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In vitro Sequence-dependent Synergistic Effect of Suramin and Camptothecin

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Suramin, a hexasulphonated naphthylurea with activity in prostatic cancer, possesses a wide variety of antitumour mechanisms of action, one of which is the inhibition of topoisomerase II. In this *in vitro* study, suramin was combined with the topoisomerase I inhibitor, camptothecin. Several suramin concentrations (0.2–3000 μM) were combined with camptothecin (0.4 pM–20 μM) in MCF-7 and PC3 human cancer cell line cultures. In addition, we studied the topoisomerase II and I gene expression by northern blot analysis, and the cell cycle distribution by flow cytometry, after exposure to suramin. While there was only an additive effect when suramin and camptothecin were added simultaneously, a remarkable synergism was obtained when camptothecin was added after a 3-day exposure to suramin. Topoisomerase II and I gene expression and the number of cells in S phase were significantly reduced after exposure to suramin. In conclusion, interaction of suramin with camptothecin is schedule-dependent and can be synergistic. These findings might help in identifying optimal combinations of suramin or other topoisomerase II inhibitors, with topoisomerase I inhibitors.

Key words: suramin, camptothecin, drug combination
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INTRODUCTION

ALTHOUGH CURRENT chemotherapy of malignant disease is mainly based on drug combination, the rationale behind the various combinations is often not very strong. Combinations of drugs with a different mechanism of action and a non-overlapping toxicity profile would be the most profitable; however, aspects of drug interaction, like synergism and antagonism, and their underlying mechanisms are still rather poorly understood in cancer chemotherapy [1]

Suramin is a hexasulphonated naphthylurea with activity in a variety of cancer cell lines and human tumour xenografts [2–4]. The mechanism of action of suramin is mainly through inhibition of a variety of growth factors and growth factor receptors. Recently, suramin has also been shown to inhibit topoisomerase II [5], a nuclear enzyme that is ubiquitous in prokaryotic and eukaryotic cells and essential for some vital cellular processes, like transcription, DNA synthesis and replication [6]. Suramin possesses antitumour activity in advanced hormone-refractory prostate cancer, adrenal cancer and indolent lymphomas [7]. Although the use of this drug is hampered by a broad array of side-effects (e.g. adrenal insufficiency, skin reactions, neurotoxicity, etc.), monitoring of plasma concentrations of suramin can avoid unnecessary toxicity, especially neurotoxicity, by reducing the peak levels of the drug and maintaining concentrations within a narrow therapeutic range [8, 9].

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Camptothecin (CPT), a cytotoxic plant alkaloid isolated from *Camptotheca acuminata*, has a broad spectrum of antitumour activity *in vivo* and *in vitro*, and it appears to be one of the most potent antitumour agents ever discovered [10, 11]. Unfortunately, phase I and initial phase II trials with the sodium salt of CPT, performed in the early 1970s, led to the abandonment of the agent because of excessive and unexpected toxicity, as well as the lack of activity. Less than a decade ago, it was discovered that the specific target of CPT is topoisomerase I, and that the formulation as sodium salt, used in the initial clinical studies, was the reason for the inactivity and probably also the excessive toxicity observed [11]. Clinical studies with two CPT derivatives, CPT-11 and topotecan, appear now to confirm the promise of the parent compound, as they are devoid of the unpredictable toxicity, which was seen with CPT, and have clear antitumour activity in several tumour types [11].

Because of lack of cross-resistance between topoisomerase I and II inhibitors [12], their combination is appealing; however, a number of reports have demonstrated antagonistic effects when the drugs are simultaneously added *in vitro* [13–15]. However, sequential administration of topoisomerase II inhibitors and topoisomerase I inhibitors has been observed to result in additive or synergistic cytotoxicity [14, 15].

