

Increased Anti-tumour Efficacy of Doxorubicin when Combined with Sulindac in a Xenograft Model of an MRP-1-positive Human Lung Cancer

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Abstract. *Background:* A number of cellular proteins, including P-glycoprotein (P-gp) and Multiple drug Resistance Protein (MRP-1), act as drug efflux pumps and are important in the resistance of many cancers to chemotherapy. We previously reported that a small number of NSAIDs could inhibit the activity of MRP-1. *Materials and Methods:* We chose sulindac as a candidate agent for further investigation as it has the most favourable efficacy and toxicity profile of the agents available for a potential specific MRP-1 inhibitor. NCI H460 cells expressed MRP-1 protein (by Western blot) and also the toxicity of doxorubicin (a substrate of MRP-1) could be potentiated in this line using non-toxic concentrations of the MRP-1 substrate/inhibitor sulindac. These cells were implanted in nude mice and the animals divided into various groups which were administered doxorubicin and/or sulindac. *Results:* Sulindac was shown to significantly potentiate the tumour growth inhibitor activity of doxorubicin in this MRP-1-overexpressing human tumour xenograft model. *Conclusion:* Sulindac may be clinically useful as an inhibitor of the MRP-1 cancer resistance mechanism.

Multi-drug resistance (MDR) is a serious clinical problem associated with standard treatment regimes used in many forms of cancer (1). Originally MDR was associated with the P-glycoprotein (P-gp) pump which can actively efflux a number of xenobiotics including many of the more widely used anticancer drugs (2). In 1992, an additional cellular efflux pump, Multi-drug Resistance Protein (MRP-1) was described in non-P-glycoprotein-overexpressing cancer cells;

other similar pumps have now been identified (3). A large amount of experimental and clinical data has been published suggesting that MRP-1-mediated resistance is clinically very significant in solid tumours and leukemias (1, 4-6). After the initial discovery of P-gp, several groups discovered and tested a number of agents which could inhibit the efflux of anti-cancer drugs by this pump (7,8). Although initial results proved promising, later clinical trials showed that P-gp-mediated resistance was a complex phenomenon and to date P-gp inhibitors have largely failed to deliver the anticipated clinical benefit (9, 10).

In the past few years investigations of these pumps have yielded several agents which can inhibit the actions of MRP-1. Recently a group in the US has published evidence of an agent, Biricodar, which can inhibit both MRP-1 and P-gp (11). Verapamil and cyclosporine A, both of which have been examined as experimental and clinical inhibitors of P-gp, are also known to inhibit MRP-1 (11, 12). However, there are currently no specific inhibitors of MRP-1 at the clinical trial stage. As part of a large scale screen for synergistic effects with anti-cancer agents, our research group discovered that particular agents taken from the general therapeutic class of compounds termed Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) could inhibit the MRP-1 pump in a variety of MRP-1-expressing cell lines (13,14). Further *in vitro* analysis of this finding indicated that this effect was not associated with the conventional mechanism of action of the NSAIDs (inhibition of the cyclooxygenase enzymes) and indeed most of the NSAID agents are inactive as MRP-1 inhibitors. Of the NSAIDs tested indomethacin, sulindac, tolmetin, acemetacin, mefenamic acid and zomepirac were able to inhibit the MRP-1 pump and acted synergistically to increase the cytotoxic effect of MRP-1 substrate anti-cancer agents in MRP-1-expressing cell lines. Of these agents, sulindac appears to have favourable characteristics as a potential MRP-1 inhibitor, since *in vitro* inhibition is evident at concentrations which are achievable in serum with standard doses of the agent (13,15), also, of the small number of

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NSAIDs which might be used, sulindac is relatively non-toxic and well tolerated, especially when used in an acute setting (16).

Development of candidate cancer chemotherapy drugs typically follows a course starting with *in vitro* exploration, followed by *in vivo* testing, usually in nude mice implanted with human tumours, and toxicological and kinetic evaluation, ultimately leading to initial evaluation in human cancer patients. To develop our finding further we examined the effect of one of the active NSAIDs, sulindac, with an MRP-1 substrate anti-cancer agent, doxorubicin (adriamycin) on implanted NCI H460 tumours in nude mice.

