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ORIGINAL ARTICLE

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Oral activity and pharmacokinetics of 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) in mice

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Abstract *Purpose*: 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), an anticancer drug with an antivascular action, has recently completed phase I clinical trials. Since oral administration has many advantages, we compared the biological activity and pharmacokinetics of DMXAA in mice following oral and intraperitoneal (i.p.) administration. *Methods*: Growth delays of Colon 38 tumours were measured in C57Bl/6 mice. Plasma concentrations of DMXAA, 5-hydroxyindole-3-acetic acid (5HIAA) as a measure of serotonin production, and nitrate as a measure of nitric oxide production, were determined by high-performance liquid chromatography. Tumour necrosis factor (TNF) concentrations in serum and tumour tissues were measured by ELISA. *Results*: The antitumour activity of DMXAA at the maximum tolerated oral dose (32.5 mg/kg) was low (4-day growth delay, no cures) compared to that (19-day growth delay, 40% cures) at the maximum tolerated i.p. dose (27.5 mg/kg). The pharmacokinetics of DMXAA in plasma, liver and tumour tissue indicated a bioavailability of 73%. Elevation of plasma 5HIAA, measured 4 h following i.p. administration of DMXAA, was linear with DMXAA dose, and the 5HIAA response to oral administration was consistent with its bioavailability. TNF concentrations increased following oral administration (30 mg/kg) and were particularly evident in tumour tissue, but were lower and less prolonged than those in response to i.p. administration at 25 mg/kg. Plasma nitrate levels were not increased following oral administration (30 mg/kg). *Conclusions*: DMXAA exhibits good bioavailability, and changes in serum TNF, tissue TNF, plasma 5HIAA and plasma nitrate, as markers of biological response, are consistent with this bioavailability. The low maximal plasma DMXAA concentration following oral administration, resulting in reduced retention of intratumoral TNF, may be responsible for the low antitumour activity.

Keywords Antivascular · Tumour necrosis factor · Serotonin · Nitric oxide

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Introduction

The anticancer agent, 5,6-dimethylxanthenone-4-acetic acid (DMXAA) developed in this laboratory has exceptional activity against transplantable murine tumours [22]. Its mechanism of action in mice is complex, involving the cessation of tumour blood flow and consequent induction of tumour haemorrhagic necrosis [28, 29]. DMXAA increases the plasma concentrations of tumour necrosis factor (TNF) [6, 18], nitric oxide [24] and serotonin [2], and each of these may be involved in some way in mediating its antivascular effect. DMXAA has now completed phase I clinical trials and shows evidence of the ability to both decrease tumour blood flow and increase the plasma concentrations of the serotonin metabolite 5-hydroxyindole-3-acetic acid (5HIAA) [10, 23].

A number of preclinical studies have demonstrated the utility of combining DMXAA with other agents, including thalidomide [5], radiation [26], bioreductive agents [8], radioimmunotherapy [17], cytotoxic drugs [20, 25] and gene therapy [12]. The possibility that future clinical trials of DMXAA will utilize combination schedules raises the question as to whether it might be administered by an oral route, which provides a number of advantages [9]. Preclinical pharmacokinetic and other studies of DMXAA have been carried out using intraperitoneal (i.p.) and intravenous (i.v.) administration [13, 16] and phase I trials have utilized a 20-min i.v. infusion. In the study reported here, we investigated the biological activity, pharmacokinetics and bioavailability of orally administered DMXAA, using the Colon 38 tumour model in mice. As well as antitumour effects, we

measured plasma and tissue concentrations of TNF, as well as plasma concentrations of 5HIAA and nitrate as measures of serotonin and nitric oxide, respectively.

Materials and methods

Drug administration

All procedures were carried out under institutionally approved guidelines. C57Bl/6 female mice (18–22 g) obtained from the Animal Resources Unit, Auckland University, were used for all experiments and housed under constant temperature and humidity. DMXAA was synthesized in this laboratory [22], dissolved in sterile water and protected from light [21]. I.p. administration utilized a volume of 10 μl/g body weight, while oral administration utilized a gavage tube and a volume of 5 μl/g body weight. For consistency, mice used for both i.p. and oral administration were fasted for 16 h prior to, and 4–6 h after, drug administration.

