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ORIGINAL ARTICLE

Liangli Zhao · Philip Kestell · Martin Philpott
Lai-Ming Ching · Li Zhuang · Bruce C. Baguley

Effects of the serotonin receptor antagonist cyproheptadine on the activity and pharmacokinetics of 5,6-dimethylxanthenone-4-acetic acid (DMXAA)

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Abstract *Background*: DMXAA (5,6-dimethylxanthenone-4-acetic acid) is a new drug synthesized in this laboratory and currently in phase I clinical trial. In mice it acts as an antivascular drug, selectively inhibiting tumour blood flow and inducing tumour haemorrhagic necrosis with resultant tumour regression. It also induces the synthesis of tumour necrosis factor (TNF), nitric oxide and serotonin. Cyproheptadine, a type 2 serotonin receptor antagonist, is known to reduce the degree of tumour necrosis-induced TNF in mice. We investigated the pharmacological interaction between a suboptimal dose of DMXAA (20 mg/kg) and cyproheptadine (20 mg/kg) using mice with Colon 38 tumours that are sensitive to DMXAA. *Methods*: Mice with or without tumours were treated with DMXAA and/or cyproheptadine. Concentrations of plasma and tissue DMXAA and the serotonin metabolite 5-hydroxyindoleacetic acid were measured by high performance liquid chromatography. TNF concentrations were measured by ELISA. *Results*: While DMXAA alone (20 mg/kg) showed little or no antitumour activity, coadministration with cyproheptadine was curative in four of five mice. DMXAA half-lives in plasma and tumour tissue were increased 5.1- and 5.6-fold, respectively, and the appearance of DMXAA glucuronides in bile was almost completely inhibited for up to 4 h. Serum TNF was low and unchanged by cyproheptadine, and plasma concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid were also not substantially changed. *Conclusion*: The augmentation by cyproheptadine of the induction of tumour response to DMXAA reflects a pharmacological interaction, leading to increased plasma and tumour half-lives, and to reduced excretion. However, serum TNF concentrations were not increased, suggesting that the increased antitumour effects are mediated by an increased local tumour response, arising from the extended tumour DMXAA concentrations.

Keywords Colon 38 · Bile · Glucuronide · Serotonin · TNF

L. Zhao · P. Kestell · M. Philpott · L.-M. Ching
L. Zhuang · B. C. Baguley (✉)
Auckland Cancer Society Research Centre,
Faculty of Medical and Health Sciences,
The University of Auckland, Private Bag 92019,
Auckland, New Zealand
E-mail: b.baguley@auckland.ac.nz
Tel.: +64-9-3737999
Fax: +64-9-3737502

Introduction

The tumour vasculature represents a current focus for new approaches to anticancer treatment. While most efforts are concerned with the inhibition of tumour angiogenesis [6], a number of studies have been directed towards the development of agents that selectively target the existing blood supply of solid tumour tissue. This approach had its beginnings in mixtures of bacterial endo- and exotoxins called ''Coley's toxins'', which were used in the early 1900s to induce tumour responses in cancer patients [31]. The antitumour effects of these toxins appears to be caused by the protein tumour necrosis factor (TNF) [8] and in experimental solid tumours is mediated by its selective vascular damage of tumour tissue [33]. Such work led to clinical trials of TNF in human cancer, and although host toxicity prevented general application, success was reported in isolated limb perfusion studies [21].

The demonstration in mice of the induction of tumour haemorrhagic necrosis and tumour regressions by flavone acetic acid (FAA) [30] and 5,6-dimethylxanthenone-4-acetic acid (DMXAA) [2, 28] raised the question of whether clinical low molecular weight antivascular agents might be developed. FAA lacked clinical activity [15] and DMXAA is currently undergoing phase I clinical trial [13, 29]. In mice, FAA and DMXAA selectively inhibit tumour blood flow [19, 37, 38], increase serum TNF [22, 25], and induce nitric oxide production [32]. DMXAA induces selective TNF production in situ in murine tumours and human tumour xenografts [14].