In this study, we conducted *in vitro* investigations on the interaction between suramin, an antitumour agent with complex multiple mechanisms of action, including inhibition of topoisomerase II activity, and CPT, a topoisomerase I inhibitor. The *in vitro* schedule of the two compounds influenced the efficacy of the combination, with only the sequential administration of suramin followed by CPT producing a striking synergistic effect.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow Laboratories (Irvine, U.K.); 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) from Serva Laboratories (Heidelberg, Germany); fetal calf serum (FCS) and Hanks' balanced salt solution without calcium, magnesium and phenol red (HBSS) from Gibco (New York, U.S.A.); trichloroacetic acid (TCA), glutamine, $MgCl_2$, KH_2PO_4 and gentamicin from Merck (Darmstadt, Germany); trypsin, (ethylenedinitrilo)-tetraacetic acid (EDTA), trypan blue, sulphorhodamine B (SRB), bovine serum albumin (BSA), EGTA, Triton X-100, phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT) from Sigma Chemical Co. (St Louis, U.S.A.); acetic acid [tris(hydroxymethyl)aminomethane] (Tris), from Baker Chemicals B.V. (Deventer, The Netherlands). Suramin (Germanin) was purchased from Bayer (Bayer Leverkusen, Germany), dissolved in 0.9% $NaCl_2$ and stored at 25 mM at −20°C. CPT (Sigma) was dissolved in dimethylsulphoxide and stored at 0.05 mM at 4°C. All other chemicals were of analytical purity.

Cell lines

The prostate cancer cell line, PC3, was obtained from the American Type Culture Collection (Rockville, U.S.A.), and the breast cancer cell line, MCF-7, was obtained from Dr K.H. Cowan (National Cancer Institute, Bethesda, U.S.A.). These tumour cell types were chosen based on demonstrated or hypothesised sensitivity [16]. The cells were grown as monolayer cultures in 20 mM Hepes-buffered DMEM supplemented with 5% heat-inactivated FCS and 1 mM L-glutamine, in 80-cm^2 flasks (Nunc, Roskilde, Denmark) in a 37°C, 5% CO_2, 95% humidified air incubator and were subcultured once a week. All cells were free of *Mycoplasma* infection.

Growth inhibition studies

Cells in exponential growth phase were harvested using 0.25% trypsin-EDTA, and resuspended in medium with 50 μg/ml gentamicin. Viable cell counts were determined by trypan blue exclusion in a haemocytometer, and if these were >90%, they were seeded in 96-well plates (Greiner Labortechnik, Solingen, Germany). The plating densities were 14 000 cells/well for PC3, and 4000 cells/well for MCF-7. After 24 h, to allow cell recovery, the drugs were added, dissolved in culture medium.

For growth inhibition studies, the SRB assay was used as described previously [17, 18]. Briefly, 50 μl/well of 50% TCA (final concentration 10%) were added to the culture and incubated at 4°C for 1 h, to precipitate the proteins and fix the cells. The plates were washed five times with water and air-dried. The cells were stained with 50 μl/well of 0.4% SRB dissolved in 1% acetic acid for 1 h, and then the plates were washed five times with 1% acetic acid to remove any unbound stain. The stained protein was solubilised in 150 μl/well of 10 mM unbuffered Tris base by shaking. The optical density was read at 540 nm using a microtitre plate reader (Titertek Multiskan MCC/340, Flow Laboratories).

Drug interaction analysis

Dose–response interactions between suramin and CPT were evaluated using the method of Chou and Talalay [19]. All data were processed by a computer programme developed by Chou and Chou (Elsevier-Biosoft). We used one fixed ratio for each suramin-CPT combination. The median dose values were determined from the median-effect plot, where the log (dose) is displayed on the x-axis and the log (fraction affected/fraction unaffected) is displayed on the y-axis. The combination index (CI) was calculated at any level by the formula:

$$CI = [(D)1/(Dx)1] + [(D)2/(Dx)2] + [\alpha(D)1,2/(Dx)1,2]$$

where $\alpha = 0$ (for mutually exclusive drugs), (D)1, (D)2 and (D)1, 2 are the doses of drug 1, 2 and their combination (in a specified ratio), and (Dx)1, (Dx)2 and (Dx)1,2 are the doses required to affect a system by x%; these doses for x% effect are calculated by the formula: $D = Dm\cdot[fa/(1-fa)]/m$, where Dm is the dose that is required to produce a median-effect, fa is the fraction affected, and m is the slope of the median-effect plot (a measure of sigmoidicity). Additive effect is seen when $CI = 1$, antagonism is when $CI > 1$ and synergy is when $CI < 1$.

