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The Synergistic and Antagonistic Effects of Cytotoxic and Biological Agents on the *In Vitro* Antitumour Effects of Suramin

R. Lopez Lopez, R.E.N. van Rijswijk, J. Wagstaff, H.M. Pinedo and G.J. Peters

Suramin has shown antitumour activity *in vitro* and *in vivo*. At plasma levels higher than 200 μM there is, however, excessive toxicity. We have, therefore, attempted to improve the antitumour effects of suramin *in vitro* by combining it with several other antitumour agents. The MCF-7 mammary carcinoma and PC3 prostate cancer cell lines were exposed continuously to suramin and the other agents for 6 days. The sulphorhodamine B (SRB) assay was used for the assessment of growth inhibition. The dose–response interactions were evaluated using the median-effect analysis with the Chou and Talalay computer programme. In the MCF-7 cell line, the combination of suramin plus doxorubicin (DXR), cisplatin (CDDP), 5-fluorouracil (5-FU) or tumour necrosis factor (TNF) resulted in synergistic growth inhibition, whilst its combination with miltefosine (HPC) was antagonistic. In the PC-3 cell line, suramin plus CDDP or TNF was synergistic, whilst its combination with DXR, 5-FU and HPC was antagonistic. All tested combinations with interferon- α (IFN- α), interferon- γ (IFN- γ) and with the combination of both IFN- α + IFN- γ were not synergistic. The synergistic effect of suramin with DXR was schedule dependent. Pretreatment (addition of DXR on day 1 and suramin on days 2–5) was additive at the IC₅₀ level, in both cell lines. Addition of DXR at day 5 was more effective than simultaneous exposure. We found a synergistic effect for the combination of suramin with CDDP and TNF in both cell lines. In addition the combination with DXR and 5-FU was synergistic in MCF-7. Sequential administration of DXR–suramin or suramin–DXR increased the growth inhibition.

Key words: suramin, prostate cancer, breast cancer, doxorubicin, cisplatin
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INTRODUCTION

SURAMIN, a polysulphonated naphthylurea, was synthesised at the beginning of this century by Bayer (Leverkusen, Germany) and was used for many years in the treatment of trypanosomiasis and onchocerciasis [1]. Recently, it has been shown to possess antitumour properties *in vitro* and *in vivo* [2].

Recently reported phase II studies have demonstrated

responses in several human neoplasms including prostate and adrenocortical cancer [3]. These responses have been observed with prolonged administration or higher doses. When the plasma levels of suramin exceeded 200 μM (300 $\mu\text{g/ml}$), there was, however, an excessive and intolerable toxicity, mainly polyneuropathy and coagulopathy.

The exact mechanism of action of suramin remains unclear.

Suramin may interfere with the action of peptide growth factors, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor β (TGF- β), epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) [4–6]. Interaction of suramin with several cellular enzymes, critical to cell proliferation and function, can lead to inhibition of DNA polymerases, protein kinase C and lysosomal enzymes, which can result in a systemic accumulation of glycosaminoglycans [7–9].

On the basis that the mechanism of action and toxicity profile of suramin is different from that of conventional cytotoxic agents, we have examined the effect of combining these more conventional agents with suramin in order to produce an improved therapeutic index. Cisplatin (CCDP), 5-fluorouracil (5-FU) and doxorubicin (DXR) were included because they are some of the most widely used chemotherapeutic agents in clinical oncology, including breast carcinoma and prostate cancer. The ether lipid, miltefosine (HPC), is an investigational agent which was studied because its mechanism of action involves interactions with the cell membrane and cell signal transduction pathways [10], which are also a target for suramin [8, 11]. Finally, the biological response modifiers, interferons- α (IFN- α) and - γ (IFN- γ) and tumour necrosis factor (TNF), have been incorporated because of their different spectrum of antitumour activity and toxicity to conventional agents. These combinations were studied in a breast cancer (MCF-7) and a prostate cancer (PC-3) cell line, which have been characterised extensively for their interaction with suramin [11, 12].