Materials and Methods

Cells. NCI H460 (human large cell lung carcinoma) cells (17) supplied by the ATCC were cultured in DMEM at 37°C using standard cell culture procedures.

In vitro toxicity testing. Cytotoxicity testing of drugs and drug combinations was measured by colourimetric assays as described previously. (13,18,19). Briefly, On Day 1, cells were seeded at 1×10^3 cells/well in a 96-well plate and left to attach overnight in a 5% CO₂ incubator at 37°C. The appropriate concentrations of drug and/or compound were added to the plate on Day 2, and the assay was terminated on Day 7. Measurement of cell number was performed using Acid Phosphatase determination. Each concentration or combination of drugs was assayed in a column of the 96-well plate (8 wells). The assays were performed in duplicate plates.

Western blotting. Cells were seeded at a density of 3×10^6 cells per 175-cm² flask two days before the experiment. COR L23 R (strongly MRP-1-positive) and COR L23S cells (MRP-1-negative by Western) were cultured in RPMI medium and were a gift from Dr. Peter Twentyman, MRC Clinical Oncology and Radiotherapeutics Unit, Hills Road, Cambridge, U.K. NCI H460 cells were cultured in DMEM. Media was removed and cells were trypsinised. Cells were washed twice with ice-cold PBS. All subsequent procedures were performed on ice. Cells were resuspended in 1ml of fresh NP-40 lysis buffer. The cells were sonicated and the lysed preparation pelleted at 13,000 r.p.m for 10 minutes. The supernatant was removed and protein concentration quantified using the Bio-Rad protein assay kit (Bio-Rad, CA, USA).

The proteins in the supernatant were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A total of 20 µg protein was diluted in 5X loading buffer and loaded onto the 5% stacking gel and 7.5% resolving gel. Molecular weight markers and protein samples were heated to 95°C for 2 minutes. The gels were run at 250V and 45mA. The gel was then equilibrated in transfer buffer. Protein was transferred from the gel to Hybond ECL nitrocellulose membranes (Amersham, CA, USA) by semi-dry electroblotting at a current of 34mA at 15V for 35 minutes. Membranes were blocked using 5% milk (Cadbury's: Marvel skimmed milk) in TBS (125mM NaCl, 20mM Tris pH 7.5) for 4 hours at room temperature. Anti-human MRP-1 monoclonal primary antibody (MRP r1, Kamiya Biomedical Company, USA) was added (1:50 dilution in TBS) to membranes overnight at 4°C. Rabbit anti-rat secondary antibody (Dako, Denmark) was added at

a 1:12,000 dilution in TBS for 2 hours at room temperature. Membranes were then washed in TBS - 0.5% Tween-20. A SuperSignal ULTRA Chemiluminescence kit (Pierce, CA, USA) was used for the development of immunoblots.

Drugs. Sulindac and doxorubicin were purchased from Sigma-Aldrich (Dublin, Ireland). Sulindac was dissolved in DMSO so that all concentrations used in the cell assays were in less than 1%DMSO. For animal experiments, all dilutions of sulindac were prepared using 1% DMSO in DMEM medium (Gibco, CA, USA) prewarmed to 37°C, as the vehicle. The dosing solutions of doxorubicin were prepared in sterile saline. The solutions were formulated freshly every 5th day and kept refrigerated until use.

Animals. Animal experiments were performed under UK Home Office regulations. Female nude (nu/nu) athymic mice were supplied by Charles River UK Ltd. and acclimatized in the holding facility for 14 days prior to beginning the study. Mice were aged 8-10 weeks and weighed in the range 22.5-32.5 g at the start of the study. Sterile irradiated Harlan Teklad 9607 R & M diet (Harlan Olac UK Ltd., Bicester, Oxon) and mains tap water (autoclaved for sterility), were provided *ad libitum*.