Tumour growth measurements

The Colon 38 tumour was implanted by transferring 1 mm^3 fragments of the tumour subcutaneously into anaesthetized (pentobarbitone, 87 mg/kg) mice. For tumour growth inhibition determinations, mice were treated with drug 8 days after implantation when the tumour volumes were approximately 60 mm^3. Tumour size was measured thrice weekly using callipers and the volumes calculated as $0.52 \times a^2 \times b$, where a and b are the minor and major tumour axes. The arithmetic means and standard error of the means were calculated for each time-point, counting cured animals as having zero tumour volume, and expressed as fractions of the pretreatment tumour volume. Growth delay was determined as the difference in the number of days required for the untreated and treated tumours to reach four times the pretreatment volume.

DMXAA pharmacokinetic studies

Mice (three per group) were treated i.p. with DMXAA (25 mg/kg) or orally with DMXAA (30 mg/kg). After 0.25, 1.5, 3, 4.5 and 6 h, blood samples (700–800 μl) were collected from the ocular sinus into heparinized tubes during halothane anaesthesia, centrifuged, and the plasma was removed and stored at –20ºC until analysis. In another experiment, tumour-bearing mice (three to six per group) were treated i.p. with DMXAA (25 mg/kg) or orally with DMXAA (30 mg/kg) and blood samples were taken after 0.25, 1, 2, 4, 8, 12 and 24 h. DMXAA plasma concentrations were measured using automated solid-phase extraction and high-performance liquid chromatography (HPLC) as previously described [14]. To measure free drug concentrations, samples of mouse plasma (300 μl) were added to a Sartorius Centrisart filters (MW cut-off 20,000 Da), and immediately centrifuged at room temperature (2800 *g*, 30 min). The ultrafiltrates (in duplicate) were injected directly (15 or 30 μl, depending on the drug concentration) onto the HPLC column.

TNF assay

Serum prepared from blood clotted overnight at 4ºC was used for determinations of TNF levels in the circulation. Tumour and liver tissues were homogenized in 1.5 ml α-MEM medium using a tissue homogenizer. The homogenates were centrifuged at 2000 *g* for 30 min at 4ºC and the supernatant was removed and recentrifuged (14,000 *g*, 30 min at 4ºC). Serum and supernatants from tissue homogenates were kept at –70ºC until use. TNF was assayed using a commercially available ELISA kit (OptEIA Mouse TNF kit; PharMingen, San Diego, Calif.) according to the manufacturer's directions. Briefly, diluent and a sample of the serum (100 μl) were added to a test well of 96-well flat-bottomed microwell plates coated with immobilized monoclonal antibody to mouse TNF. After allowing any TNF present in the sample to react with the antibody, the wells were washed and then biotinylated polyclonal antibody to TNF added, followed by peroxidase-labelled streptavidin. Substrate (1,2-phenylenediamine dihydrochloride) was added and the reaction stopped with acid and the colour intensity determined at 450 nm.

5HIAA assay

Plasma (50 μl) pooled from three animals per group 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 50% acetonitrile/Milli-Q water. 5HIAA was eluted with 800 μl acetonitrile/water (80:20 v/v) and aliquots (200 μl) of the resulting eluates were injected onto the HPLC column. Chromatography was performed using a Luna C18(2) column (Phenomenex, Torrence, 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%). 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.

Nitrate assay

Plasma (200 μl) was diluted with Milli Q water (1:1 or 1:5 v/v), transferred into a Micron Centrisart micropartition device (20,000 Da), and centrifuged (30 min, 2000 *g*). Separation of nitrate in the ultrafiltrates was achieved by ion-exchange chromatography using a Dionex IonPac AS4A-SC column fitted with an ion exchange guard column packed with the same material. The eluent, comprising 32 m*M* boric acid and 32 m*M* sodium hydrotetraborate, was pumped at a flow rate of 1.0 ml/min. Absorbance at 214 nm using a UV-VIS Detector Pro Star 320 was measured and data were collected and processed using Unicam 4880 software.