Flow cytometry

Cells after being cultured for 3 and 6 days were detached with 0.2% EDTA, harvested, resuspended in culture medium, washed twice with phosphate-buffered solution (PBS) and fixed in 70% ethanol at 4°C. Alcohol-fixed cell cultures were kept at 4°C until analysis. Cells were then washed with ice-cold PBS and counted (2×10^6 cells/sample). The cells were resuspended in RNase (Sigma) (0.5 mg/ml) and stained with propidium iodide (Sigma) (200 μg/ml) in a final volume of 200 μl, and incubated for 30 min at 37°C. The distribution of DNA per cell content was measured in 15 000 cells/sample by flow cytometry, using a fluorescence-activated cell sorter (FACSTAR^{plus}, Becton-Dickinson, Etten-Leur, The Netherlands). DNA histogrammes were analysed using the 'DNA Cell-Cycle Analysis Software – Ver. C' (Becton-Dickinson).

RNA extraction and northern blotting

RNA was extracted by cell lysis with guanidine isothiocyanate and centrifugation in a caesium chloride gradient [20]. Ten micrograms of total RNA were electrophoresed on a 1% agarose-formaldehyde gel and then transferred to a nylon membrane (GenescreenPlus, E.I. du Pont de Nemours Co., Boston, U.S.A.). Prehybridisation and hybridisation conditions were as recommended by the supplier. A 1.8-kb human p170 topoisomerase II cDNA fragment (ZII-1.8) [21], and a 0.7-kb human topoisomerase I cDNA fragment [22] were kindly provided by Dr Leroy Liu (Baltimore, U.S.A.). A 1.3-kb probe of rat GAPDH cDNA was kindly provided by Dr Carol Thiele (Bethesda, U.S.A.) [23]. The probes were [α-^{32}P]dCTP (Amersham, Buckinghamshire, U.K.) labelled with a random primer kit (Bethesda Research Laboratory, Rockville, U.S.A.). The final wash of northern blots was at 60°C for 30 min in 2× SSC (0.3 M sodium chloride and 0.03 M sodium citrate) and 1.0% SDS. Densitometry of exposed films was performed to determine the levels of topoisomerase gene expression, which were normalised for RNA loading by dividing the absorbance of the topoisomerase transcript by that of the GAPDH gene.

RESULTS

Growth inhibition studies

Cells were continuously exposed to the drugs for 6 days. The concentration of suramin which gave 50% growth inhibition (IC_{50}) was 60 ± 17 μM (mean ± standard error of the mean in three different experiments, S.E.M.) and 271 ± 38 μM for MCF-7 and PC3 cell lines, respectively. The IC_{50} for CPT was 18 ± 2 nM for MCF-7 and it was higher than 10 nM for PC3.

When the combination of both agents was simultaneous for 6 days (Figure 1) an additive effect was observed, while when CPT was added from day 3 to 6 to the culture in the presence of suramin for 6 days, we observed a remarkable synergistic effect in both cell lines (Figures 2 and 3). Under these conditions, the 50% growth inhibition was reached at suramin concentrations of 14 and 30 μM, concurrent with CPT 14 and 150 nM, in MCF-7 and PC3 cell lines, respectively. In contrast, when CPT was added on day 1 for the first 3 days, together with 6 days of exposure to suramin, an antagonistic effect was observed (not shown).

Flow cytometric studies

Flow cytometry analysis showed that, after 3 days of exposure to suramin, the number of cells in G_0/G_1 phase increased in a dose-dependent manner in PC3 cells, coupled with reduction of cells in G_2/M phase (Table 1). After 6 days of drug incubation, the same trend was observed, although a more striking shift of cells in G_1 and S phases, as compared to control cells was observed, than that observed after 3 days of incubation (Table 1).

