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ORIGINAL ARTICLE

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Modulation of the pharmacokinetics of the antitumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in mice by thalidomide

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Abstract *Background*: 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), an investigative drug currently in clinical trial, acts on tumour vasculature through the induction of cytokines. Coadministration of thalidomide, a modulator of cytokine production, potentiates the antitumour activity of DMXAA against the murine Colon 38 carcinoma in mice. We wished to determine whether alteration of the pharmacokinetics of DMXAA by thalidomide could provide an explanation for this potentiation. *Results*: Coadministration of thalidomide to Colon 38 tumour-bearing mice significantly ($P < 0.05$) increased the elimination half-life ($t_{1/2}$) of DMXAA in plasma (413 μmol/l), liver (132 μmol/l), and spleen (77 μmol/l), and significantly ($P < 0.05$) increased DMXAA concentrations in Colon 38 tumour tissue (0.25–4.5 h). L-Thalidomide had a greater effect on DMXAA elimination ($P < 0.01$) than did D-thalidomide or the racemate. Coadministration of thalidomide increased the area under the concentration-time curve (AUC) of DMXAA by 1.8-fold in plasma, liver and spleen, and by 3.0-fold in tumour. Bile from mice given thalidomide and DMXAA contained substantially lower amounts of the glucuronide metabolite of DMXAA (DMXAA-G) than did bile from mice given DMXAA alone. *Conclusion*: Glucuronidation is a major excretory pathway for DMXAA in the mouse. Thalidomide, probably as the L-form, decreases the rate of elimination of DMXAA from plasma, spleen, liver and tumour by altering the rate of glucuronidation. The reduction in the elimination of DMXAA by thalidomide may lead to a selective increase in exposure of tumour tissue to drug, providing a basis for its potentiation of antitumour activity.

Key words HPLC · Colon 38 · Tumor · Bile · Glucuronide

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Introduction

Thalidomide (α-(*N*-phthalimido)glutarimide, Fig. 1), best known for its sedative and teratogenic properties, has a diversity of activities including modulation of cytokine production [31], host immunity [14, 17] and inhibition of angiogenesis [10]. It is undergoing clinical trial as an angiogenesis antagonist for cancer therapy [26] and is also being investigated for use in a number of diseases in which autoimmunity is a factor, such as leprosy [35], rheumatoid arthritis [16], Crohn's disease [34, 44], Behçet's disease [15], graft-versus-host disease [41, 42], pruritus [36], sarcoidosis [3] and multiple myeloma [37]. The inhibition of tumour necrosis factor-α (TNF) [25] and other immune-modulating cytokines [23] by thalidomide is thought to mediate these immunosuppressive and antiinflammatory effects.

We have previously demonstrated that a further effect of thalidomide is to potentiate the activity of the experimental antitumour agent, 5,6-dimethylxanthenone-4-acetic acid (DMXAA, Fig. 1). DMXAA [30] has demonstrated excellent preclinical activity against transplantable murine tumours with an established vasculature [1] and coadministration of thalidomide with DMXAA increases cure rates against Colon 38 tumours in mice [8]. The mechanism of action of DMXAA, which is currently in phase I trial, is complex and involves cytokine induction [28], vascular shutdown [21], modulation of host immunity [7] and inhibition of

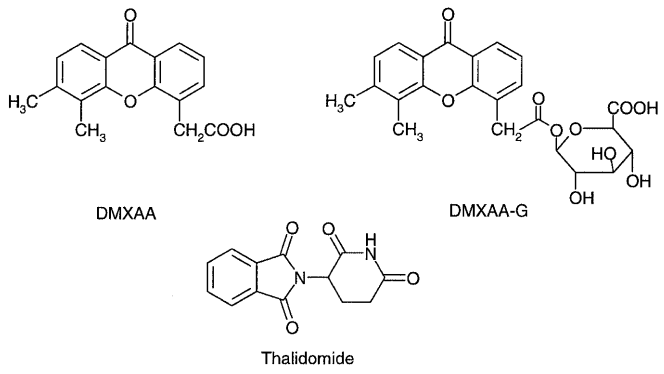

Fig. 1 Chemical structures of DMXAA, thalidomide and DMXAA-G

angiogenesis (Z. Cao and L.-M. Ching, unpublished results).