Serotonin (5-hydroxytryptamine), a vasoactive amine with multiple physiological functions including the induction of tumour necrosis [4, 23], appears to have a significant role in the action of antivascular agents. We have also previously found that FAA and DMXAA increase plasma concentrations of serotonin with maximal concentrations attained after approximately 4 h, which is subsequent to the maximal increase in plasma TNF [5]. Coadministration of the type 2 serotonin receptor antagonist cyproheptadine has been shown to inhibit tumour haemorrhagic necrosis induced by TNF in the Meth A fibrosarcoma [23] and the Colon 38 adenocarcinoma [4], suggesting a functional relationship between TNF and serotonin. Coadministration of cyproheptadine (20 mg/kg) has also been shown to delay tumour necrosis induced by maximal single doses of DMXAA and FAA, but its effect on the antitumour properties of DMXAA and FAA could not be measured because of increased toxicity [4].

In the study reported here we showed that coadministration of cyproheptadine unexpectedly potentiated the antitumour activity of a lower dose (20 mg/kg) of DMXAA. We investigated the pharmacological interaction of cyproheptadine and DMXAA, and demonstrated that cyproheptadine strongly inhibited the plasma clearance of DMXAA. We also examined the effects of the drug combination on the synthesis of 5-hydroxyindoleacetic acid (5HIAA) and TNF.

Materials and methods

Drugs and dosing

DMXAA was synthesized in this laboratory [28], formulated in sterile water, protected from light [27] and administered intraperitoneally (20 mg/kg; 10 μl/g body weight). Cyproheptadine and HIAA were obtained from Sigma (St. Louis, Mo.). Cyproheptadine was dissolved in 30% aqueous ethanol and administered intraperitoneally (20 mg/kg; 5 μl/g body weight).

Mouse studies

C57BL/6 and (C57BL/6×DBA/2)F_1 mice (20–30 g) were housed under constant temperature and humidity using sterile bedding, water and food. The Animal Ethics Committee of the University of Auckland approved all animal procedures. Colon 38 tumours were transferred subcutaneously to C57BL/6 mice by implantation of 1-mm^3 fragments under anaesthesia (pentobarbitone 90 mg/kg). In tumour histology experiments, female C57BL/6 mice with tumour volumes of approximately 250 mm^3 were treated with DMXAA (20 mg/kg), and the tumours removed after 24 h, fixed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The percentage necrosis was determined using a grid marked at 0.4-mm intervals as previously described [2]. In growth delay experiments, female DBA/2×C57BL/6 mice F_1 hybrid mice were treated 8 days after implantation (tumour volumes approximately 60 mm^3). Tumour diameters were measured with callipers and tumour volumes calculated as $0.52 \times a^2 \times b$, where a and b were the minor and major tumour axes [3].

Blood samples (200–300 μl) for pharmacokinetic studies (DMXAA and 5HIAA) were collected from male C57BL/6 mice in heparinized tubes during terminal halothane anaesthesia, centrifuged, and the plasma removed and stored in the dark at –20°C until analysis. After cervical dislocation, other tissues used for analysis were removed, homogenized in 10 m*M* ammonium acetate buffer (pH 5.5) and stored at –20°C until analysis.

TNF was measured in C57BL mice, which were treated with DMXAA with or without cyproheptadine. At the indicated times these were anaesthetized with halothane, and blood was removed by cardiac puncture, coagulated overnight in the dark in microcentrifuge tubes on ice, and centrifuged (15,000 *g*, 10 min). Serum was removed and assayed on ELISA plates (OptEIA murine TNF-α kit; PharMingen, San Diego, Calif.) with appropriate TNF standards (40–5000 pg/ml) according to the manufacturer's directions.