Xenografts. NCI H460 cells were resuspended in sterile PBS at an approximate density of 5×10^7 cells/ml. Nude (athymic) mice were injected subcutaneously with 0.1 ml cell suspension in the right flank. The mice were examined routinely for the appearance of tumours. Mice were allocated to groups on day 9 after tumour implantation. Treatment was also initiated on this day. There were 6 treatment groups with 12 mice per group. The groups consisted of the following:

Group	Treatment	Dose
1	Vehicle	0.5 ml <i>i.p.</i>
2	Doxorubicin	0.5 mg/kg <i>i.p.</i>
3	Sulindac	30 mg/kg <i>i.p.</i>
4	Doxorubicin and Sulindac	0.5 mg/kg and 30 mg/kg <i>i.p.</i>
5	Doxorubicin	0.75 mg/kg <i>i.p.</i>
6	Doxorubicin and Sulindac	0.75 mg/kg and 30 mg/kg <i>i.p.</i>

All dosing solutions were combined prior to administration. The dose volume for all groups was 0.5 ml per mouse, irrespective of body weight (therefore doses stated in mg/kg are an approximation based on a 25 g average mouse weight). Mice were dosed (*i.p.*) daily for 5 days followed by a 2-day dosing holiday. Groups 1, 2, 3 and 5 were then dosed for a further 7 days prior to sacrifice, and groups 4 and 6 for a further 11 days prior to sacrifice. Tumour size was measured regularly from the start of treatment and animals were sacrificed as soon as tumour size was judged to be in danger of causing distress. Three mice from each group were taken for pathological study of certain organs: liver, kidney, heart, lungs, ovaries, stomach, duodenum, jejunum, caecum, colon, rectum and sternum.

Immunocytochemistry. Two tumours from each of treatment groups were analysed for MRP-1 protein expression by immunocytochemistry. This analysis was performed according to the method of Hsu *et al.* (20) using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (ABC) plus an appropriate secondary antibody. Tissue sections were dewaxed in xylene, rehydrated in graded alcohols and placed in Tris Buffered Saline

(TBS) / 0.05% (v/v) Tween 20. Endogenous peroxidase activity was quenched using 3% (v/v) H₂O₂. All slides were blocked for non-specific staining with 20% (v/v) normal rabbit serum. MRP-r1 monoclonal antibody (21) was applied to each sample diluted 1/25 in TBS/0.1% (v/v) Tween 20. Sections were incubated overnight at 4°C followed by a 30-minute incubation with biotinylated rabbit anti-rat (1/500 dilution in TBS/ 0.1% (v/v) Tween 20). Finally, Vectastain Elite ABC reagent (HRP conjugated) (Vector Laboratories, UK, PK-7100) was applied using the peroxidase substrate 3'-3 diaminobenzidine tetrahydrochloride (DAB) as a chromogen. All tissue sections were lightly stained with Harris's haematoxylin, Following dehydration, slides were cleared in xylene and mounted in DPX (BDH, UK). Negative control slides consisted of sections immunostained as outlined but primary antibody was omitted.

Data analysis. Calculations of relative tumour volumes and plots of tumour growth curves were performed. Tumour volume was calculated by the formula $(W^2 \times L)/2$, where W is the tumour measurement at the widest point and L is the tumour dimension at the longest point. Relative tumour volume (RTV) was calculated using the tumour volume on the first day of treatment, e.g. tumour volume on Day 3/tumour volume on Day 0. Day 0 was the day of treatment initiation. Tumour growth delay was calculated as the time taken for the mean relative tumour volume of the treatment group to reach 6 times the start volume minus the time taken for the mean relative tumour volume of the control group to reach 6 times the start volume. Specific growth delay was calculated using the doubling time of the vehicle-treated control group (growth delay at RTV6/control tumour doubling time). Two mice allocated to groups at the start of study were not included in the final analysis of results. The tumour of mouse 50 (Group 1) was retrospectively deemed not to be an established tumour ("no take"). Mouse 30 (Group 3) died on the 6th day from the start of dosing.

Statistical analysis. The data from all of the groups on the study were compared using one-way analysis of variance for each day of tumour measurement whilst the control group remained on study. As the variances within the groups differed significantly between groups, a Kruskal-Wallis analysis was used. A Dunn's post test was used to allow comparison of data between all groups.