DMXAA induces systemic [28] and intratumoral TNF, activating both host and some tumour cells in the tumour tissue to synthesize TNF [6, 18]. Thalidomide decreases the induced serum and hepatic TNF production but increases and sustains intratumoral TNF synthesis [2]. While the enhanced antitumour activity caused by coadministration of thalidomide correlates with the enhanced intratumoral TNF activity [2], the mechanism involved is unclear. As part of our investigation into the basis for the synergy between the two agents, we examined the effect of thalidomide on the plasma and tissue pharmacokinetics of DMXAA in mice. We demonstrated that the biliary excretion is the major route of elimination of DMXAA in mice, as has been shown in rats [43], and that this route is inhibited by coadministration of thalidomide. We also compared the activity of the L- and D-stereoisomers of thalidomide with that of the racemic mixture.

Materials and methods

Animals and therapeutic agents

For all experiments we used 8–12-week-old female C57Bl/6 mice purchased from the Department of Laboratory Animal Sciences, Otago Medical School, Dunedin. All animals were housed under conditions of constant temperature and humidity according to institutional ethical guidelines. Colon 38 tumours were passaged subcutaneously every 2–3 weeks by subcutaneous implantation of tumour fragments (1 mm^3) into one flank of anaesthetized mice (pentobarbitone 90 mg/kg). Experiments were carried out when tumour diameters were 5–10 mm. DMXAA, synthesized as the sodium salt at the Auckland Cancer Society Research Centre [29], was dissolved in sterile water and administered by intraperitoneal (i.p.) injection to mice (10 µml/g body weight). L-Thalidomide, D-thalidomide and the racemic mixture (*rac*-thalidomide), synthesized at Celgene Corporation (Warren, N.J.), were dissolved in dimethylsulphoxide (40 mg/ml) and similarly administered (2.5 µl/g body weight). All compounds were >99% pure when analysed by thin-layer chromatography. Authentic DMXAA acyl glucuronide (DMXAA-G) was isolated from rat bile according to a previously described method and characterized by mass spectrometry [43]. All other chemicals or solvents were of analytical grade.

Pharmacokinetic studies

Groups of non-tumour-bearing mice (three to six animals per group) were treated i.p. with DMXAA (10 or 25 mg/kg) alone or coadministered with L-thalidomide, D-thalidomide or *rac*-thalidomide (100 mg/kg). After 0.25, 1.5, 3, 4.5 and 6 h, mice were anaesthetized with halothane and blood (700–800 µl) collected through the ocular sinus into heparinized tubes. Plasma was separated by centrifugation and stored at −20 °C until analysis.

Groups of tumour-bearing mice (three to six animals per group) were treated i.p. with DMXAA (10 or 25 mg/kg) alone or coadministered with thalidomide (100 mg/kg), either as one of the stereoisomers or the racemic mixture. After 0.25 h, animals were anaesthetized and blood samples were taken and stored at −20 °C. Animals were immediately killed by cervical dislocation, and tumours were excised, blotted to remove any fluid, weighed, homogenized in 1 ml ammonium acetate buffer and stored at −20 °C until assay.

Groups of tumour-bearing mice (three to six animals per group) were treated i.p. with DMXAA (25 mg/kg) either alone or with *rac*-thalidomide (100 mg/kg, i.p.). After 0.25, 1.5, 3, 4.5, 6, 16 and 24 h, blood samples were taken and the animals killed. Spleen, liver and tumour were excised, weighed and homogenized in ammonium acetate buffer (1 ml). Gall-bladder samples were homogenized, centrifuged to provide bile and corrected for the weight of gall-bladder tissue. Bile and homogenates were stored at −20 °C.

DMXAA assay

A specific reverse-phase high-pressure liquid chromatography (HPLC) assay was developed to measure DMXAA concentrations in plasma and bile and in homogenates of liver, spleen and tumour, using automated solid-phase extraction and 2,5-dimethylxanthenone-4-acetic acid as an internal standard. Aliquots of plasma or homogenate (200 µl) were mixed with an equal volume of 10 m*M* ammonium acetate buffer (pH 5.5) in a glass tube containing the internal standard solution (50 µl, 20 µmol/l). Plasma proteins were precipitated using ice-cold acetonitrile/methanol (3:1 v/v). After centrifugation (3000*g*, 10 min, 4 °C), the supernatants were added to ammonium acetate buffer (9 ml) and loaded automatically onto 1 ml/100 mg preconditioned (1 ml acetonitrile/methanol, 3:1 v/v, and 1 ml Milli Q water) C18 Bond Elut columns (Varian, Harbor City, Calif.). This was accomplished using an automated sample preparation with an extraction column system (ASPEC, Gilson Medical, Middleton, Wis.). The columns were washed with Milli Q water (1 ml) and the compounds of interest eluted using 1 ml acetonitrile/methanol (3:1 v/v).