Expression of the topoisomerase genes

The PC3 and MCF-7 cell lines were plated in 80-cm^2 flasks at 0.1×10^6 cell/ml in 10 ml of medium. After 24 h, to allow cell recovery, suramin was added at different concentrations. The IC_{50} after 6 days of continuous exposure was 44 ± 9 μM for MCF-7 and 164 ± 25 μM for PC3, as obtained by cell counting. The cells were harvested on day 3, or on day 6 for the following

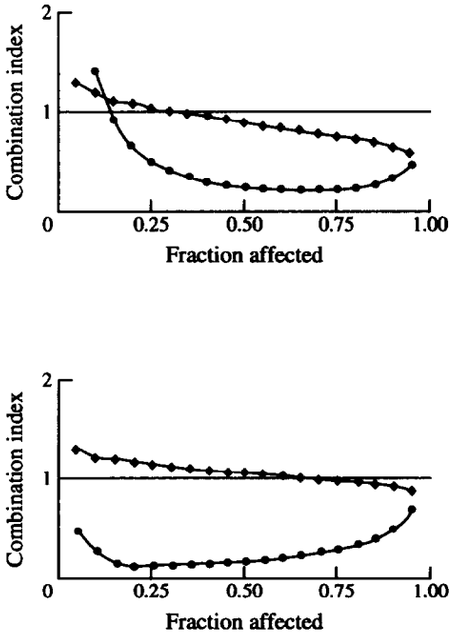

Figure 2. The Chou and Talalay plot evaluating synergy of suramin combination in MCF-7 (top panel) and PC3 (bottom panel) cell lines. Suramin and CPT added simultaneously on day 1 for days 1–6 (◆). Suramin added on day 1 for days 1–6, and CPT on day 3 for days 3–6 (●). The value of the combination index indicates CI = 1 (additive effect, CI > 1 (antagonistic effect) and CI < 1 (synergistic effect). Values represent the mean value of each fraction affected in three independent experiments.

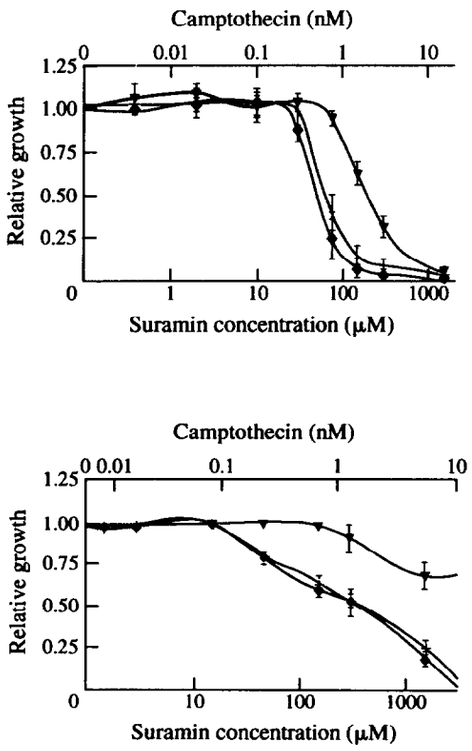

Figure 1. Growth inhibition curves of MCF-7 (top panel) and PC3 (bottom panel) cell lines. The cells were cultured for 6 days with suramin (+), CPT (▼) or a combination of both (◆). The fixed ratios (suramin: CPT) were 10^5 : 1 for MCF-7 and 30000 : 1 for PC3. Values represent mean ± S.E.M. of three different experiments.

experiments where higher number of cells were required than for cytotoxicity assays.

The expression of topoisomerase I and II were analysed in MCF-7 and PC3 cells cultured with different suramin concentrations (Figure 4). The topoisomerase I and II transcripts were detected in both cell lines and were of the expected molecular size. The level of expression of both topoisomerase genes was decreased in a dose dependent manner in cells treated continuously with suramin for 6 days, as compared to control cells grown in absence of suramin for the same period (Table 2). Northern blot analysis showed a 50% reduction of expression of topoisomerase I and II in both cell lines after exposure for 6 days to the suramin concentration which inhibited 50% growth in cytotoxicity assays.

DISCUSSION

Our results show that suramin and CPT, a topoisomerase I inhibitor, have remarkable synergism *in vitro*, when they are sequentially administered. In fact, only when CPT was added after a 3-day exposure to suramin, was a synergistic effect observed, with only a marginally additive effect seen when the two drugs were added simultaneously, and an antagonistic effect observed when CPT was added on the first 3 days of the 6-day suramin exposure.