Pharmacokinetic and statistical analysis

The area under the concentration-time curve (AUC) of drug concentration was calculated as a function of time using the log trapezoidal rule. The elimination rate constant (K_{el}) was obtained by fitting all of the points on the concentration-time profile to an exponential function, and the elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$. C_{max} was the maximum concentration measured. Statistical significance was assessed by Student's *t*-test, with $P < 0.05$ being considered as significant.

Results

Antitumour activity of DMXAA following oral and i.p. administration

The maximum tolerated dose (MTD) for oral administration was 32.5 mg/kg and the optimal dose was defined as 30 mg/kg. Similarly, the MTD for i.p. administration was 27.5 mg/kg and the optimal dose was defined as 25 mg/kg. We first compared growth inhibition of Colon 38 tumours in C57Bl/6 mice. Oral administration of DMXAA (30 and 32.5 mg/kg) induced

tumour growth delays of 4 and 3 days, respectively, with no cures (Fig. 1). In contrast, DMXAA administered i.p. at 25 or 27.5 mg/kg provided mean tumour growth delays of 13 and 19 days, respectively, and cure rates of 40% in each group (Fig. 1).

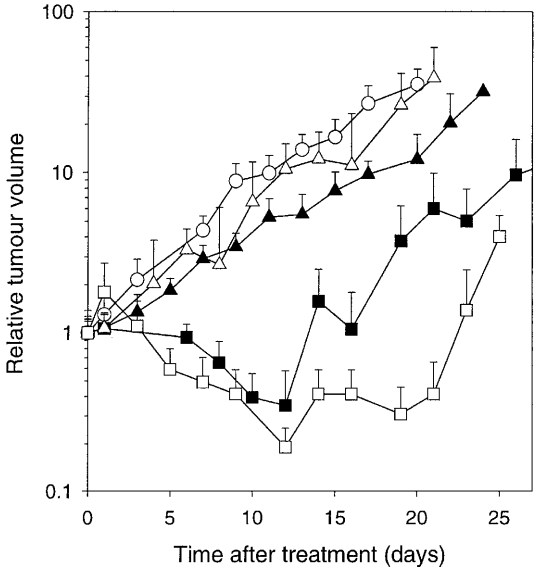


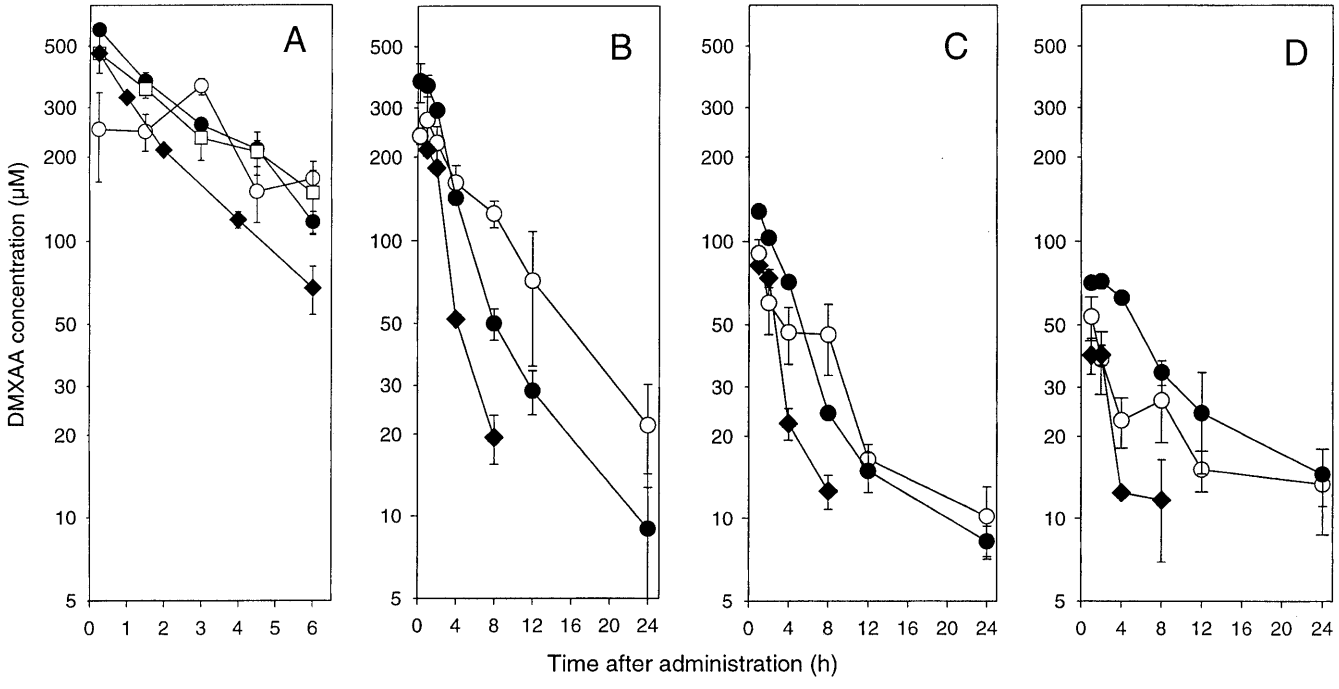
Fig. 1 Growth of subcutaneous Colon 38 tumours with no treatment (*open circles*) or following administration of DMXAA 30 mg/kg orally (*closed triangles*), 32.5 mg/kg orally (*open triangles*), 25 mg/kg i.p. (*closed squares*) or 27.5 mg/kg i.p. (*open squares*). Each point represents the arithmetic mean ± SEM of five tumours

Plasma and tissue pharmacokinetics