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 BRL (Paisley, U.K.); trichloroacetic acid (TCA), glutamine and gentamycin from Merck (Darmstadt, Germany); trypsin, (ethylenedinitrilo)-tetraacetic acid (EDTA), trypan blue, sulphorhodamine B (SRB) from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.); acetic acid and [tris(hydroxymethyl)aminomethane] (Tris) from Baker Chemicals B.V. (Deventer, The Netherlands).

Drugs

Suramin (Germanin) was purchased from Bayer (Bayer Leverkusen, Germany) and stored as a 25 mM solution in 0.9% NaCl at -20°C . Hexadecylphosphocholine (Miltefosine) (HPC) was kindly donated by Dr P Hilgard (Asta Pharma, Germany) and was also stored as a 25 mM solution at -20°C . CCDP (Bristol Myers, Brussels, Belgium) was stored at 2.4 mM in 0.9% NaCl at $+4^{\circ}\text{C}$. DXR was purchased from Farmitalia Carlo Erba (Nivelles, Belgium) and stored as a 3.45 mM solution in water at -20°C . 5-FU, from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) was stored at 0.01 M in phosphate buffered saline (PBS) at $+4^{\circ}\text{C}$. Recombinant human TNF (Knoll AG, Ludwigshafen, Germany, batch number EA-9013) was stored at

1 $\mu\text{g}/\mu\text{l}$ in 0.9% NaCl at -20°C . Recombinant human IFN- γ (with a specific activity of $2\text{--}6 \times 10^6$ U/mg) and recombinant IFN- α were kindly provided by Boehringer Ingelheim (Alkmaar, The Netherlands) and stored at 10^6 U/ml and at 5×10^6 U/ml, respectively, at -20°C .

Cell lines

The prostate cancer cell line PC-3 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, Maryland, U.S.A.). 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 cm² flasks (Nunc, Roskilde, Denmark) in a 37°C , 5% CO₂, 95% humidified air incubator, and were subcultured once a week as previously described [11, 12]. All cells were free of mycoplasma infection, as tested by Hoechst staining of a Mycoplasma-sensitive indicator strain of monkey kidney cells.

Growth inhibition studies

Cells in exponential growth phase were harvested by 0.25% trypsin-EDTA, resuspended in medium with 50 $\mu\text{g}/\text{ml}$ gentamycin. Viability was determined by Trypan blue exclusion using a haemocytometer; in case of $> 90\%$ viability, cells were counted with an automatic counter (Sysmex, CC-110, Tokyo, Japan) and seeded in 96-well plates (Greiner Labortechnik, Solingen, Germany). The plating densities for PC3 were 14 000 cells/well and for MCF-7 4 000 cells/well. After 24 h, to allow cell recovery, the drugs were added dissolved in culture medium. For 6 days, without any medium change, the cells were exposed to suramin, to one of the agents of interest or to the combination of both. Cell growth was exponential under these conditions. Chemosensitivity was determined at day 7 with the SRB assay [13].

Different experiments were carried out to study the schedule dependence of the suramin combination with DXR. For pretreatment with DXR, the cells were plated at day 0; after recovery at day 1, DXR was added for 1 day and then removed by aspiration and then replaced by suramin for 3 additional days. Subsequently, the cells were cultured in drug-free medium for 2 days and the SRB assay performed. For the posttreatment schedule, the cells were cultured from day 2 to 5 in medium with suramin, which was removed on day 5. Subsequently the cells were treated with DXR for 1 day. At day 6, the wells were aspirated and drug-free medium was added for 1 additional day after which the SRB assay was performed.