The eluates were evaporated to dryness using a centrifugal evaporator (Jouan, St. Nazaire, France) and the residues reconstituted in 200 µl mobile phase. Aliquots (50 µl) of these solutions were automatically injected into the chromatograph (Waters WISP 712B sample injector and Model 510 pump; Waters Associates, Milford, Mass.) with a fluorescence detector (Shimadzu Model RF-530; Shimadzu, Kyoto, Japan) with excitation and emission wavelengths set at 345 and 409 nm, respectively, and a LUNA C18(2) 5 µ*M* 100 × 4.6 mm stainless steel column. Data acquisition and integration was achieved using a Unicam 4880 chromatography data system (Unicam, Cambridge, UK). Compounds were eluted from the column (retention time of DMXAA and internal standard were 5 and 7 min, respectively) using a mobile phase of ammonium acetate buffer and acetonitrile (3:1 v/v) at a flow rate of 2 ml/min. Calibration human plasma samples were prepared by adding DMXAA to plasma over the concentration range 0.125–100 µmol/l. The peak-height ratios of DMXAA to the internal standard were plotted against concentrations of DMXAA calibration standards and the best fit straight line obtained by linear regression analysis. Quantitation of DMXAA in mouse plasma was achieved by calculating the peak-height ratio in these samples and using the equation obtained from the best fit of the linear regression analysis.

The relative recoveries and coefficients of variation (CV) for the intraassay accuracy and precision were 90–110% and 5–8% ($n = 8$), respectively, over the concentration range 0.125–100 μmol/l. Interassay accuracy and precision was also acceptable with similar relative recoveries (90–110%) and CVs (5–8%, $n = 8$). The limit of quantitation was 0.125 μmol/l, and concentrations greater than 100 μmol/l were diluted with 10 m*M* ammonium acetate buffer to within the assay range. Quality control plasma samples were prepared using mouse plasma or tissue homogenate with added DMXAA concentrations of 400, 100 and 20 μmol/l and were stored at −20 °C. These were included in each analysis and found to be stable over a period of 4 months, since the relative recoveries in eight assays during that time ranged from 90 to 105% with CVs of 5–8%.

Alkaline hydrolysis of bile

Aliquots (200 μl) of bile were treated with 500 μl 0.5 *M* sodium hydroxide and incubated in darkness at 37 °C for 24 h. The hydrolysates were adjusted to pH 5.5 with 260 μl 1*M* acetic acid and 9 ml 10 m*M* ammonium acetate buffer (pH 5.5) containing 50 μl internal standard solution. These were transferred to preconditioned 1 ml/100 mg C18 Bond Elut columns and the amount of DMXAA determined as described above.

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. Concentration and half-life values are expressed as the mean ± SEM. Statistical significance was assessed by Student's *t*-test with $P < 0.05$ being considered as significant.

Results

Effect of thalidomide on plasma and tissue DMXAA pharmacokinetics

Non-tumour-bearing mice were treated with DMXAA (25 mg/kg), alone or together with D-thalidomide, L-thalidomide or *rac*-thalidomide (100 mg/kg) (Fig. 2). Coadministration of thalidomide slightly increased maximum plasma DMXAA concentrations although the increases for individual experiments were not statistically significant. In contrast, plasma $t_{1/2}$ values were significantly longer ($P < 0.05$) in mice coadministered each form of thalidomide, as compared to mice administered DMXAA alone. Furthermore, the plasma $t_{1/2}$ value for mice coadministered L-thalidomide was significantly longer ($P < 0.01$) than that for mice coadministered D-thalidomide or *rac*-thalidomide (Table 1). The DMXAA AUC was found to increase by 1.6-fold for *rac*-thalidomide, 1.7-fold for D-thalidomide and 1.8-fold for L-thalidomide. A significant increase in plasma $t_{1/2}$, together with a 1.9-fold increase in DMXAA AUC, was observed when L-thalidomide (100 mg/kg) was coadministered with a smaller dose (10 mg/kg) of DMXAA (Table 1).