In order to investigate the basis for the low antitumour response following oral administration of DMXAA, the plasma pharmacokinetics of DMXAA were compared following administration i.p. (20 or 25 mg/kg), i.v. (25 mg/kg) or orally (30 mg/kg) in non-tumour-bearing mice. C_{max} values (at 15 min), elimination half-lives ($t_{1/2}$) and AUC_{0-6} values were measured and found to be similar following i.p. or i.v. administration (25 mg/kg) (Fig. 2, Table 1). Following oral administration (30 mg/kg) the C_{max} occurred at a later time (3 h) and was 24% lower than with i.v. administration (25 mg/kg). The $t_{1/2}$ was also 2.8-fold longer and the AUC_{0-6} 12% was lower than that following i.v. administration, and on this basis the oral bioavailability was calculated to be 73%.

DMXAA was also administered i.p. (20 and 25 mg/kg) and orally (30 mg/kg) to Colon 38 tumour-bearing mice (Table 1). Again, oral administration produced a lower plasma C_{max}, which occurred at a later time (1 h) (Fig. 2, Table 1). The $t_{1/2}$ was 2.3-fold longer following oral administration (30 mg/kg) than following i.p. administration (25 mg/kg), and the AUC_{0-24} was 11% higher, reflecting the lower rate of elimination. Notably, the C_{max} value following DMXAA administration (25 mg/kg i.p.) was significantly lower ($P < 0.001$) in tumour-bearing animals than in non-tumour-bearing

Fig. 2A–D DMXAA concentration-time profiles in mice following DMXAA administration at doses of 25 mg/kg i.v. (*open squares*), 25 mg/kg i.p. (*closed circles*), 30 mg/kg orally (*open circles*) or 20 mg/kg i.p. (*closed diamonds*). Each point represents the arithmetic mean ± SEM of three animals. **A** Plasma from non-tumour-bearing mice. **B** Plasma from Colon 38 tumour-bearing mice. **C** Liver tissue from Colon 38 tumour-bearing mice. **D** Tumour tissue



animals (Fig. 2, Table 1). We therefore measured free drug C_{max} values and found them to be 8.06 ± 0.13 and 7.52 ± 0.36 μmol/l, respectively. The free drug fraction in non-tumour-bearing mice (1.3%) was therefore significantly lower ($P < 0.05$) than that in tumour-bearing mice (2.2%).