The SRB assay was performed as described previously [12, 13] with the method of Skehan and colleagues [14]. Briefly, TCA (final concentration 10%) was added to the cells to precipitate the proteins and fix the cells. After washing and air drying, the cells were stained with 0.4% SRB dissolved in 1% acetic acid, washed 5 times with 1% acetic acid, and air-dried. The protein stain was solubilised with 10 mM unbuffered tris base and the optical density measured using a 510 nm filter, using a microtitre plate reader (Titertek Multiskan MCC/340, Flow Laboratories). Previously, we demonstrated that neither suramin [12], nor most of the other drugs [13] interfered with the SRB assays. Similar effects of these drugs were observed for both the MTT and SRB assays [12, 13]. In every experiment in control wells, the interaction between drugs and the assay was always included as a blank.

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Synergy analysis

In order to have a robust, objective method for evaluation of the interactions between suramin and the other agents, we used the median-effect analysis, as described by Chou and Talalay [15], using a computer programme developed by Chou and Chou (Elsevier-Biosoft). For this purpose, a fixed ratio for each suramin-drugs combination was used. The median dose values were determined from the median-effect plot where the x-axis = $\log(\text{dose})$ and the y-axis = $\log(\text{fraction affected/fraction unaffected})$. The combination index (CI_x) was calculated at any level by the formula: $CI_x = [(D)1/(Dx)1] + [(D)2/(Dx)2] + [\alpha(D)1,2/(Dx)1,2]$ where $\alpha = 0$ (for mutually exclusive drugs), and $(D)1$, $(D)2$ and $(D)1,2$ were the doses of drug 1, 2 and their mixture (in specific ratios), and $(Dx)1$, $(Dx)2$ and $(Dx)1,2$ were the doses required to affect a system $x\%$; these doses for $x\%$ effect are calculated from the formula: $D = D_m [fa/(1-fa)]/m$, where D_m is the dose that was required to produce a median effect and m was the slope of the median-effect plot (a measure of sigmoidicity). The value of CI_x indicated $CI_x = 1$ (an additive effect), $CI_x > 1$ (an antagonistic effect) and $CI_x < 1$ (a synergistic effect). This method also enabled us to evaluate the interaction of the drug with isobolograms. In all cases, the analysis of the combination index was confirmed by the isobologram.

RESULTS

For all agents, the concentration which gave 50% growth inhibition (IC_{50}) was first determined in both cell lines. With the exception of HPC, the MCF-7 cell line was two to 10 times more sensitive to all drugs than the PC-3 cell line (Table 1).

The ratio between the concentration of drugs was based on the IC_{50} value. Drugs were combined at their approximate IC_{50} values and a range of concentrations below and above were chosen in the same fixed ratio. We chose this evaluation because it allowed us to simulate a range of concentrations for both drugs. This method would also allow us to use a computerised, objective evaluation of the interaction between both drugs. In addition to the median-effect analysis, we also evaluated the results using isobolograms. This analysis led to the same results as were obtained with CI_x (data not shown). In the same experiment, we always included controls with suramin and the studied drug alone. In MCF-7 cells, with continuous exposure over 6 days, the combination of suramin with DXR, 5-FU or CDDP ($CI_x = 0.69, 0.45$ and 0.49 , respectively at 0.5 of the fraction affected) produced synergy, whilst its combination with

