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ORIGINAL ARTICLE

Shufeng Zhou · James W. Paxton · Philip Kestell
Malcolm D. Tingle · Lai-Ming Ching

In vitro and in vivo kinetic interactions of the antitumour agent 5,6-dimethylxanthenone-4-acetic acid with thalidomide and diclofenac

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Abstract *Background*: Previous studies have demonstrated that coadministration of L-thalidomide with the novel antitumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) results in an increased area under the plasma concentration-time curve (AUC) of DMXAA, suggesting an explanation for the observed increase in the antitumour activity. The aims of this study were to investigate the effects of L-thalidomide on the in vitro metabolism of DMXAA in mouse and human liver microsomes using diclofenac as positive control, to examine the effects of L-thalidomide and diclofenac on the plasma protein binding of DMXAA in vitro, and to investigate whether the in vivo interactions can be predicted from in vitro data, particularly in humans. *Methods*: Mouse and human liver microsomes were used to investigate the effects of L-thalidomide and diclofenac on DMXAA metabolism. The resulting in vitro data were extrapolated to predict in vivo changes in DMXAA, which were then compared with the results of in vivo mouse pharmacokinetic interaction studies. The protein binding of DMXAA in mouse and human plasma was determined using ultrafiltration followed by HPLC. *Results*: Diclofenac at 100 μ*M* caused significant inhibition of glucuronidation (>70%) and 6-methylhydroxylation (>54%) of DMXAA in mouse and human liver microsomes. In vivo diclofenac (100 mg/kg i.p.) resulted in a 24% and 31% increase in the plasma DMXAA AUC, and a threefold increase in $T_{1/2}$ ($P < 0.05$) in male and female mice, respectively. In contrast, L-thalidomide at 100 μ*M* had no inhibitory effect on DMXAA metabolism in vitro in either species, except for a decrease of about 25% in 6-methylhydroxylation in mice. L-Thalidomide at 500 μ*M* resulted in further significant decreases in 6-methylhydroxylation in mice (30–60%) and human (30%) microsomes. Coadministration of L-thalidomide in male mice resulted in a 23% increase in DMXAA AUC and a twofold increase in $T_{1/2}$ ($P < 0.05$). Neither L-thalidomide nor diclofenac at 50 or 500 μ*M* had any significant effect on the in vitro plasma protein binding of DMXAA (500 μ*M*) in mouse or human plasma. Based on our in vitro inhibition studies, we predicted a 20% increase in DMXAA AUC in mice with concomitant diclofenac, but little or no effect (< 5%) with L-thalidomide. *Conclusion:* Both L-thalidomide and diclofenac increased the plasma DMXAA AUC in mice. In the case of diclofenac, this appeared to be due to direct competitive inhibition of DMXAA metabolism, but this mechanism does not appear to be appropriate for L-thalidomide. From the in vitro human inhibition studies, it appears unlikely that concurrent diclofenac will cause an increase in the plasma AUC of DMXAA in patients. However, the effect of L-thalidomide on DMXAA could not be readily predicted from the in vitro data. Our study demonstrated that a predictive model based on direct inhibition of metabolism is appropriate for diclofenac-DMXAA interactions, but is inappropriate for the prediction of L-thalidomide-DMXAA interactions in mice and humans in vivo.

Key words DMXAA · L-Thalidomide · Drug interaction · Glucuronidation

S. Zhou · J. W. Paxton (✉) · M. D. Tingle
Department of Pharmacology and Clinical Pharmacology,
The University of Auckland School of Medicine,
Private Bag 92019, Auckland, New Zealand
E-mail: j.paxton@auckland.ac.nz
Tel.: +64-9-3737599
Fax: +64-9-3737556

P. Kestell · L. -M. Ching
Auckland Cancer Society Research Centre,
The University of Auckland School of Medicine,
Private Bag 92019, Auckland,
New Zealand