We also compared liver and tumour tissue pharmacokinetics following oral or i.p. administration of DMXAA. Oral administration gave lower concentrations of DMXAA in tumour tissue at all times. The rate of clearance from the tumour following i.p. or oral administration was similar, but the C_{max} (54 μmol/l) and AUC (484 μmol·h/l) after oral administration was lower than the C_{max} (72 μmol/l) and AUC (772 μmol·h/l) determined after i.p. administration (Fig. 2, Table 1).

5HIAA response

The time-dependence of plasma 5HIAA was compared for i.p (25 mg/kg) and oral (30 mg/kg) administration. Plasma 5HIAA rose steadily up to 24 h following i.p. administration of DMXAA, but following oral dosing there was an increase to 4 h and a decline at later times (Fig. 3A). The dependence of the plasma 5HIAA concentrations on dose was also measured 4 h after i.p.

Table 1 Pharmacokinetic parameters with different routes of DMXAA administration

Sample	Dose (mg/kg)	Route	C_{max} (μmol/l)	$t_{1/2}$ (h)		AUC (μmol·h/l)	
				0–6 h	0–24 h	0–6 h	0–24 h
Non-tumour-bearing mice ($n = 3$)							
Plasma	30	Oral	358 ± 25	9.2	–	1392	–
	20	i.p.	413 ± 20	2.1	–	1095	–
	25	i.p.	570 ± 21	2.7	–	1715	–
	25	i.v.	470 ± 12	3.3	–	1595	–
Tumour-bearing mice ($n = 3$–5)							
Plasma	30	Oral	271 ± 70	6.3	5.5	1120	1977
	20	i.p.	213 ± 6.4	1.6	–	590	–
	25	i.p.	374 ± 61	2.8	3.8	1302	1777
Liver	30	Oral	90 ± 11	5.9	7.4	319	684
	20	i.p.	82 ± 2.6	1.9	–	244	–
	25	i.p.	128 ± 1.3	3.5	5.7	468	737
Tumour	30	Oral	54 ± 9.6	3.4	11.9	177	484
	20	i.p.	39 ± 5.7	2.1	–	128	–
	25	i.p.	72 ± 1.4	4.3	9.1	321	772

