The stimulation of growth in NCI-H187 cells was confirmed by an increase of cells in S-phase and cells staining positive to Ki-67, a marker of cell proliferation. The antigen recognised by the Ki-67 monoclonal antibody accumulates predominantly during progression of cells into S phase, and its rate of synthesis accelerates during the second half of this phase [33]. Our results support the fact that suramin resulted in an increase of cells located between the late S, M or G2 phases in NCI-H187 cells. Although increased proliferation was observed in NCI-H187 exposed to suramin, no increase of topoisomerase IIα gene expression was detected by northern blotting or immunohistochemistry. This might be due to the low sensitivities of both methods in detecting relatively small differences of expression. Topoisomerase II activity can, in fact, vary from very low levels, as in quiescent cells, to high levels, as in proliferating cells [34]. Since the NCI-H187 cell line is highly proliferating and has a high topoisomerase expression [20], it might well be that small differences of expression at these higher levels could not be detected.

The stimulatory effect of suramin at relatively low concentrations has been described in several types of human tumour cell lines, including breast, prostate, squamous cell carcinoma and lung cancer cell lines [5, 27, 35–37]. In the study by Morocz and associates [37], one lung adenocarcinoma cell line out of 17 lung cancer cell lines tested was highly stimulated by suramin exposure. In this cell line, growth was stimulated by 2.6-fold, in comparison with control cells, up to a suramin concentration of 300 μg/ml; remarkably, even at the highest suramin concentration of 1500 μg/ml, DNA synthesis was still more than twice that of the control.
The stimulatory effect of low suramin concentrations on the proliferation of cancer cells, and in particular of lung cancer cells, should be considered seriously in planning clinical trials with this drug. The concentrations of suramin found to stimulate growth in cell lines are, in fact, easily reached after suramin administration has been stopped [38]. As the half-life of suramin in plasma is exceptionally long (around 40-50 days) [38], the duration of a relatively low plasma concentration, able to stimulate growth in some cell lines, can be sufficiently long to provoke tumour growth acceleration.

The stimulation of growth has been shown to be mediated through the EGF receptor (EGFR) in the KB cell line [27], the cells being induced to release membrane-bound TGFα, resulting in activation of EGFR. Moreover, it was recently reported that a large proportion of a broad panel of SCLC cell lines express the EGFR [28].

In the human SCLC cell line, NCI-H187, no effect was observed by EGF, GM-CSF, TGF-β or IGF-II either alone or in combination with suramin. EGF and IGF-II did not display any effect either alone or in combination with suramin on NCI-N417 cells growing in 10% FCS or in HITES serum-free medium. NCI-N417 cells are known to express EGFR [28]. EGF, GM-CSF and IGF-II have been shown to be able to modulate cell growth in lung cancer cell lines [17, 39, 40] and TGF-β has recently been shown to be expressed in a number of SCLC cell lines, along with its receptor [41]. The IGF-II gene is more widely expressed than the IGF-1 gene in human lung cancer cell lines [40].

The expression of GRP was not elicited by exposure of NCI-H187 cells to suramin. GRP expression can be increased in cell lines which already express this gene, for example, after exposure to laminin, the major component of the extracellular matrix [42]. The NCI-H187 cell line, however, does not constitutively express GRP even when assessed by sensitive methods [29].

Since suramin is highly protein bound [43], mainly to albumin [44], and it has been suggested that suramin inhibitory effects are dependent on the free suramin concentrations [21], we performed additional experiments in NCI-N417 cells adapted to grow in different FCS concentrations and in HITES serum-free medium. Interestingly, suramin was 18 times more cytotoxic in NCI-N417 cells growing in 2% FCS than in 10% FCS, but only 3.3 times more cytotoxic when grown in HITES serum-free medium. The higher IC50 values obtained in cells grown in 10% FCS and in HITES were due to a great degree to the stimulatory effect that suramin had at low concentrations on these cells. Concentrations of serum might be important, as stimulation of cell proliferation was observed in NCI-N417 growing in 10% FCS and was greatest in HITES serum-free medium. It might be that critical concentrations of an as yet unidentified 'growth factor' contained in FCS may be necessary to potentiate the action of suramin on proliferation of cells. This factor, together with the differential cytotoxicity based on protein concentration in the medium, may be responsible for the modulation of the effects on the growth of these cell lines. The addition of BSA, although producing an increase in the IC50 in HITES-grown NCI-N417 cells, as shown for other cell lines [21], did not alter the stimulatory effect of suramin at low concentrations, indicating that the binding might be less relevant at these relatively low concentrations.