High performance liquid chromatography

DMXAA concentrations in plasma and in tissue homogenates were measured using automated solid-phase extraction and high-performance liquid chromatography (HPLC) in subdued light as previously described [17]. Plasma concentrations of 5HIAA were determined as follows. Working standards of 5HIAA (40, 30, 20, 10, 7.5, 5, 3, 2, 1.5, 1, 0.5 and 0.25 μ*M*) were prepared in 0.1% ascorbic acid and used to make plasma quality controls (50, 500 and 1500 n*M*), which were stored at –20°C in polyethylene tubes containing 0.1% ascorbic acid. These were stable for short periods at 4°C and for long periods at –20°C. Blood samples were centrifuged (1000 *g*, 10 min) and the plasma was stored in polyethylene tubes containing ascorbate (–20°C) until the time of assay. Plasma samples (50 μl) were mixed with 0.1 *M* HCl containing 0.01% ascorbic acid (2 ml) and transferred to 1 ml/100 mg C18 Bond Elut columns which had been conditioned with acetonitrile (1 ml) and Milli-Q water (1 ml). After washing with 1 ml Milli-Q water, 5HIAA was eluted with 800 μl acetonitrile/water (20:80, v/v) into 12×100 mm glass tubes. Aliquots (200 μl) of the resulting eluates were directly injected into the HPLC. Conditioning, loading, washing and final elution of the C18 columns were performed as described for DMXAA. Chromatography was performed using a Luna C18(2) 5 μm 75×4.6 mm column (Phenomenex, Torrance, Calif.) equilibrated overnight with mobile phase containing 0.14 *M* potassium phosphate buffer, pH 4.5, methanol (15%), acetonitrile (5%) and cetyltrimethylammonium bromide (0.004%), and a Waters WISP 712B automatic sample injector with a Model 510 pump (Waters Associates, Milford, Mass.) with a flow rate of 2 ml/min. 5HIAA was detected using a Model 400 electrochemical detector (EG & G, Princeton Applied Corporation, N.J.; sensitivity range 1 nA) with a glassy carbon working electrode, a NaCl/AgCl reference electrode and a working potential of +400 mV.

Pharmacokinetic analysis

The area under the concentration-time curve (AUC) of drug concentration as a function of time was calculated using the log trapezoidal rule over a period of up to either 6 h or 16 h. Half-life ($t_{1/2}$) was calculated as 0.693/elimination rate constant. C_{max} was the maximum concentration achieved. Statistical significance was assessed by Students *t*-test with $P < 0.05$ being considered as significant.

Results

Effects of cyproheptadine on tumour and host responses to DMXAA

Experiments were carried out in Colon 38-bearing mice using a suboptimal dose of DMXAA (20 mg/kg). DMXAA alone had little additional effect on the degree of tumour haemorrhagic necrosis at 24 h, but its combination with cyproheptadine (20 mg/kg) unexpectedly

caused a high degree of necrosis (Fig. 1). Cyproheptadine alone had no significant effect. The result was reflected in the in vivo tumour response: while DMXAA or cyproheptadine alone (20 mg/kg) had little effect, combination of the two agents provided cures in four out of five mice (Fig. 2).

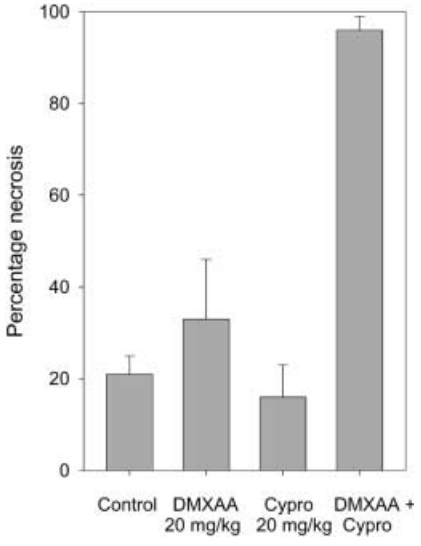

Fig. 1 Effect of DMXAA (20 mg/kg), cyproheptadine (20 mg/kg) or DMXAA plus cyproheptadine on the degree of haemorrhagic necrosis of subcutaneous Colon 38 tumours measured 24 h after treatment

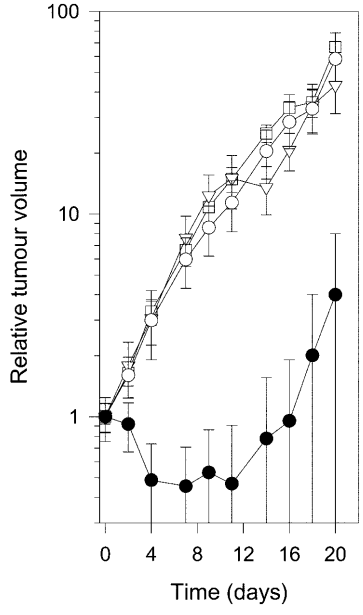

Fig. 2 Growth of subcutaneous Colon 38 tumours either with no treatment (❑) or following administration (day 0) of DMXAA (20 mg/kg) alone (○), cyproheptadine (20 mg/kg) alone (▽) or DMXAA plus cyproheptadine (●). Each point represents the arithmetic mean ± SEM from five mice. In the group treated with both drugs there were cures in four of five mice and the mean of all mice is calculated