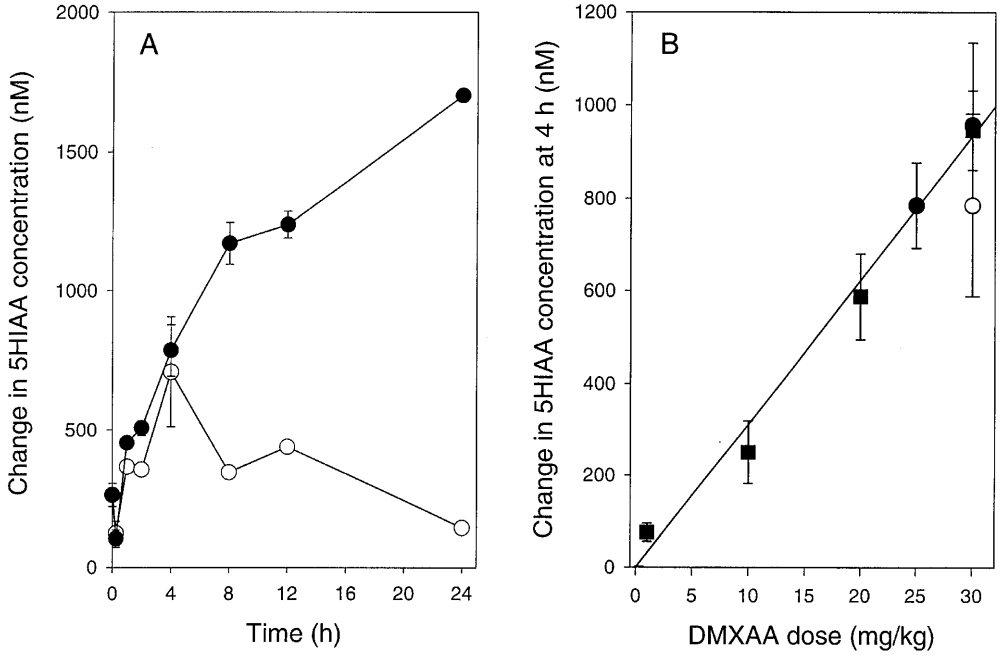


Fig. 3 **A** Plasma 5HIAA concentrations in Colon 38 tumour-bearing mice after administration of DMXAA at a dose of 25 mg/kg i.p. (*closed circles*) or 30 mg/kg orally (*open circles*). **B** Plasma 5HIAA concentrations in non-tumour-bearing mice (*closed squares*) and Colon 38 tumour-bearing mice (*closed circles*) 4 h after treatment with DMXAA either i.p. (*closed squares*, *closed circles*) or orally (*open circles*). Points represent arithmetic means ± SEM of at least three animals

administration of DMXAA to non-tumour-bearing or Colon 38-bearing mice. A linear dependence was observed, with little difference between non-tumour-bearing and tumour-bearing mice (Fig. 3B).

TNF response

TNF concentrations were measured in serum, liver and tumour tissue at different times after oral (30 mg/kg) and i.p. (20 and 25 mg/kg) administration of DMXAA. No significant increases in TNF concentration were observed in liver tissue for any of the measurement time-points. Changes in serum concentrations were maximal 4 h after i.p. administration, but because of variations between individual mice the increases were not statistically significant for any time-point (Fig. 4). Significant increases in TNF were observed for both oral and i.p. administration in Colon 38 tumour tissue (Fig. 4), and the increases were maximal 4 h after i.p. administration and 2 h after oral administration. The maximal response following 30 mg/kg orally was comparable with that following 20 mg/kg i.p. (Fig. 4).

Plasma nitrate response

Plasma nitrate levels increased up to 24 h in tumour-bearing mice following administration of DMXAA both i.p. (25 mg/kg) and orally (30 mg/kg) Fig. 5. The effect was significant in both cases but was ninefold higher following i.p. administration.

Discussion

We showed that DMXAA had only a small antitumour effect when administered at its single oral MTD (32.5 mg/kg; Fig. 1). In order to determine the reason for the low activity, we examined the plasma pharmacokinetics of DMXAA following oral, i.p. and i.v. dosing (Fig. 2). The calculated oral bioavailability based on AUC (0–6 h) was 73%. Although the MTD for oral and i.p. administration (25 and 32.5 mg/kg, respectively) was compatible with this bioavailability, the antitumour activity was not. An oral dose of 32.5 mg/kg should provide equivalent antitumour activity to an i.p. dose of 24 mg/kg, but since an i.p. dose of 25 mg/kg cured 40% of mice, factors other than the plasma AUC must have limited the antitumour activity of orally administered DMXAA.

Following treatment of mice with DMXAA, changes occurred in plasma 5HIAA, serum TNF and plasma nitrate, suggesting that these might have utility as