Results

Expression of MRP-1 and enhancement of doxorubicin cytotoxicity by sulindac in NCI H460. A Western blot of NCI H460 cell extract demonstrated the presence of MRP-1 protein (Figure 1). The results in Figure 2 illustrate that non-toxic concentrations of sulindac can increase the cytotoxicity of doxorubicin in this cell line, in a dose-dependent manner consistent with the presence of active MRP-1.

Effect of doxorubicin and sulindac on growth of NCI H460 cells in nude mice. These experiments were designed to evaluate the impact of sulindac on doxorubicin-induced growth retardation of NCI H460 cells growing as a tumour

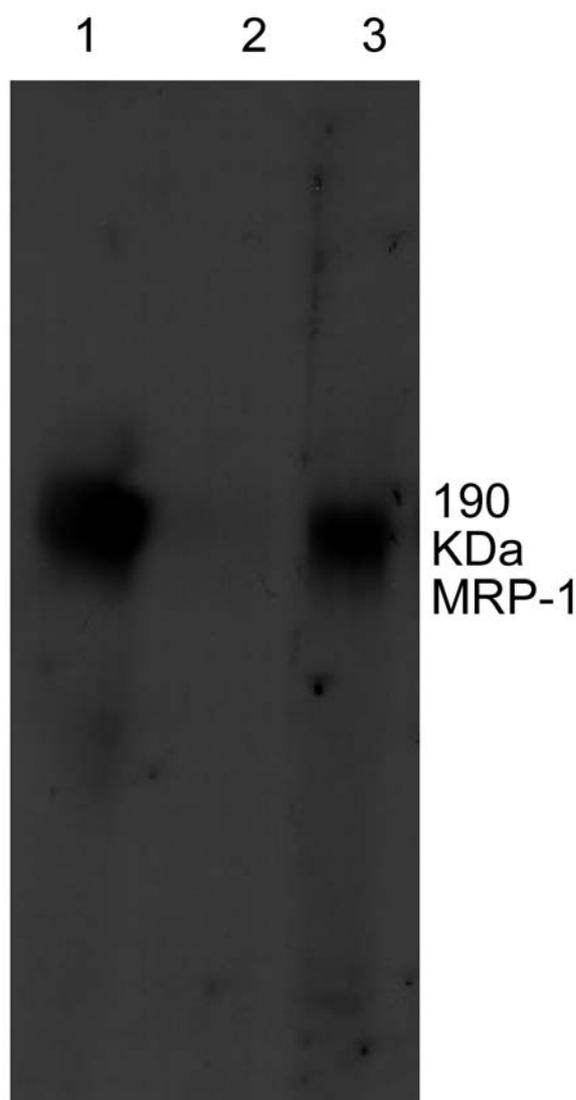


Figure 1. Western analysis of cell lines for MRP-1 protein expression using MRP-r1 antibody. Lane 1 COR L23R cells (highly MRP-1-expressing), Lane 2 COR L23S cells (very low MRP-1 expression), Lane 3 NCI H460 cells.

xenograft in nude mice. No difference was observed between the mean tumour volumes of the treatment groups on Day 0, the day of randomization. The mean relative tumour volumes for the treatment groups from each measurement day are presented graphically in Figure 3. Tumour growth delays were calculated from the graphs at mean relative tumour volume 6 (RTV6), comparing treatment groups with the control group. Tumour growth delay and specific growth delay data are presented in Table I. The doubling time (from a mean relative tumour volume of 4 to a mean relative tumour volume of 8) of the NCI-H460 vehicle control group in this study was 5.4 days.