Colon 38 tumour-bearing mice were treated with DMXAA (25 mg/kg) alone or together with D-thalidomide, L-thalidomide or *rac*-thalidomide (100 mg/kg). The DMXAA concentrations at 0.25 h were similar to those obtained for non-tumour-bearing mice (Table 1). To extend this study, the effect of *rac*-thalidomide (100 mg/kg) on plasma pharmacokinetics of DMXAA (25 mg/kg) in liver, spleen and tumour tissue was measured (Fig. 3). The plasma $t_{1/2}$ and AUC values were similar to those obtained for non-tumour-bearing mice (Table 2). Cotreatment with thalidomide caused significantly higher ($P < 0.05$) DMXAA $t_{1/2}$ values in plasma, liver and spleen of animals as compared to those treated with DMXAA alone. While the C_{max} for plasma, liver and spleen occurred at 0.25 h, that for tumour tissue was at 6 h and declined thereafter. Over the range 0.25–4.5 h, plasma DMXAA concentrations were significantly higher ($P < 0.05$) in mice coadministered thalidomide (Fig. 3D; Table 2). Overall, *rac*-thalidomide increased the DMXAA AUC 1.8-fold in plasma, liver and spleen, and 3-fold in tumour (Table 2).

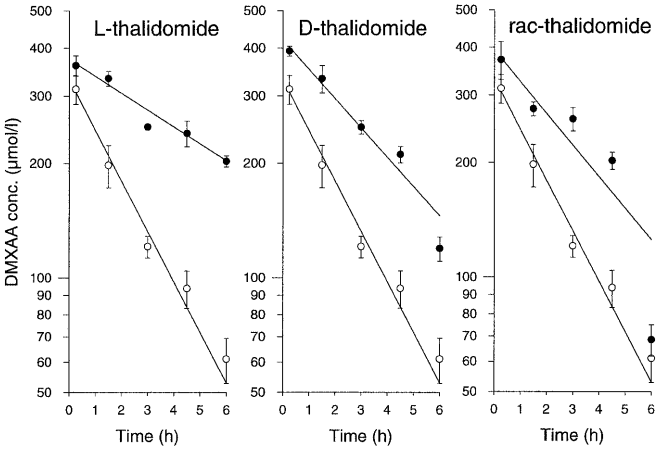

Fig. 2 Plasma DMXAA concentration-time profile in non-tumour-bearing mice measured up to 6 h after treatment with DMXAA, either alone at 25 mg/kg (○), or coadministered with L-thalidomide, D-thalidomide, or *rac*-thalidomide, each at 100 mg/kg (●). Each point represents the mean ± SEM from three to nine mice. The line indicates the least-squares regression

Effect of thalidomide on DMXAA metabolism

The increased plasma and tissue levels of DMXAA following coadministration of thalidomide suggested that thalidomide was affecting either the metabolism or the excretion of DMXAA. Since the major elimination pathway of DMXAA involves glucuronidation and biliary excretion [19], bile samples from mice treated with DMXAA, with or without thalidomide, were analysed by HPLC. Typical chromatograms are shown in Fig. 4. The major biliary component, with a retention time of 2.5 min (Fig. 4B), was identified as the acyl glucuronide metabolite DMXAA-G. Identification was based on coelution with authentic DMXAA-G (Fig. 4A) and on its complete conversion to DMXAA under alkaline hydrolysis conditions (Fig. 4C).

Table 1 Effect of administration of thalidomide isomers (100 mg/kg) on DMXAA pharmacokinetic parameters

Treatment	Non-tumour-bearing mice				Tumour bearing mice	
	$C_{0.25h}$ plasma (μmol/l)[a]	$t_{1/2}$ (h)	$AUC_{(0-6h)}$ (μmol · h/l)	AUC ratio[b]	$C_{0.25h}$ plasma (μmol/l)[a]	$C_{0.25h}$ tumour (μmol/kg)[a]
DMXAA (25 mg/kg)[c]	312 ± 27	2.3 ± 0.3	880 ± 81		350 ± 35	31.0 ± 4.3
+ L-thalidomide	360 ± 22	6.8 ± 0.9*	1542	1.8	393 ± 121	54.3 ± 4.2*
+ D-thalidomide	393 ± 12	3.9 ± 0.4*	1520	1.7	342 ± 36	60.3 ± 4.8*
+ *rac*-thalidomide	371 ± 42	3.6 ± 0.6*	1381	1.6	425 ± 20	62.7 ± 7.6*
DMXAA (10 mg/kg)	126 ± 12	1.7 ± 0.2	298			
+ L-thalidomide	157 ± 12	3.2 ± 0.6*	577	1.9		