Suramin has shown wide *in vitro* tumour cell growth inhibition activity [2–4, 24]. In clinical trials, its use is hampered by the presence of severe non-haematological side-effects, which can be largely reduced by appropriate drug plasma monitoring [8, 9]. Suramin has definitive antitumour activity in hormone-refractory advanced prostate cancer [25]. The combination of suramin with other drugs with a different mechanism of action

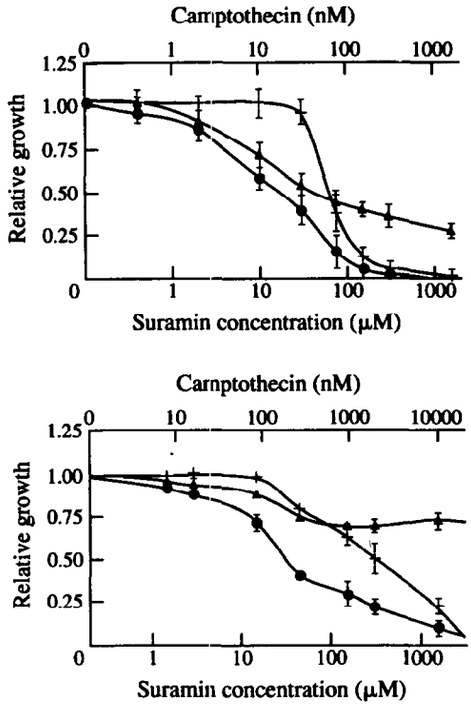

Figure 3. Growth inhibition curves of MCF-7 (top panel) and PC3 (bottom panel) cell lines. Cells exposed to suramin on days 1–6 (+). CPT (▲) was added on day 3, for days 3–6. In the combination of suramin and CPT (●), suramin was added on day 1 and CPT on day 3. All plates were read on day 6. The fixed ratio (suramin : CPT) was 1000 : 1 for MCF-7 and 150 : 1 for PC3. Values represent mean ± S.E.M. of three different experiments.

and toxicity profile (i.e. myelotoxicity) appears attractive and feasible. Inded, suramin has shown synergistic effect when combined with doxorubicin *in vitro* and *in vivo* [26].

Suramin has recently been shown to inhibit topoisomerase II activity [5], an effect which adds to a variety of other actions that suramin exerts on cells, of which the inhibition of growth factor activity is the most important [27].

CPT is a phase-specific drug, and a specific topoisomerase I inhibitor, whose activity is very much dependent on the proliferative state of cells, exerting maximal cytocidal effect in S phase and dividing cells [28]. Although the combination of topoisomerase II with topoisomerase I inhibitors is appealing,

Table 1. DNA cell cycle analysis of PC3 cells

		Suramin (μM)			
		0	15	90	150
G_1	Day 3	57 ± 1.5	59.5 ± 3.5	71.5 ± 3.5	72.5 ± 6.5
	Day 6	58 ± 2.3	67 ± 4.3	77 ± 0.7	80 ± 0.7*
S	Day 3	33 ± 4	29.5 ± 1.5	27.5 ± 4.5	26.5 ± 6.5
	Day 6	33 ± 3.3	23 ± 3.8	17 ± 2.5	15 ± 1.8
G_2-M	Day 3	9.5 ± 2.5	11 ± 1	1.5 ± 0.5	1.5 ± 0.5
	Day 6	9 ± 1.5	10 ± 0.7	6 ± 2.4	4.5 ± 1.5

PC3 cells were harvested after being exposed to suramin for 3 to 6 days. Values represent mean ± S.E.M. of four different experiments. *Significantly different from control cells ($P < 0.05$, Student's t-test).