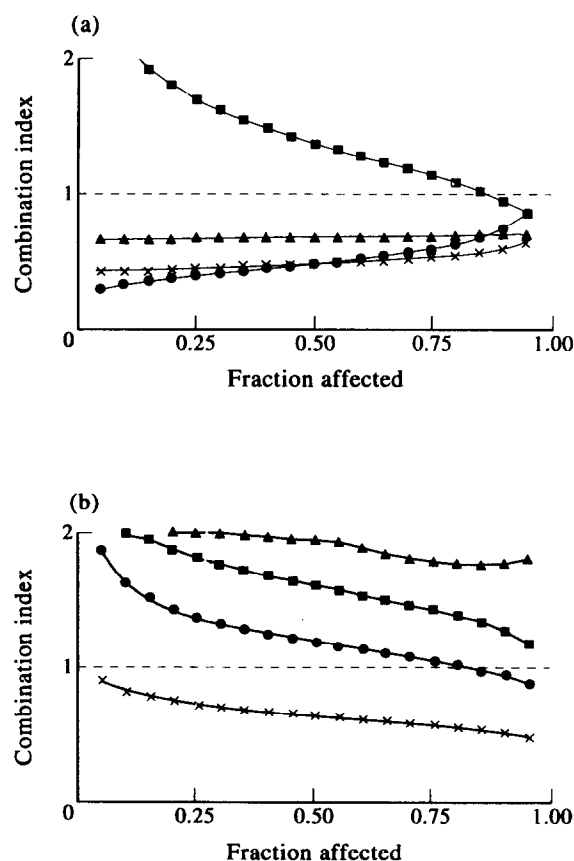


Figure 1. The Chou and Talalay plot evaluating synergy of the suramin combinations with CDDP (x), DXR (Δ), 5-FU (●) and HPC (■) in the MCF-7 cell line (a) and in the PC-3 cell line (b).

HPC resulted in antagonism ($CI_x = 1.38$) (Figure 1a). For the prostate cancer cell line, PC-3, suramin plus CDDP was synergistic ($CI_x = 0.64$) whilst its combination with DXR, 5-FU and HPC produced antagonism ($CI_x = 1.9, 1.18$ and 1.57 , respectively) (Figure 1b).

The combinations of suramin plus IFN- α , IFN- γ and both IFNs together were also studied. No growth inhibition by either IFN or a combination of both IFNs was observed in either cell line at a concentration up to 3000 U/ml. The combination of suramin with the IFNs resulted in a decrease in the growth inhibitory effect of suramin alone, with CI_x always being > 1 (antagonistic) (Table 2).

TNF was tested in both cell lines at concentrations ranging from 0.15 pM to 6 μ M. The MCF-7 cell line was very sensitive with an IC_{50} of 0.15 nM whilst in PC-3 cells TNF produced no growth inhibition with concentrations up to 6 μ M. Suramin plus TNF was, however, markedly synergistic with the effect being more pronounced in the PC-3 cell line (Figure 2).

The schedule dependence of suramin combined with DXR was examined in more depth, as described above. We performed control experiments without aspiration steps in order to ensure that the cell loss was minimal. This method was reproducible and the percentage of cells dissipated was $< 10\%$. Under these new culture schedules, the cells were cultured with suramin for 3 days (from day 2 to 5 of the culture) and then were allowed to recover for 2 days. As expected, under these conditions, the IC_{50} of suramin increased and was 83 and 432 μ M for MCF-7 and PC-3, respectively. For cells exposed exclusively on day 1 to DXR and allowed to recover for 5 days, the IC_{50} was 0.11

Table 1. Sensitivity of MCF-7 and PC-3 to cytotoxic agents

Drugs (μ M)	MCF-7	PC-3
Suramin	31 ± 2	317 ± 15
DXR	0.09 ± 0.002	0.178 ± 0.02
5-FU	0.056 ± 0.01	0.384 ± 0.1
CDDP	0.266 ± 0.02	0.6 ± 0.1
HPC	27 ± 3	14 ± 0.4
TNF	0.0001	> 6

The cells were continuously exposed to the cytotoxic agents for 6 days and then the SRB assay was performed. Values in μ M represent the mean $IC_{50} \pm$ standard error of the mean (SEM) of four different experiments. DXR, doxorubicin; 5-FU, 5-fluorouracil; CDDP, cisplatin; HPC, miltefosine; TNF, tumour necrosis factor.