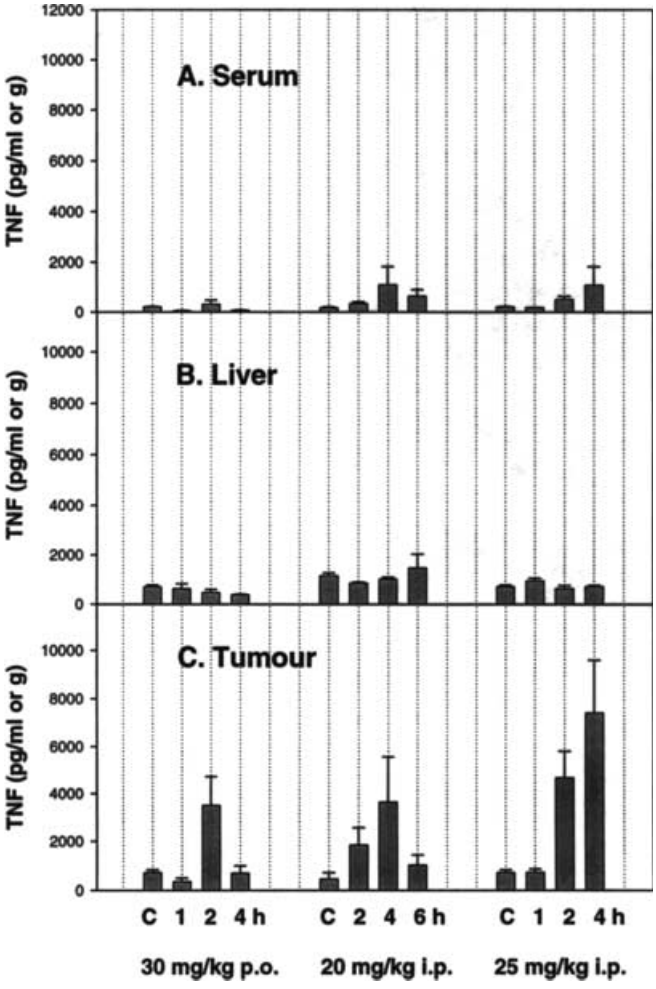


Fig. 4A–C TNF in serum (**A**), liver (**B**) and tumour (**C**) in Colon 38 tumour-bearing mice after treatment with DMXAA either orally (30 mg/kg) or i.p. (20 or 25 mg/kg). Results are expressed as picograms TNF per millilitre serum or per gram tissue. Mean ± SEM of three mice per group

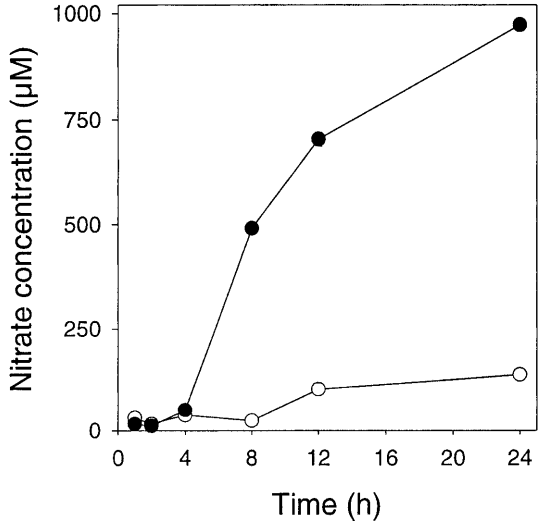


Fig. 5 Plasma nitrate concentrations in Colon 38 tumour-bearing mice after administration of DMXAA either orally at 30 mg/kg (*open circles*) or i.p. at 25 mg/kg (*closed circles*). Each point represents the mean of duplicate determinations of plasma pooled from three animals

surrogate markers of response. The 5HIAA response appears to reflect the release of serotonin from platelets into plasma as a consequence of vascular effects [1, 2], and has been observed in clinical trials of DMXAA [23]. 5HIAA plasma concentrations in mice, measured 4 h after i.p. administration, were almost linearly related to dose (Fig. 3B), providing a means of comparing the biological response of i.p. and orally administered DMXAA. The response to an oral dose of 30 mg/kg at 4 h resembled that of an i.p. dose of 24 mg/kg, comparable to that expected on the basis of its bioavailability. However, the 5HIAA response following oral dosing was not sustained in the same way as that found for i.p. dosing (Fig. 3A). The serum TNF responses reported here, determined using an ELISA assay, were low (Fig. 4), and the responses to oral DMXAA (30 mg/kg) and i.p. DMXAA (20 and 25 mg/kg) were not significantly different from each other. This reflects the small number of animals used and the degree of variability of TNF production among individual animals. The plasma nitrate response, because of its steep dose-response relationship, could also not be used as a sensitive gauge of the biological response. Nevertheless, it is clear that plasma concentrations of TNF and nitrate rose in a dose-dependent fashion in response to DMXAA, confirming previous data [18, 24].