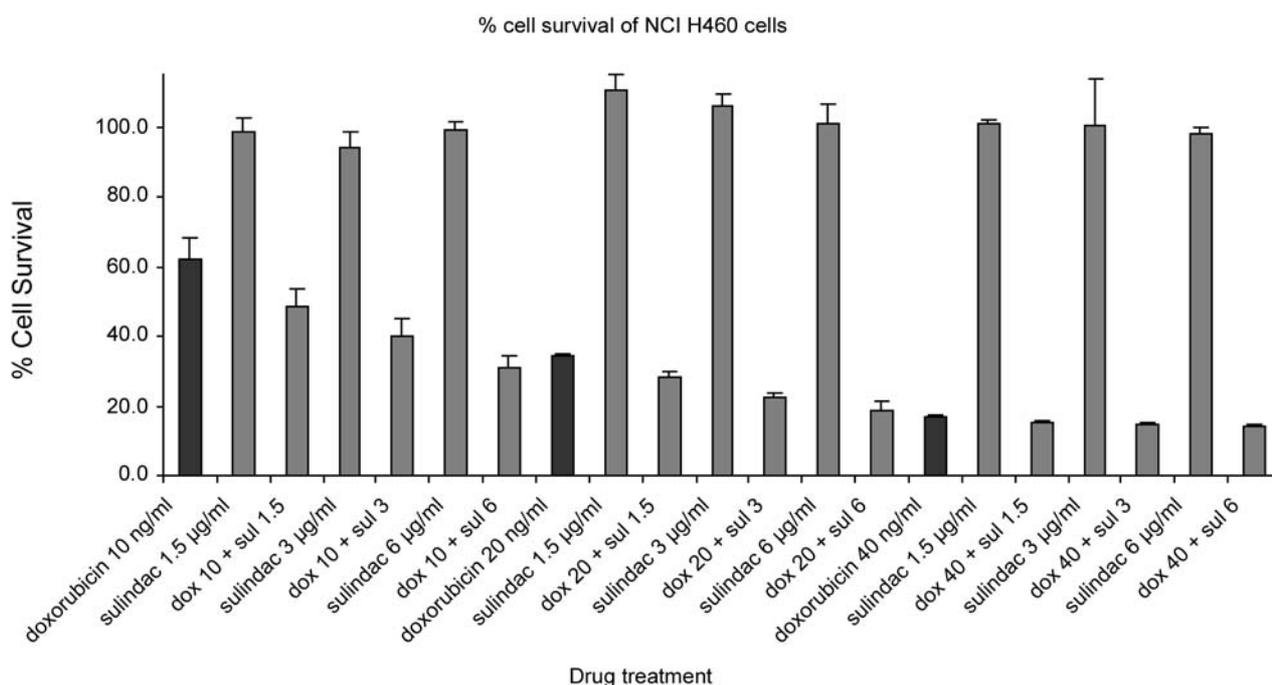


Figure 2. *In vitro* combination toxicity data for doxorubicin and sulindac using the MRP-1-expressing NCI H460 cell line.

Treatment with the reference substance (doxorubicin) as a single agent had a measurable but not statistically significant effect on the growth of the NCI-H460 tumours when administered at either 0.5 or 0.75 mg/kg daily for 12 administrations. A growth delay of 2.3 and 2.0 days, respectively, was observed for these dose levels, calculated at RTV6.

Treatment with sulindac (30 mg/kg), alone, daily for 12 administrations, again had a measurable but not statistically significant effect on the growth of the NCI-H460 tumours compared to the vehicle control. A tumour growth delay of 4.0 days was observed when compared to the control.

When sulindac was co-administered with doxorubicin (0.5 or 0.75 mg/kg), statistically significant effects on the growth of the NCI-H460 tumours were observed compared to the vehicle-treated control. For both dose levels of doxorubicin, when co-administered with the test article sulindac (30 mg/kg), tumour growth was significantly retarded at day 10 ($p < 0.05$), day 12 ($p < 0.05$) and day 14 ($p < 0.01$). The tumours of these 2 groups continued to be measured until Day 18, when the animals were sacrificed. The animals of the control group were sacrificed at Day 14 (for ethical reasons) and therefore statistical comparisons after Day 14 could not be made. Growth delays to a relative tumour volume of 6 were 6.7 and 7.0 days for the 0.5 and 0.75 mg/kg doxorubicin in combination with sulindac groups, respectively (Groups 4 and 6).