We have previously shown that administration of DMXAA causes dose-dependent increases in the plasma concentrations of TNF [25], serotonin and the serotonin metabolite 5HIAA [5]. The effects of coadministration of cyproheptadine (20 mg/kg) on 5HIAA and TNF concentrations were measured at various times after administration of DMXAA (Fig. 3). Significant increases in 5HIAA were observed at early times after administration and maximal concentrations were observed after 4 h. Coadministration of cyproheptadine (20 mg/kg) extended the 5HIAA response slightly but did not affect its magnitude. In contrast, TNF, measured by ELISA, was below the minimum detectable concentration (40 pg/ml) following administration of DMXAA (20 mg/kg) with or without cyproheptadine. The analysis was extended to a DMXAA dose of 30 mg/kg which did induce TNF with a maximum 3 h after drug administration. Coadministration of cyproheptadine delayed the onset of TNF production (to 4 h) but did not affect its magnitude (Fig. 3). Since the mice were significantly hypothermic because of the toxicity of this combination, the delayed onset may have been due to a lowered body temperature.

Effect of cyproheptadine on the plasma and tissue pharmacokinetics of DMXAA

To determine whether a pharmacokinetic interaction was responsible for the potentiation of DMXAA anti-

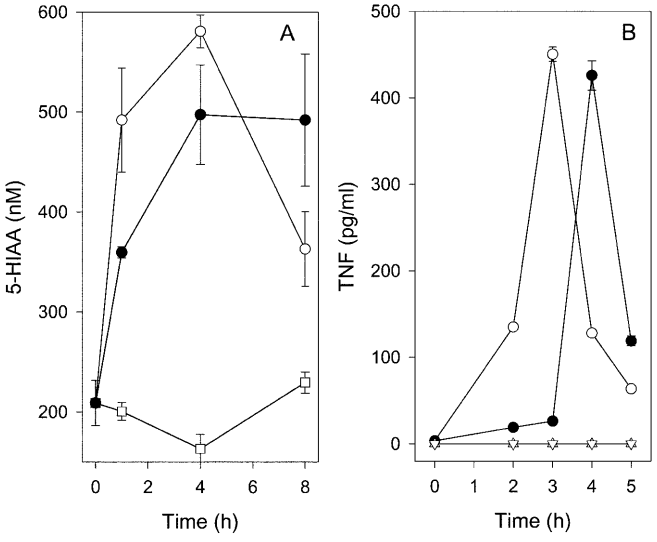

Fig. 3A, B Effect of coadministration of cyproheptadine (20 mg/kg) on 5HIAA and TNF production in non-tumour-bearing animals. **A** Mice were treated with DMXAA (20 mg/kg) alone (○), DMXAA plus cyproheptadine (20 mg/kg; ●) or solvent alone (❑) and plasma was prepared at the indicated times for 5HIAA analysis. **B** Mice were treated with DMXAA (20 mg/kg) alone (Δ), DMXAA (20 mg/kg) plus cyproheptadine (20 mg/kg; ▽), DMXAA (30 mg/kg) alone (○) or DMXAA (30 mg/kg) plus cyproheptadine (20 mg/kg; ●), and serum was prepared at the indicated times for TNF measurement. Each point represents the mean ± SEM from three mice

tumour activity by cyproheptadine, plasma, liver, spleen and Colon 38 tumour concentrations of DMXAA were measured up to 8 h after administration of DMXAA (20 mg/kg) with or without cyproheptadine (Fig. 4). Coadministration of cyproheptadine (20 mg/kg) caused no statistically significant change in C_{max} values but dramatically increased the drug half-life. The plasma half-life was increased 5.1-fold, with a corresponding 2.2-fold increase in plasma AUC, while the tumour tissue half-life was increased 5.6-fold with a corresponding 2.5-fold increase in AUC (Table 1).