Introduction

Coadministration of thalidomide (α-(*N*-phthalimido)glutarimide, Fig. 1) with the novel antitumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA, Fig. 1), currently completing phase I evaluations, causes a dramatic potentiation of the antitumour effects of DMXAA in mice [5, 6]. Further studies have found that

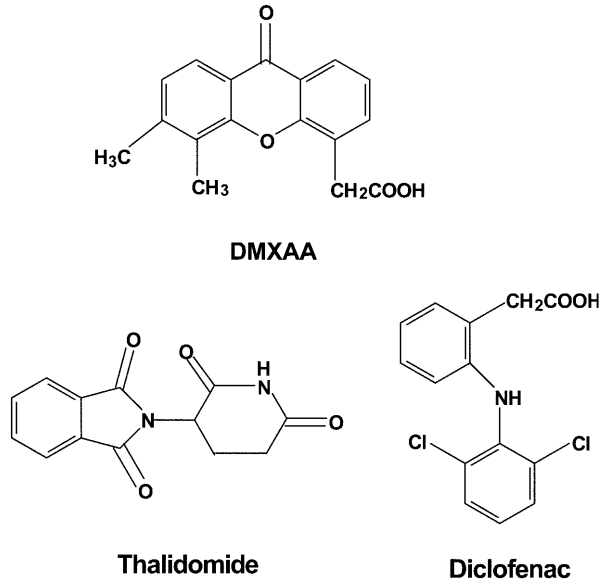

Fig. 1 The chemical structure of DMXAA, thalidomide and diclofenac

coadministration of L-thalidomide has a greater effect than either D-thalidomide or the racemate on DMXAA's pharmacokinetics, causing an increase in the area under the plasma concentration-time curve (AUC) of DMXAA by 80% in both nontumour-bearing and tumour-bearing female mice [16], providing a basis for the improved antitumour activity. The mechanisms responsible for the pharmacokinetic interactions have not been investigated. Studies have indicated that DMXAA is extensively metabolized mainly by glucuronidation, but also by 6-methylhydroxylation, with the major metabolites, DMXAA acyl glucuronide (DMXAA-G) and 6-methylhydroxyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) excreted in the bile and urine [15, 33]. Uridine diphosphate glucuronosyltransferase (UGT) 1A9 and UGT2B7, as well as cytochrome P450 (CYP) 1A2 have been shown to be involved in the metabolism of DMXAA [18, 36].

The aims of this study were to investigate the effects of L-thalidomide on metabolism of DMXAA in mouse and human liver microsomes, to examine the effects of L-thalidomide on the plasma protein binding of DMXAA in vitro, and to investigate whether the in vitro data would be predictive of in vivo changes in pharmacokinetics, particularly in humans. Diclofenac (Fig. 1), an arylacetic nonsteroidal antiinflammatory agent, is a potent inhibitor of DMXAA glucuronidation [18] and 6-methylhydroxylation [36] in human liver microsomes, and was used in these studies as a positive control.

Materials and methods

Chemicals and reagents

DMXAA and 2,5-dimethylxanthenone-4-acetic acid (SN24350, as internal standard) were synthesized in the Auckland Cancer Society Research Centre (ACSRC) [24]. DMXAA was protected from light exposure to avoid degradation [25]. L-Thalidomide was synthesized by Dr. Palmer (ACSRC), as described previously [2]. Authentic DMXAA-G and 6-OH-MXAA were isolated and purified by a solid-phase extraction method from the bile and urine of rats treated with DMXAA, and their structure confirmed by mass spectrometry and [^{1}H] nuclear magnetic resonance [15]. Diclofenac was purchased from Sigma-Aldrich (Auckland, New Zealand). NADPH and UDPGA were purchased from Roche Diagnostics NZ (Auckland). All other reagents were of analytical or HPLC grade as appropriate.

Animals

Male and female C57Bl/6 mice (25–32 g) were housed under conditions of constant temperature, lighting and humidity according to institutional guidelines. Sterile food and water was available ad libitum. All animal procedures were approved by the Animals Ethics Committee of the University of Auckland.