Specific growth delays were calculated and the results are presented in Table I. These values were determined using

the time taken for the mean relative tumour volume of the control group to increase from 4 to 8. This doubling time of the control tumours was 5.4 days. Both combination groups of doxorubicin and sulindac slowed the growth of the tumours by more than 1 control tumour doubling time (1.24 and 1.30, for 0.5 and 0.75 mg/kg doxorubicin plus sulindac, respectively). The doxorubicin alone-treated groups both slowed the tumour growth by less than half of one doubling time (0.43 and 0.37, for 0.5 and 0.75 mg/kg doxorubicin, respectively).

Pathological examination of xenografted mice after treatment. Pathology was performed on mice from each treatment group in the study. Analysis revealed few gross findings, all of which could be expected to occur sporadically in this age and strain of mice. Histopathology revealed some variation in lymph node morphology, but this could be expected in nude mice. Again, there were no findings which could consistently be attributed to any treatment regime.

MRP-1 expression in NCI H460 xenografts. Analysis of a small number of tumours from the animals for the presence of MRP-1 indicated that the protein was expressed in these tumours. Typical findings are illustrated in Figure 4. No clear trends in protein expression were observed when the staining characteristics were compared among the limited number of tumours analysed.

Table I. Tumour growth delay and specific growth delay

Treatment	Growth delay (days)	Specific growth delay
Doxorubicin (0.5 mg/kg)	2.3	0.43
Sulindac (30 mg/kg)	4.0	0.74
Dox (0.5 mg/kg) + Sulindac (30 mg/kg)	6.7	1.24
Doxorubicin (0.75 mg/kg)	2.0	0.37
Dox (0.75 mg/kg) + Sulindac (30 mg/kg)	7.0	1.30

Growth delay was calculated at relative tumour volume 6.

Vehicle control group mean tumour volume doubling time was calculated as the time taken to reach mean relative tumour volume 8 from mean relative tumour volume 4. In this study, the control tumour doubling time was 5.4 days.

Specific growth delay was calculated as tumour growth delay/vehicle control tumour volume doubling time.

Discussion

As a prelude to human studies, we examined the *in vitro* and *in vivo* activity of sulindac against a cell line which would also be capable of forming xenograft tumours in nude mice. The Western blot analysis indicates that NCI-H460 cells express a measurable amount of MRP-1 protein. Previous studies from our group have indicated that the NSAID, sulindac, can inhibit the ability of MRP-1 to efflux substrate drugs, such as doxorubicin, and the combination cytotoxicity experiments presented here support the hypothesis that sulindac can potentiate doxorubicin cytotoxicity in MRP-1-expressing cells. This potentiation was also evident in the impact of the combined therapy on the NCI H460 tumour growing in nude mice.

Treatment of mice bearing NCI-H460 *s.c.* tumours with doxorubicin (0.5 and 0.75 mg/kg *i.p.*), daily from Day 0 to 4 and Day 7 to 13, had a clear effect on the mean tumour volume but the effect was not statistically significant. This was anticipated as the dose of doxorubicin used was chosen so as to provide a measurable effect on tumour growth without eradicating the tumour (so that any potentiation by sulindac could be measured more easily).

Treatment with sulindac (30 mg/kg *i.p.*) by the same schedule also appeared to cause a measurable delay in the growth of the xenograft. Surprisingly, the magnitude of this effect appeared to be greater than the effect of doxorubicin (either concentration) but again there was no statistically

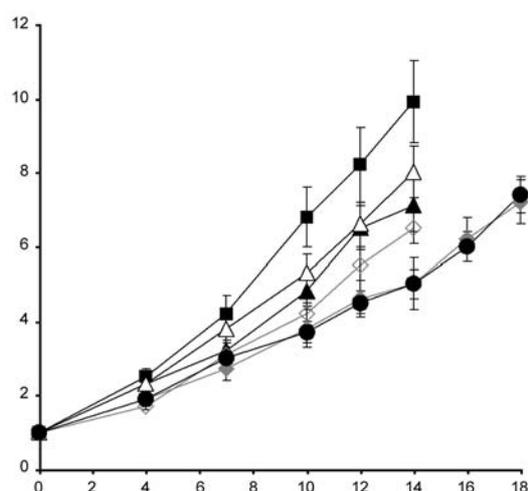


Figure 3. Growth of NCI-H460 human lung tumour xenograft following treatment with doxorubicin and sulindac.