Effect of cyproheptadine on the biliary excretion of DMXAA

DMXAA is metabolized in mice primarily to the glucuronide, which is excreted in bile [17]. To determine whether decreased excretion was responsible for the decreased clearance of DMXAA, mice were treated with DMXAA with or without cyproheptadine, and at various times later the mice were killed and the gall bladders removed. Homogenates were assayed before and after alkaline hydrolysis to provide estimates of unchanged DMXAA and DMXAA glucuronide. In tumour-bearing mice, coadministration of cyproheptadine reduced DMXAA glucuronide concentrations to less than 9% of that of mice treated with DMXAA alone (Table 2), but since gall bladders were completely empty between 4 and 8 h after administration, sampling was precluded. The experiments were therefore repeated in non-tumour-bearing mice. Coadministration of cyproheptadine reduced DMXAA glucuronide concentrations to very low levels between 1 and 4 h after administration. The concentration of unchanged DMXAA was initially higher after coadministration of cyproheptadine, but had decreased 2 and 4 h after administration (Fig. 5, Table 2).

Discussion

Coadministration of the type 2 serotonin receptor antagonist cyproheptadine (20 mg/kg) dramatically increased the degree of tumour necrosis at 24 h (Fig. 1) and tumour growth delay (Fig. 2) of a submaximal dose of DMXAA (20 mg/kg). This result extends a long list of agents, including thalidomide [9, 17], radiation and cytotoxic drugs [34, 35], serotonin [4, 19] and bioreductive drugs [10], that interact productively with DMXAA to increase its antitumour effect. Coadministration of cy-

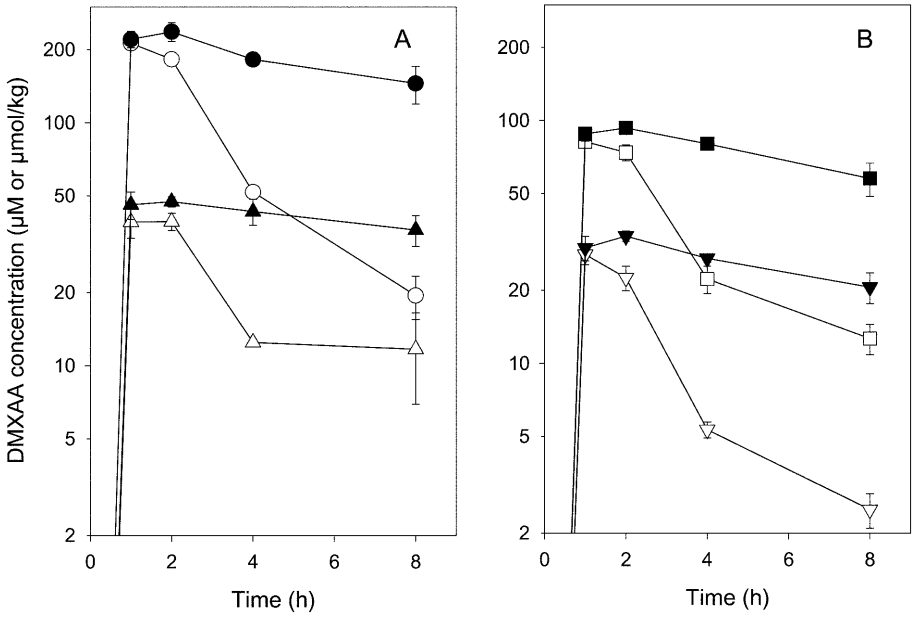

Fig. 4A, B DMXAA pharmacokinetics in plasma (●, ○) and tumour (▲, △) (**A**), and in liver (■, □) and spleen (▼, ▽) (**B**). Colon 38 tumour bearing mice were treated with DMXAA (20 mg/kg; *open symbols*) or DMXAA (20 mg/kg) plus cyproheptadine (20 mg/kg; *filled symbols*). Each point represents the mean ± SEM from three mice

Table 1 Plasma and tissue pharmacokinetic parameters in tumour-bearing mice following administration of DMXAA (20 mg/kg) with or without cyproheptadine (20 mg/kg) (*Cypro*)