Mouse and human plasma and liver microsomal preparation

Fresh heparinized blood was obtained from C57Bl/6 mice (both male and female) and healthy humans. Plasma was separated by centrifugation at 1000 *g* for 15 min. Microsomes were prepared by differential centrifugation of livers from humans ($n = 3$) and C57Bl/6 mice ($n = 12$ for each gender) as described previously [26]. The human liver samples were donated by three patients (HL12, HL13 and HL14) who underwent liver resection for metastasis of colon cancer. Histological examination of the resected liver samples ensured the use of healthy liver tissue. Ethical approval was obtained from the Northern New Zealand Research Ethics Committee and written informed consent was obtained for liver tissue to be used for research. Livers and microsomes were stored at –80 °C until used. Microsomal protein concentration was determined by the bicinchoninic acid method [28]. The CYP content was determined as described previously [21].

In vitro metabolism inhibition studies

The effects of L-thalidomide and diclofenac (100 and 500 μ*M*) on DMXAA glucuronidation and 6-methylhydroxylation in mouse and three sources of human liver microsomes (HL12, HL13 and HL14) were investigated using optimized incubation conditions [36]. Typical incubations (total volume 200 μl) for DMXAA glucuronidation contained liver microsomal protein (0.1 mg/ml), 10 m*M* UDPGA, 5 m*M* $MgCl_2$, 0.1 mg/ml D-saccharic acid 1,4-lactone, Brij 58 (0.1–0.4:1 ratio of Brij 58 to microsome, w/w), L-thalidomide or diclofenac, and DMXAA in 0.1 *M* phosphate buffer (pH 6.8). D-Saccharic acid 1,4-lactone was used to inhibit the activity of *β*-glucuronidase in microsomes. Typical incubations (total volume 200 μl) for 6-methylhydroxylation contained 1 mg/ml liver microsome, 5 m*M* $MgCl_2$, 0.5 m*M* NADPH, L-thalidomide or diclofenac, and DMXAA in 0.1 *M* phosphate buffer (pH 7.4). L-Thalidomide and diclofenac were dissolved in dimethyl sulphoxide (DMSO, final concentration 1% v/v) for initial inhibition studies. The concentrations of DMXAA were 140 μ*M* for glucuronidation in both the mouse and human microsome incubations, and 240 or 20 μ*M* for 6-methylhydroxylation in the mouse and human microsome incubations, respectively [36]. These values correspond to the apparent K_m values for each metabolic pathway in the mouse and human.

The reactions were initiated by the addition of NADPH or UDPGA as appropriate. Preincubation was performed in triplicate in the presence of inhibitor and cofactor (NADPH or UDPGA) for 0, 5 or 15 min prior to the addition of DMXAA at 37 °C in a shaking waterbath. Incubations were stopped by cooling on ice and adding two volumes of an ice-cold acetonitrile/methanol mixture (3:1 v/v) containing 2 μ*M* internal standard, and vortexing vigor-

ously. Mixtures were centrifuged (3000 *g* for 10 min) to remove the precipitated microsomal protein. The supernatant was removed and evaporated under nitrogen gas and the residue reconstituted with mobile phase and injected into the HPLC system. Control incubations with 1% DMSO showed no significant effect on liver microsomal DMXAA glucuronidation, but caused a 22% reduction in 6-methylhydroxylation of DMXAA. L-Thalidomide and diclofenac were also incubated with microsomes and UDPGA or NADPH in the absence of DMXAA to determine whether there were any chromatographic peaks interfering with the quantitation of DMXAA metabolites. The effects of each drug on the formation of DMXAA-G and 6-OH-MXAA were compared with the control values determined from incubations in the presence of DMXAA alone, and expressed as a percentage of the control values. When significant inhibition was observed, indicating a therapeutically relevant K_i value, further kinetic studies were undertaken.