*$P < 0.05$ vs control without thalidomide
[a] Plasma and tumour concentrations of DMXAA at 0.25 h
[b] Ratio = $AUC_{(0-6h)}$ DMXAA + thalidomide/$AUC_{(0-6h)}$ DMXAA
[c] Mean ± SE from three experiments

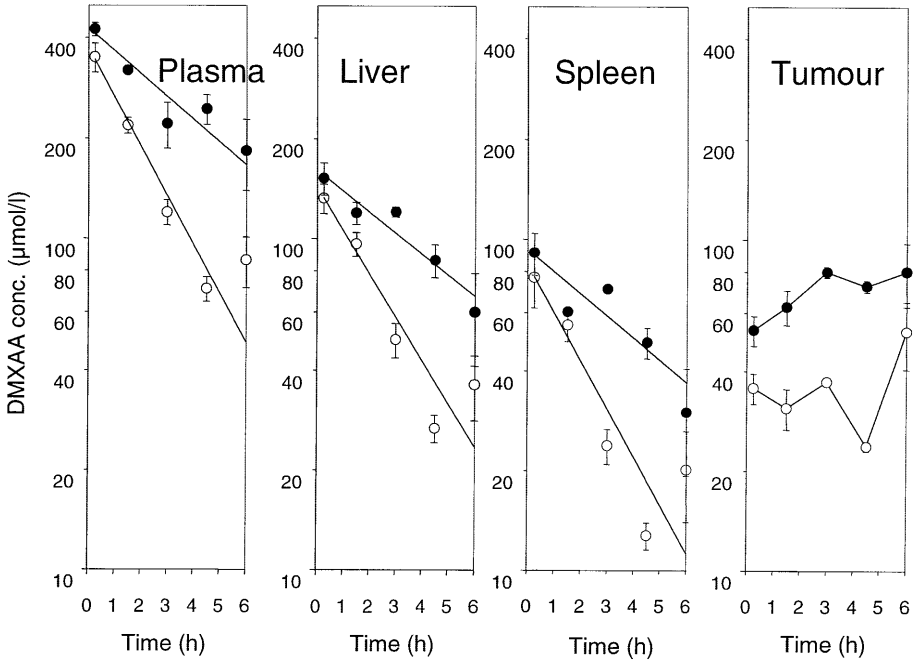

Fig. 3 Mean DMXAA concentration-time profile in plasma, liver, spleen and Colon 38 tumour of mice measured up to 24 h after treatment with DMXAA, either alone at 25 mg/kg (○), or coadministered with *rac*-thalidomide at 100 mg/kg (●). Each point represents the mean ± SEM from three to six mice. Data are shown for time-points up to 6 h and the line indicates the least-squares regression for the data to 24 h

DMXAA-G was quantitated by comparison of biliary concentrations of DMXAA before and after alkaline hydrolysis. Parent DMXAA biliary concentrations increased from 192 ± 45 μmol/l to 372 ± 171 μmol/l over the first 1.5 h after administration, and then decreased slowly to 253 ± 108 μmol/l at 24 h. In contrast, DMXAA-G concentrations increased dramatically from 115 ± 95 μmol/l to 865 ± 40 μmol/l over the same 1.5-h period then gradually fell to 205 ± 55 μmol/l at 24 h (Fig. 5). Thalidomide did not significantly alter parent DMXAA biliary concentrations, but between 1.5 and 4.5 h significantly ($P < 0.05$) decreased biliary DMXAA-G concentrations. DMXAA-G was barely detectable in some biliary samples (Fig. 4D,E), reflecting the reduced DMXAA-G production in thalidomide-treated animals. DMXAA bile-to-plasma ratios were found to rise progressively between 0 and 24 h after drug administration. Ratios measured 0.25, 3, 6, 16 and 24 h after administration of DMXAA alone (25 mg/kg) were 0.46, 2.5, 4.1, 37 and 380, respectively. Ratios after administration of DMXAA plus thalidomide (100 mg/kg) were 0.57, 1.0, 2.8, 19 and 13, respectively.