Tissue	C_{max} (μmol/l or /kg)[a]		$t_{1/2}$ (h)		AUC (μmol·h/l or /kg)[b]		AUC ratio
	– Cypro	+ Cypro	– Cypro	+ Cypro	– Cypro	+ Cypro	
Plasma	213 ± 6.4	237 ± 21	1.9	9.7	642	1410	2.2
Liver	82 ± 2.6	93 ± 2.9	2.4	10	272	581	2.1
Spleen	28 ± 2.6	33 ± 1.6	1.9	11	78	201	2.6
Tumour	39 ± 3.2	47 ± 2.3	3.2	18	153	381	2.5

[a] Means ± SEM
[b] Determined over the range 0–8 h

proheptadine causes a substantial change in the plasma and tissue pharmacokinetics of DMXAA (Fig. 4, Table 1) and the 2.5-fold rise in AUC of tumour tissue provides a likely basis for its potentiation of the antitumour activity of DMXAA. Pharmacokinetic interactions are also involved in combinations of DMXAA with thalidomide [17], emphasizing the importance of pharmacokinetics in the analysis of the effects of drug combinations.

Coadministration of cyproheptadine, as well as strongly inhibiting the initial plasma and tissue clearance of DMXAA (Fig. 4), almost completely inhibited the biliary secretion of DMXAA glucuronides (Fig. 5). The mechanism of this effect is still unknown. Glucuronidation in the liver is the principal mechanism by which DMXAA is eliminated from the mouse [16, 24], suggesting three possible mechanisms: prevention of liver uptake, inhibition of DMXAA glucuronidation and inhibition of secretion of DMXAA glucuronides into bile. Access of drugs to the liver parenchymal cells requires passage through the fenestrated endothelium and the Space of Disse in the liver sinusoid, and the fenestrated endothelium can be altered by drug treatment [12, 36]. The in vitro effect of cyproheptadine on the glucuronidation of DMXAA by liver enzymes has been measured and found to be minimal [18]. Excretion of drug glucuronides is accomplished by transport proteins of the multiresistant protein (MRP) family [20]. Since cyproheptadine is active in overcoming the multidrug resistance phenotype of doxorubicin-resistant P388 leukaemia cells [26], another possible explanation is that it affects the activity of MRP proteins. The macroscopic (mottled, discoloured) appearance of livers from mice treated with cyproheptadine plus DMXAA, as opposed to mice treated with either drug alone (results not shown), is consistent with some type of hepatotoxic effect.

Coadministration of cyproheptadine, despite dramatically decreasing the plasma clearance of DMXAA (Fig. 4), had little effect on either plasma 5HIAA concentrations or serum TNF concentrations in response to DMXAA (Fig. 3). The ELISA used has somewhat lower sensitivity than the bioassay used previously [25] but is more specific, and showed no significant increase in serum TNF in response to DMXAA at this dose. Although surprising, the lack of dependence of TNF production on the AUC of DMXAA is consistent with in vitro studies indicating that it is the maximal concentration of DMXAA, rather than the duration of exposure, that determines the TNF response (M. Philpott, unpublished observation). The question of whether the production of 5HIAA is dependent on the C_{max} or the AUC of DMXAA has not been resolved, but the data in Fig. 3 suggest that the situation may be similar to that with TNF.

Many studies have linked the elevation of serum TNF to its antitumour effects [1]. The present results demonstrate that the induction of Colon 38 tumour necrosis and cures in mice occur in the absence of a detectable increase in serum TNF. This unexpected result is consistent with two previous observations. The first is that coadministration of thalidomide, while suppressing serum TNF concentrations augments the antitumour activity and in doing so increases the amount of tumour tissue-associated TNF [7]. The second is that DMXAA

Table 2 Effect of coadministration of cyproheptadine (20 mg/kg) (*cypro*) on biliary concentrations of DMXAA and DMXAA glucuronide 2 h after administration of DMXAA (20 mg/kg)

	DMXAA	DMXAA + cypro
Tumour-bearing mice ($n=3$)		
DMXAA (μM)	126 ± 23	154 ± 39
DMXAA glucuronide (μM)	350 ± 102	30 ± 17*
Non-tumour-bearing mice ($n=3$)		
DMXAA (μM)	355 ± 100	200 ± 13
DMXAA glucuronide (μM)	691 ± 189	9.5 ± 2.7*