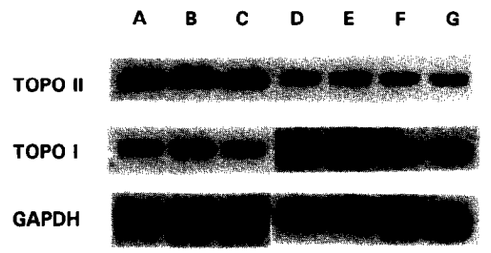

Figure 4. Topoisomerase I and II gene expressions in MCF-7 and PC3 cell lines. Lane A, MCF-7, control; lane B, MCF-7 after exposure of 4.5 μM suramin; lane C, MCF-7 after exposure of 45 μM suramin; lane D, PC3, control; lane E, PC3 after exposure of 15 μM suramin; lane F, PC3 after exposure of 100 μM suramin; lane G, PC3 after exposure of 150 μM suramin. Total RNA (10 μg) was fractionated in a 1% agarose-formaldehyde gel, transferred to a nylon filter, and hybridised to a ^{32}P-labelled topoisomerase II probe. The same filter was stripped and sequentially rehybridised with topoisomerase I and GAPDH probes.

some reports have demonstrated antagonistic effects in hamster lung fibroblasts, human leukaemia cell lines and colon carcinoma cells, when the drugs were simultaneously added *in vitro* [13–15]. This antagonistic effect could be explained by the inhibition on nucleic acid synthesis which is required to convert topoisomerase II–DNA adducts into cytotoxic lesions [13–15]. Nevertheless, interestingly, sequential administration of topoisomerase II inhibitors and topoisomerase I inhibitors resulted in additive or synergistic cytotoxicity [14, 15].

Based on possible synergistic interaction between VP16, a topoisomerase II inhibitor, and CPT-11, a water-soluble CPT derivative, a phase I trial with this combination has been performed [29]. In this study, first VP16 and then CPT-11 were given for 3 consecutive days. A remarkable antitumour activity was observed, which definitely excludes the possibility of an antagonistic effect of this combination.

In an attempt to understand the mechanism underlying the synergistic effect observed in our study, with the sequential administration of suramin and CPT, we investigated whether a possible increase of the expression of topoisomerase I gene might have been responsible for this effect. Interestingly, the gene expression of both topoisomerase II and I genes was reduced

Table 2. Relative levels of expression of topoisomerase I and II genes

Cell line	Suramin (μM)	Topoisomerase I	Topoisomerase II
MCF-7	0	1 ± 0.11	1 ± 0.07
	4.5	0.83 ± 0.12	1.02 ± 0.06
	45	0.57 ± 0.08	0.47 ± 0.12
PC3	0	1 ± 0.17	1 ± 0.08
	15	1.09 ± 0.15	1.4 ± 0.15
	100	0.51 ± 0.04	0.37 ± 0.04
	150	0.47 ± 0.11	0.46 ± 0.04

Relative expression of the topoisomerase genes was determined by densitometry of northern blot autoradiograms and normalised by the GAPDH gene expression. Relative gene expression is reported in arbitrary units, a value of 1 being assigned to the expression level obtained in MCF-7 and PC3 growing in absence of suramin for 6 days. The cells were exposed at different suramin concentrations for 6 days. Values are the means ± S.E.M. of three different experiments.

after a 6-day exposure to suramin and was suramin dose-dependent, reaching a maximum of 50% reduction in gene expression at the highest suramin concentration tested. These results were paralleled by a reduction of S phase cell compartment after 3 and 6 days of cultures in the presence of suramin, while the non-proliferating compartment increased. Because CPT is an S phase-specific antitumour drug, this finding was unexpected. Obviously the mechanism underlying the synergistic effect observed between suramin and CPT does not involve modifications of expression of topoisomerase genes. A similar result has been obtained with the drug combination of 2'-deoxy-5-azacytidine with topotecan [30]. In this study, the sequential administration of the two drugs resulted in marked synergism *in vitro* and *in vivo*, but no correlation was observed between the synergistic effect and topoisomerase I levels of activity, which actually decreased.

In conclusion, suramin and CPT can exert a synergistic effect, provided that suramin preceeds CPT. The mechanism of synergy does not involve modifications of expression of the topoisomerase genes, and remains unexplained. The implication of this finding may be important, in the light of possible *in vivo* combinations between suramin or other topoisomerase II inhibitors and CPT derivatives.

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