Discussion

These studies indicate that coadministration of *rac*-thalidomide or L- or D-thalidomide significantly increases the plasma and tissue $t_{1/2}$ values of DMXAA, and causes 1.8-fold increases in AUC in plasma and normal tissues. The L-isomer of thalidomide increased the elimination half-life of DMXAA to a significantly greater extent ($P < 0.01$) than did the D-isomer or the racemate (Table 1). Although the optical isomers of thalidomide rapidly interconvert after in vivo administration [12], attenuating the difference between the isomers, the data are consistent with the L-isomer having the greater effect on DMXAA elimination. Non-racemizable derivatives

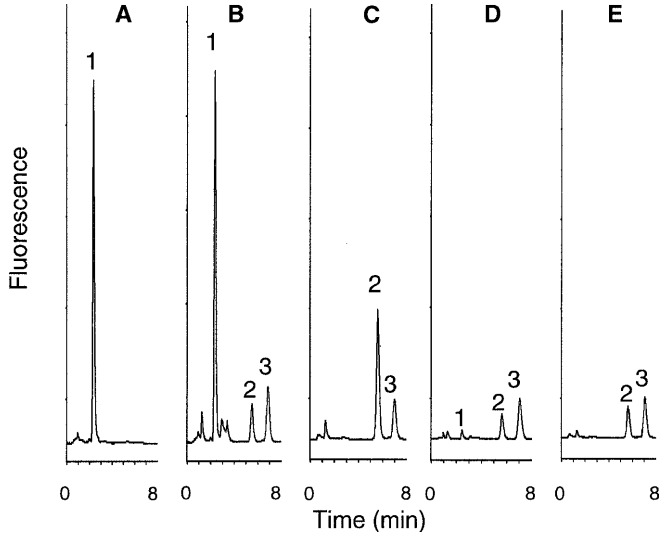

Fig. 4A–E Chromatograms of bile collected 1.5 h after administration of DMXAA either alone (25 mg/kg) or in combination with thalidomide at 100 mg/kg (**A** authentic DMAA-G, **B** bile from mouse treated with DMXAA, **C** bile from mouse treated with DMXAA after alkaline hydrolysis, **D** bile from mouse treated with DMXAA and thalidomide, **E** bile from mouse treated with DMXAA and thalidomide after alkaline hydrolysis; *peak 1* DMXAA-G, *peak 2* DMXAA, *peak 3* internal standard)

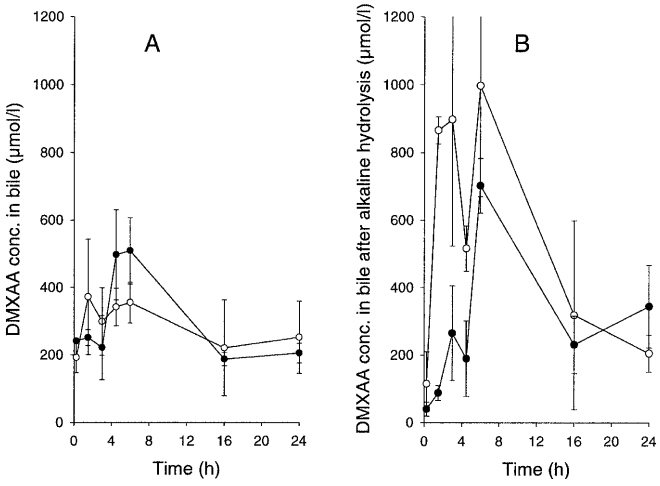

Fig. 5A,B Mean concentrations of parent DMXAA (**A**) and DMXAA after alkaline hydrolysis (**B**) in bile from mice treated with DMXAA either alone at 25 mg/kg (○) or coadministered with *rac*-thalidomide at 100 mg/kg (●). Each point represents the mean ± SEM from three mice

of thalidomide have been used to demonstrate that the L-isomer is the more biologically active species [27, 39]. Differences in the half-lives of the isomers in humans have been documented, with the D-isomer eliminated three times more slowly than the L-isomer [12]. However, even if it is eliminated more rapidly, the active L-isomer would have a greater effect than the D-isomer or the racemic form because the D-isomer has to be interconverted to be active.

The effects of thalidomide on plasma pharmacokinetics were compared in tumour-bearing and non-tumour-bearing mice because DMXAA toxicity is higher in tumour-bearing mice [8]. Although no significant difference in the pharmacokinetic parameters was found (Tables 1 and 2), thalidomide markedly affected tumour concentrations of DMXAA (Fig. 3), and caused a threefold increase in AUC (Table 2). Tumour concentrations remained almost constant for 6 h after administration, consistent with previous findings demonstrating that DMXAA selectively halts tumour blood flow [45], effectively preventing efflux of drug from the tissue. The higher concentrations of DMXAA in tumour tissue induced by thalidomide thus correlate with its improvement of the antitumour response to DMXAA [8].