However, it should be considered that the concentration of proteins contained in RPMI 1640 plus 10% FCS is only approximately 10% of that normally present in human blood. Taking this into consideration and the fact that in patients an optimal concentration in plasma of suramin is between 150 and
Figure 6(a). k-DNA decatenation assay of nuclear extracts of NCI-N417 cell line. (A–D) Different suramin concentrations. (A) Control (without suramin); (B) suramin 10 μM; (C) suramin 40 μM; (D) suramin 80 μM. Lanes 1–6 have different protein concentrations. Lane 1: 100 ng/μl; lane 2: 50 ng/μl; lane 3: 25 ng/μl; lane 4: 12.5 ng/μl; lane 5: 6.25 ng/μl; lane 6: control (no protein). (b) k-DNA decatenation assay of nuclear extracts of NCI-H460 cell line. (A–F) Different suramin concentrations. (A) Control (no suramin); (B) suramin 10 μM; (C) suramin 20 μM; (D) suramin 30 μM; (E) suramin 40 μM; (F) suramin 80 μM. Lanes 1–4 have different protein concentrations. Lane 1: 50 ng/μl; lane 2: 25 ng/μl; lane 3: 12.5 ng/μl; lane 4: control (no protein).

250 mg/l (100 μM = 143 mg/l), it can be expected that only in a minority of cases will it be possible to reach a plasma concentration able to inhibit growth of lung cancer cells. In fact, only in one cell line that we tested were such concentrations able to cause 50% growth inhibition.

Suramin inhibited topoisomerase II activities extracted from two human lung cancer cell lines. However, despite a 6-fold difference in suramin cytotoxicity between the two cell lines tested, there was no appreciable difference in inhibition of decatenating activity by suramin. Although inhibition of topoisomerase II activity occurs, it remains to be established whether suramin cytotoxic activity is topoisomerase II mediated. In contrast to other topoisomerase II inhibitors, suramin has not been shown to induce DNA fragmentation [16], and DNA cleavage appears to be an important step in topoisomerase II-mediated cell kill induced by topoisomerase II inhibitors [45]. Furthermore, in the panel of studied cell lines, no correlation was observed between levels of expression of topoisomerase II [20] and cytotoxicity to suramin, in contrast to the rather good correlation with other topoisomerase II inhibitors (e.g. doxorubicin, etoposide). Moreover, no correlation between suramin cytotoxicity and cytotoxicity to other topoisomerase II inhibitors was observed in this study [20]. Factors other than topoisomerase inhibition may be responsible for this lack of correlation.

In conclusion, a wide range of suramin concentrations induced growth inhibition of human lung cancer cell lines, but only in one was the IC50 in the range of concentrations that can be reached in plasma of patients. Although suramin inhibited topoisomerase II activity in the human cell lines tested, this might represent a secondary mechanism of suramin cytotoxicity. As stimulatory action was observed with suramin in two cell lines, properly designed clinical trials are mandatory to correctly assess the anti-tumour activity of suramin and its possible accelerating effect on tumour growth. The mechanism employed by suramin to stimulate growth of some lung cancer cell lines remains to be elucidated.

5. Olivier S, Formento JL, Fischel JL, Etienne MC, Milano G.


Acknowledgements—G.J. Rubio is the recipient of a fellowship from the National Academy of Medicine of Buenos Aires, Argentina. We gratefully thank Dr G. Astaldi-Ricotti for provision of monoclonal antibodies to topoisomerases.