*$P < 0.05$

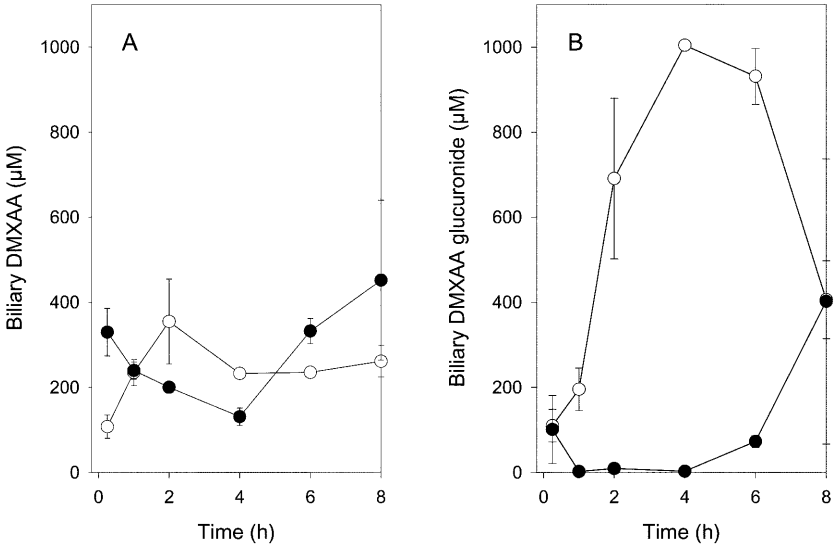

Fig. 5A, B Effect of coadministration of cyproheptadine (20 mg/kg) on biliary concentrations of DMXAA (**A**) and DMXAA glucuronide (**B**). Non-tumour-bearing mice were treated with DMXAA alone (20 mg/kg; ○) or DMXAA plus cyproheptadine (20 mg/kg; ●). Each point represents the mean ± SEM from three mice

(30 mg/kg) induces haemorrhagic necrosis of Colon 38 tumours in TNF knockout mice, and although serum TNF was absent, TNF in the tumour, derived from the tumour cells themselves, was present. Taken together, these results suggest that elevation of tumour tissue TNF, rather than serum TNF, is necessary for the induction of Colon 38 tumour necrosis. We are currently investigating whether DMXAA-cyproheptadine combinations modulate tumour concentrations of TNF.

Paradoxically, the antitumour activity of DMXAA against Colon 38 tumours is augmented both by serotonin [4, 19] and by the serotonin receptor antagonist cyproheptadine (Fig. 2). The mechanisms of these two effects may be different, and with different kinetics. Administration of a high dose of DMXAA (30 mg/kg) induces high levels of serum TNF [25]. Coadministration of cyproheptadine prevents the induction of both tumour necrosis and nitric oxide synthesis by administered TNF [4, 23]. Serotonin is known to bind serotonin receptors (mainly type 2) on the vascular endothelium and thereby increase vascular permeability to macromolecules [11], thus facilitating the extravasation of proteins into tissues. The concentration of serotonin (0.5 μM) in mouse plasma [5] is likely to be sufficient to increase vascular permeability such that administration of cyproheptadine, by preventing binding of serotonin to endothelial cell receptors, will decrease vascular permeability to macromolecules. Thus, coadministration of cyproheptadine may prevent the extravasation of TNF into tumour tissue (to induce necrosis) and liver tissue (to induce nitric oxide synthase). Conversely, reduction of vascular permeability by cyproheptadine might also potentiate the effects of local production of cytokines within tumour tissue by preventing their release into the circulation.

In conclusion, the results described here suggest that DMXAA might induce tumour necrosis in two ways, firstly by stimulating synthesis of circulating TNF that subsequently extravasates to tumour tissue [1], and secondly by a local tumour reaction, probably involving in situ TNF production [14]. There are potentially important implications of these results for the clinical trial of DMXAA. Firstly, significant rises in serum TNF may not be necessary for an antitumour effect, since under the appropriate conditions local reactions of tumour tissue may be sufficient to induce antitumour activity. It will thus be important to measure tissue concentrations of TNF or other cytokines as markers of a response to DMXAA. Secondly, if the main action of cyproheptadine is to extend the half-life of DMXAA, then administration of DMXAA by infusion, to mimic the pharmacokinetics demonstrated in Fig. 4, should be considered. We are currently extending our studies in mice to investigate these possibilities.

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