Key: Vehicle control - full black square (■)
 Doxorubicin 0.5mg/kg - full black triangle (▲)
 Doxorubicin 0.75 mg/kg - open triangle (△)
 Sulindac 30 mg/kg - open rhombus grey line (◇)
 Doxorubicin 0.5mg/kg plus sulindac 30 mg/kg - full grey rhombus (◆)
 Doxorubicin 0.75 mg/kg plus sulindac 30 mg/kg - full black circle (●)

significant difference when compared to the vehicle-treated control group.

These results suggest that sulindac itself may have some impact on tumour growth resulting from the NCI-H460 cells. This activity was not anticipated from the *in vitro* data; anti-tumour effects of sulindac have, however, been observed in animal models and there are a number of potential explanations. Anti-tumour effects of sulindac have been observed with DMH-induced tumours in rats in a previous study where doses of 10 mg/kg reduced the rate of development and growth of colon tumours (22). Case reports have also shown that sulindac can cause regression of benign colonic adenomas in patients with Familial Adenomatous Polyposis (FAP) (23,24) and sulindac is being investigated clinically as a potential chemopreventative agent in such conditions (25).

Sulindac, its primary biological metabolites and certain analogues have been demonstrated to have pro-apoptotic actions (the majority of which are independent of cyclooxygenase inhibitory activity) (26-29). Several of these *in vitro* studies have used very high concentrations of sulindac or metabolites; the 30 mg/kg dose used here, however, represents approximately 80 times a conventional human dose which produces typical sustained circulating plasma levels of 10 μ M or more (15). The experimental conditions used may have brought the concentration of sulindac and metabolites to levels where these *in vitro* findings are relevant to the tumours in the mice.