Table 2. Combination index (CI_x) of suramin with cytotoxic and biological agents

Drugs (μM)	Fixed ratio	MCF-7	PC-3
DXR	200:1	0.69	1.9
5-FU	2000:1	0.45	1.18
CDDP	2000:1	0.49	0.64
HPC	10:1	1.38	1.57
TNF	400:1	0.41	0.24
IFN-α	1:1	1.3	1.3
IFN-γ	1:1	1.2	1.6
IFN-α + γ	1:1:1	1.7	1.8

Values represent the CI_x of suramin in combination with different agents for 3 different experiments. The CI_x is expressed at the IC₅₀ level (0.5 of fraction affected). The fixed ratio suramin:agent is expressed in μM:μM, except for IFN where it is μM:U/ml. CI_x > 1 mean antagonism, CI_x = 1 is additive and CI_x < 1 is synergism. Abbreviations as for Table 1. IFN, interferon.

and 0.3 μM for MCF-7 and PC-3 cell lines, respectively. As anticipated, cells exposed to DXR at day 5 of the culture were less sensitive and the IC₅₀ was higher, 0.92 and 12 μM for MCF-7 and PC-3 respectively, because there were more cells in the cultures. In contrast to the simultaneous continuous exposure of

suramin and DXR (Figure 3), there was a synergistic effect with DXR given on day 5 (posttreatment) in both cell lines (CI_x = 0.51, 0.46 for MCF-7 and PC-3, respectively). DXR given on day 1 (pretreatment) followed by suramin caused a synergistic effect only at higher concentrations and antagonistic at lower concentrations of both drugs, with the CI_x at 50% fraction affected by approximately 1 (additive).

DISCUSSION

In this paper, the *in vitro* effects of combining suramin with conventional cytotoxic and biological response modifiers are reported. In the most sensitive breast cancer cell line, MCF-7, synergistic growth inhibition of suramin combined with DXR, CDDP, 5-FU or TNF was observed at all concentrations tested. In the prostate cancer cell line, PC-3 synergism was only observed when suramin was combined with CDDP or TNF for a continuous period of 6 days.

Fruehauf and colleagues [16] have reported a synergistic effect of suramin in combination with DXR or TNF in PC-3 cells. The data presented here confirm the results with TNF, but demonstrated antagonism for the combination of suramin with DXR in this cell line. These contradictory results are likely to be due to differences in plating densities and culture conditions rather than variability in cell line sensitivity, since the growth modulatory effects of suramin, DXR or TNF alone were similar to those observed in the current studies [16]. Liu and colleagues [17] have also observed marked synergism of growth inhibition with the combination of suramin with TNF and IFN-γ in the PC-3 cell line, although in a different experimental system. Recently, Berthois and associates [18] reported an antagonistic effect of DXR by suramin; in this study, cells were exposed simultaneously to the two drugs, but the effect of DXR was only studied and observed at one concentration of suramin. However, the effect was not confirmed by using, for example, isobolograms. As demonstrated in the present paper with the median-effect plots, the interaction of DXR and suramin is schedule- and concentration-dependent; a synergistic effect for both cell lines was only observed when DXR was added after suramin.

DXR, CDDP and 5-FU are all in routine clinical use and have significant antitumour activity in breast cancer, and some activity in prostate cancer [19,20]. Both CDDP and 5-FU can be given by continuous infusion with plasma levels above the IC₅₀ for MCF-7 and PC-3 [21, 22]. Considering the clinical activity of CDDP in these diseases, further investigation combination with suramin might be worthwhile. The DXR concentrations used in