A number of studies suggest that a critical determinant of the antitumour response is the concentration and persistence of TNF in tumour tissue. Endotoxin caused almost no growth delay of Colon 38 tumours, and while it caused a large increase in tumour TNF, this was not sustained after 2 h. In contrast, DMXAA (25 mg/kg), as well as having a strong antitumour effect, caused a sustained increase in tumour TNF for over 4 h [3]. Coadministration of thalidomide with DMXAA (25 mg/kg), while decreasing plasma TNF concentrations, increases the concentration and persistence of tumour TNF [3], concomitantly increasing activity against Colon 38 tumours [4]. Coadministration of the serotonin type-2 receptor antagonist cyproheptadine with DMXAA (20 mg/kg) also increases the concentration and persistence of tumour TNF (L. Zhao, unpublished data) and increases antitumour activity [27]. Thus, the data in Fig. 4 suggest that oral administration of DMXAA increased tumour TNF but this increase was not sustained for as long as that observed following i.p. administration. One possible reason for this is an alteration in DMXAA pharmacokinetics in tumour tissue.

The main difference in the plasma pharmacokinetics in tumour-bearing mice treated with DMXAA either orally (30 mg/kg) or i.p. (25 mg/kg) was not in AUC but in C_{max} (Table 1). Initial drug concentrations were much lower than those observed following i.p. administration, probably because of slow uptake from the gut. This leads to reduction of both C_{max} and AUC in tumour tissue, providing a potential explanation for the lowered antitumour activity. DMXAA causes a selective, dose-dependent inhibition of tumour blood flow [15, 28] and since this occurs before the detection of any changes in serum or tumour TNF, it is likely to be caused by a direct effect on tumour vasculature [1]. The induced changes in tumour blood flow can trap drugs or proteins within the tumour tissue, as demonstrated for a radioimmunotherapeutic agent [17] and the drug melphalan [20]. TNF is synthesized by both host and tumour cells within Colon 38 tumour tissue [6, 11] and, by an analogous argument, can be trapped in the tissue by reduced blood flow. The reduced initial plasma concentrations of DMXAA following oral administration might therefore result in a smaller direct effect on tumour blood flow, allowing TNF synthesized in the tumour to be released into the circulation rather than to be trapped within the tumour.

It is of interest that the maximal plasma concentrations of DMXAA in tumour-bearing mice were on average 64% of those in non-tumour-bearing mice (Table 1). For the two i.p. doses used, this was statistically significant ($P < 0.05$). The free drug C_{max} concentrations, however, were similar, indicating that the differences reflect changes in protein binding. DMXAA is highly protein-bound in mice [16] and assuming a plasma volume of 6.3% of body weight, approximately 43% of the administered dose in non-tumour-bearing mice, and 32% of the administered dose in tumour-bearing mice, is bound to plasma proteins.

In conclusion, the results raise the issue of the relative importance of C_{max} and AUC in determining antitumour efficacy. DMXAA appears to act on target cells to activate the NF-κB transcription factor [7, 19], but such activation is transient and may require drug to be present only over a short time period. Preliminary experiments in this laboratory, as well as previously published data [18], indicate that repeated dosing of DMXAA over a 24-h time period is not superior to administration of a single dose and therefore that the antitumour effect of DMXAA is not a direct function of AUC. The relationships among C_{max}, AUC and antitumour effect have considerable relevance to further clinical studies and require further investigation.

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