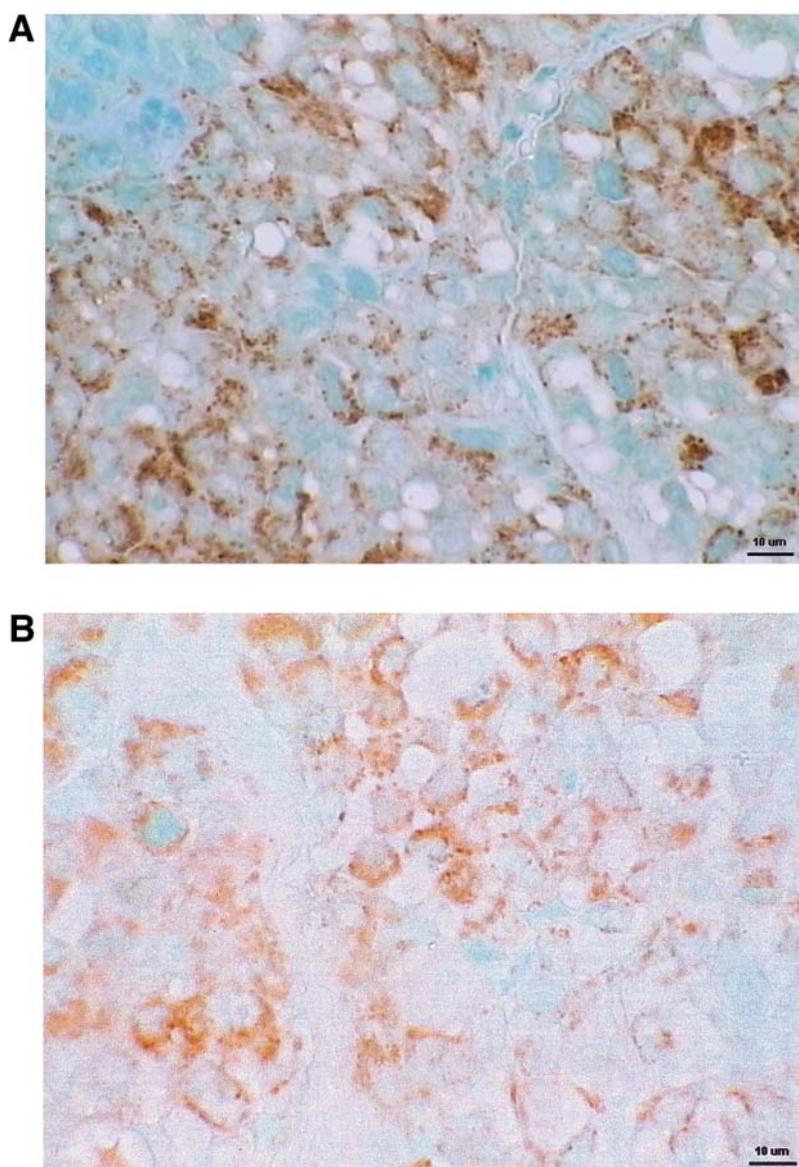


Figure 4. Immunohistochemical analysis of MRP-1 protein expression in NCI H460 tumour xenograft sections identified with the MRP-1-specific Mab, MRP-1 (brown). Original objective magnification 60X, scale bar = 10µm. (a) tumour xenograft from 0.75 mg/kg doxorubicin-treated mouse (b) tumour xenografts from 0.75mg/kg doxorubicin plus sulindac-treated mouse.

The second isoform of cyclooxygenase (COX-2) has a number of important roles in tumour biology (30) and may have particular importance in the angiogenic process (31). To this end, sulindac has also been tested in clinical trials where it has demonstrated the ability to inhibit the development of cancerous polyps of the colon of patients suffering the disease FAP, which predisposes sufferers to an early death from colon cancer (24,25). Sulindac and selected metabolites have been shown to possess anti-angiogenic properties in model systems at concentrations which should be achievable in models such as the one used in this paper (32,33). A strong anti-angiogenic effect might cause a delay in the rate of tumour growth.

In the experiment reported here, treatment with a combination of doxorubicin (0.5 or 0.75 mg/kg *i.p.*) and sulindac (30 mg/kg *i.p.*), co-administered daily from day 0 to 4 and 7 to 17, significantly reduced the mean tumour volume compared to the vehicle-treated control on days 10 ($p < 0.05$), 12 ($p < 0.05$) and 14 ($p < 0.01$). This suggests that sulindac is able to potentiate the anti-tumour activity of doxorubicin in this animal model. This is in agreement with our *in vitro* data but the observation of anti-tumour efficacy in the sulindac alone treatment group suggests that the inhibition seen in the combination group may be due to more than just MRP-1 inhibition. Since there is no information on the toxicological implications of using

sulindac in combination with doxorubicin, we also wanted to make maximum use of the animal data to see if there were any signs of unanticipated toxicity to the whole animal. Both gross pathological and histopathological examinations, carried out on animals from all treatment groups at the termination of the study, revealed no findings which could be directly attributed to any of the treatment regimes. Although pathological examination is not necessarily a sensitive measure of toxicity, it does suggest that there are no unexpected severe toxicological consequences likely to be associated with simultaneous use of sulindac and doxorubicin.

In summary, we have demonstrated the presence of MRP-1-associated drug resistance in NCI-H460 cells. This resistance mechanism could be blocked in these cells *in vitro* using sulindac, which led to a potentiation of the toxicity of doxorubicin without using toxic concentrations of sulindac. This potentiation was also evident when NCI-H460 cells were implanted into nude mice. However, examination of the data suggests that the potentiation associated with sulindac in this model is possibly through more than one mechanism; potentially inhibition of MRP-1-mediated doxorubicin resistance coupled with other activities such as anti-angiogenesis which has been described for sulindac. On the basis of the activity and lack of toxicity to non-cancerous tissue observed here, a phase I trial of this combination with a view to possible circumvention of MRP-1-mediated drug resistance appears to be warranted and is now underway.

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