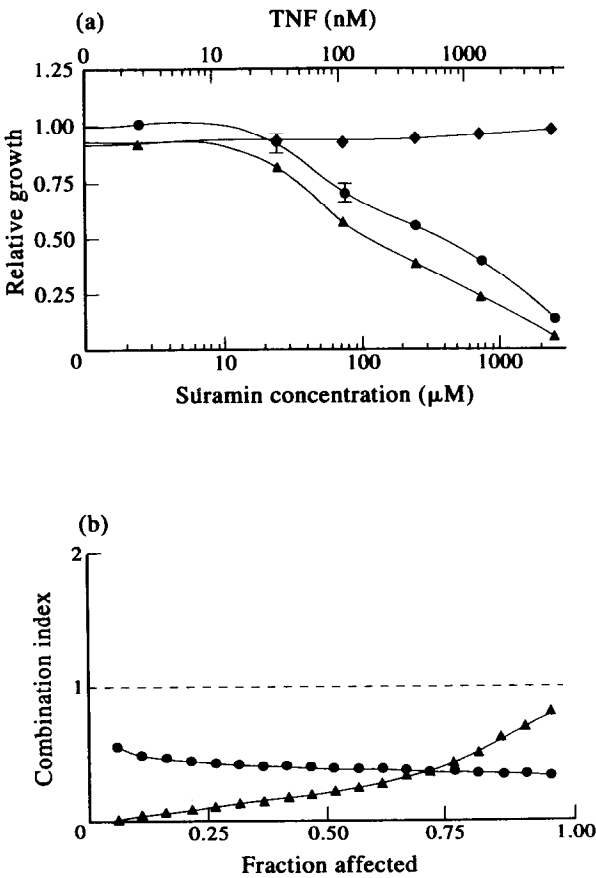


Figure 2. (a) PC-3 dose-effect curves of suramin (●), TNF (◆) and the combination of both (▲). (b) The Chou and Talalay plot evaluating synergy of the suramin combination with TNF for MCF-7 cell line (●) and PC-3 cell line (▲).

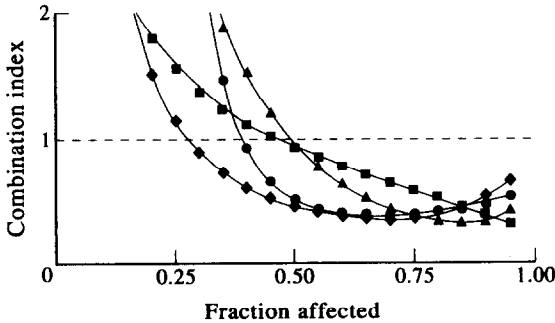


Figure 3. The Chou and Talalay plot evaluating synergy of 1 day pretreatment with DXR and then 3 days of suramin for MCF-7 (■) and for PC-3 (▲). The synergy plot for 1 day posttreatment with DXR in suramin-free medium following 3 days of suramin exposure for MCF-7 (●) and for PC-3 (◆) are also given.

our experiments would not be maintained in patients for 6 days, since the peak plasma concentration following an intravenous bolus injection does not exceed 15 μM at the standard dose of 50 mg/m^2 [23], and the administration of 30 mg/m^2 DXR on 3 successive days resulted in 3 h DXR levels of approximately 0.09 μM on day 3 [24]. The schedule of administration appears to be an important determinant of efficacy and toxicity for many drugs given alone or in combination [25]. We have studied the effect of pre- and post-treatment on the cell lines with DXR, resulting in a synergistic effect when the cells were exposed for 3 days to suramin and then exposed to DXR for 1 additional day in suramin-free medium. When the cells were pretreated for 1 day prior to 3 days of suramin, there was an antagonistic effect at lower concentrations, an additive effect at the IC_{50} and synergism at higher concentrations for both drugs. The scheduling of the suramin-DXR combination has been studied in tumour-bearing Ehrlich carcinoma mice by Osswald and Youssef [26]. The suramin-DXR combination resulted in a small inhibition of tumour growth, but was extremely toxic causing death in 6 out of 15 mice. The DXR-suramin combination acted synergistically without significant toxicity. Although this is in line with our *in vitro* results, more *in vivo* studies have to be performed.

Suramin has a complicated mechanism of action [27, 28], mediated by its interference with growth factors and inhibition of a number of enzymes. These properties of suramin as well as its high binding to plasma proteins [12, 29] and its extraordinarily slow total plasma elimination, with a terminal half-life about 45 days [30, 31], should be taken into consideration when optimising the combinations with suramin in cancer patients.

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