Plasma protein binding assay

L-Thalidomide or diclofenac was dissolved in ethanol at concentrations of 5 and 50 m*M*. DMXAA (500 μ*M*) was added to plasma from mice or humans ($n = 3$), followed by L-thalidomide or diclofenac (1:100 dilution), and the mixture was incubated for 30 min at 37 °C with shaking. A 400-μl aliquot was then transferred to a Centrisart ultrafiltration device (20,000 molecular weight cut-off; Sartorius Corporation, Germany), centrifuged (2000 *g* for 30 min) at 37 °C, and the DMXAA concentration in the ultrafiltrate determined by HPLC. A 100-μl sample of ultrafiltrate was mixed with 50 μl 0.1 *M* phosphate buffer (pH 7.4) containing 10 μ*M* internal standard, and 50 μl injected into the HPLC system. A 100-μl aliquot of plasma was also taken to determine the total DMXAA concentration. The aliquot was mixed with 50 μl methanol containing 20 μ*M* internal standard, followed by 0.4 ml ice-cold acetonitrile/methanol (3:1 v/v). After centrifugation (2500 *g* for 15 min) to remove precipitated proteins, the supernatant was removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 μl mobile phase, and 50 μl was injected into the HPLC system. Preliminary experiments demonstrated that the addition of ethanol at a final concentration of 1% (v/v) had no significant effect on the plasma protein binding of DMXAA.

Drug administration and sampling

Female and male mice ($n = 18$ per group) were treated with DMXAA alone, or DMXAA with diclofenac pretreatment. In order to reduce the total number of mice for ethical reasons, only male mice were used for L-thalidomide, as the previous study using this protocol had been carried out in female mice [16]. DMXAA was dissolved in sterile water and administered by an intraperitoneal (i.p.) injection at 25 mg/kg. L-Thalidomide and diclofenac were dissolved in DMSO and injected i.p. at 100 mg/kg (1 μl/g body weight) 15 min before DMXAA administration. Controls were injected with DMSO at 1 μl/g body weight. All experiments were conducted in subdued light. Blood was collected in heparinized tubes via the ocular sinus ($n = 3$ for each time-point) at 0.25, 1.5, 3, 4.5, 6 and 8 h following drug administration. Plasma was separated immediately by centrifugation and stored at –20 °C until assayed.

HPLC assay

The determination of DMXAA, DMXAA-G and 6-OH-MXAA in plasma and microsomes has been described previously [15, 35]. Briefly, for DMXAA, plasma samples were diluted with 1 m*M* ammonium acetate buffer (pH 5.5) and extracted using an automatic solid-phase extraction system (ASPEC; Gilson, Middleton, Wis.). DMXAA was eluted with 1 ml methanol, and the eluents collected and evaporated under nitrogen. The residues were reconstituted with 200 μl mobile phase and injected into the HPLC system. The latter consisted of model 510A pumps with a WISP 712B sample injector and a Radial Compression Module (Waters Associates, Milford, Mass.) fitted with a Hypersil C18, 5 μm, 100 × 4.6 mm Luna column (Phenomenex Company, Torrance, Calif.) protected by a Luna C18 guard column. The mobile phase (flow rate 1.9 ml/min) was 10 m*M* ammonium acetate buffer/acetonitrile (24:76 v/v) adjusted to pH 5.0 with acetic acid. Fluorescence detection was performed using a Shimadzu RF-530 at excitation and emission wavelengths of 345 and 409 nm, respectively (supported by Unicam 4880 data processing software).

The HPLC system for determining DMXAA-G and 6-OH-MXAA in microsomal incubations consisted of a solvent delivery system, a Model SF250 fluorescence detector (excitation and emission wavelengths 345 and 409 nm, respectively), a Model 460 autosampler, and a Model D450 data processing system (all from Kontron Instrument Company, Milan, Italy). A Luna C18 guard column and a 5 μm Spherex analytical column (150×4.6 mm; Phenomenex) were used. The mobile phase was the same as for DMXAA but at a flow rate of 2.8 ml/min. All HPLC methods had acceptable accuracy (85–115% of true values) and precision (intra- and interassay coefficients of variation < 15%). Assay specificity was indicated by the absence of interfering chromatographic peaks in microsomal samples from all species and in incubations with L-thalidomide.