The data in Fig. 5 indicate the presence of substantial amounts of both parental DMXAA and DMXAA-G in bile. Most xenobiotics for which biliary excretion is an important route of elimination have bile-to-plasma ratios between 10 and 1000 [20]. The present studies demonstrate that the bile to-plasma ratio for DMXAA increases progressively with time to 380 at 24 h, indicating that biliary excretion in the mouse, as in the rat, is the major excretory route of DMXAA. By analogy with the related drug flavone acetic acid [4, 5], DMXAA-G may be hydrolysed in the gut to release DMXAA, which is then reabsorbed to give rise to a secondary peak of DMXAA in the blood (Fig. 5A).

Table 2 Plasma and tissue pharmacological parameters following administration of DMXAA (25 mg/kg), with or without *rac*-thalidomide (100 mg/kg), to Colon 38 tumour-bearing mice

Tissue	Treatment	$C^{a}_{0.25h}$ (µmol/l or µmol/kg)	$t_{1/2}$ (h)	$AUC_{(0–6h)}$ (µmol · h/l)	$AUC_{(0–24h)}$ (µmol · h/l)	AUC ratio[b]
Plasma	DMXAA	413 ± 20	1.9 ± 0.3	963	1280	
	DMXAA + *rac*-thalidomide	425 ± 44	4.4 ± 0.6*	1580	2300	1.8
Liver	DMXAA	132 ± 14	2.3 ± 0.3	366	529	
	DMXAA + *rac*-thalidomide	152 ± 17	4.7 ± 0.7*	628	939	1.8
Spleen	DMXAA	77 ± 15	2.1 ± 0.4	198	299	
	DMXAA + *rac*-thalidomide	91 ± 13	4.5 ± 0.8*	350	527	1.8
Tumour	DMXAA	38 ± 5.5		205	345	
	DMXAA + *rac*-thalidomide	63 ± 6.1*		628	1020	3.0

*$P < 0.05$ vs control without thalidomide
[a] Plasma and tumour concentrations of DMXAA at 0.25 h
[b] Ratio = $AUC_{(0–24h)}$ DMXAA + thalidomide/$AUC_{(0–24h)}$ DMXAA

The decrease in biliary DMXAA-G concentrations following coadministration of thalidomide with DMXAA suggests that thalidomide either directly or indirectly interferes with either the glucuronidation of DMXAA or its transport from the hepatocyte into the bile by a transporter protein. One possible candidate for competitive inhibition by thalidomide, or its hydrolysis product, is a glucuronosyl transferase. Another target is the family of canalicular multispecific organic anion transporter proteins, which are reported to mediate the biliary excretion of the camptothecin derivative CP-11 [9].

Since thalidomide is a known to be an immunomodulatory agent [23, 25] and there is a large body of evidence demonstrating the downregulation of drug metabolism by cytokines and other immune factors [24, 33], it might be considered that thalidomide acts by this mechanism. However, similar pharmacokinetic effects were observed using a dose of DMXAA (10 mg/kg) that is well below its threshold for cytokine induction, suggesting that the alterations in the metabolism or elimination of DMXAA by thalidomide are independent of cytokine production. Studies to investigate the effect of thalidomide on glucuronosyl transferase enzyme activities in the glucuronidation of DMXAA and the involvement of canalicular multispecific organic anion transporter proteins in DMXAA-G biliary excretion are in progress.

Thalidomide is currently proposed for use in the treatment of a number of diseases including cancer [11, 13, 26, 38] and HIV infection [22] where polypharmacy is common. Several studies have shown that there are no drug interactions between thalidomide and ethinyl oestradiol or norethindrone, and that the efficacy of oral contraceptives in the prevention of pregnancy and teratogenicity are not affected [32, 40]. The present demonstration that the pharmacokinetics of DMXAA are significantly altered by coadministration of thalidomide suggests that it would be useful to investigate the effect of thalidomide on the efficacy and pharmacokinetics of other coprescribed drugs. In the case of the combination of DMXAA and thalidomide, the alteration in pharmacokinetics is not associated with an increase in toxicity and the combination results in a beneficial increase in antitumour activity.

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