Determination of pharmacokinetic parameters

Pharmacokinetic parameters were calculated using standard model-independent pharmacokinetic formulae using a Prism 3.0 package (GraphPad Software Company, Calif.). The elimination half-life ($T_{1/2}$) was calculated as 0.693/k, where k is the elimination rate constant calculated from the terminal linear portion of the plasma log concentration-time curve. The AUC was calculated using the log trapezoidal rule without extrapolation to infinity. The plasma clearance of DMXAA following i.p. administration was calculated as the total administered dose/AUC. Statistical significance was assessed using a Student's *t*-test at $P < 0.05$.

Prediction of drug interaction based on in vitro data

Interactions were predicted as follows. An interaction in vivo was considered to be likely if the following was true [11]:

$$[I]/K_i > 0.2 \tag{1}$$

where $[I]$ is the maximal unbound inhibitor concentration, and K_i is the inhibition constant. For competitive inhibition, the expected inhibition can be calculated from the following equation [11]:

$$\%\text{Inhibition}(R) = \frac{[I]}{[\text{I}] + K_i \times (1 + [S]/K_m)} \times 100 \tag{2}$$

where R is the degree of inhibition of the metabolic pathway, and $[S]$ the maximal unbound therapeutic substrate concentration. Diclofenac is ≥99.0% bound to plasma proteins in all species [3]. Thalidomide is 50–60% bound to plasma protein, mainly α_1-acid glycoprotein [32]. The plasma unbound fraction of DMXAA is 4.61% (at 540 μ*M*) and 12.3% (at 2000 μ*M*) in mice and humans, respectively [37].

The increase (R_c) in steady-state concentration or AUC as a result of the drug interaction can be calculated as follows [11]:

$$R_c = \frac{1}{f_h \times f_m \times [1/(1 + [I]/K_i)] + 1 - (f_h \times f_m)} \tag{3}$$

where f_h is the fraction of hepatic clearance in total clearance, and f_m is the fraction of a metabolic pathway in hepatic clearance. Approximate f_h and f_m values for DMXAA in mice and humans can be estimated on the basis of previous studies [16, 33]. Totals of 52% and 28% of the DMXAA were recovered as acyl glucuronide and unchanged form, respectively, from isolated perfused rat liver, and 0.6% was excreted in the urine in the in vivo studies. Thus

approximately 71.4% of the DMXAA was metabolized. Of the fraction metabolized, 73% (i.e. 52% of the total dose) was formed by acyl glucuronidation and 27% (i.e. 19%) by 6-methylhydroxylation in the rat, assuming that only the liver is responsible for the DMXAA metabolism and only two metabolic pathways (glucuronidation and 6-methylhydroxylation) are involved. In mice, 7.8% [15] of the total DMXAA dose is excreted as the unchanged form. As the urinary metabolite profile in the mouse is similar to that in the rat, f_m was assumed to be similar to that of the rat, resulting in an approximate f_h, f_m (glucuronidation) and f_m (6-methylhydroxylation) of 92.2%, 73% and 27% (i.e. 67% and 25% of the total dose), respectively. In the urine of a patient treated with DMXAA, 2.4%, 35.9% and 5.5% is excreted as unchanged DMXAA, DMXAA-G and 6-OH-MXAA [14]. Thus approximate f_h, f_m (glucuronidation) and f_m (6-methylhydroxylation) values are 97.6%, 86.7% and 13.3% (i.e. 85% and 13% of the total dose), respectively.

Results

Inhibition of in vitro DMXAA metabolism

Diclofenac at 100 μ*M* caused significant inhibition of DMXAA glucuronidation (70–85%) and 6-methylhydroxylation (54–65%) with apparent K_i values of 54 and 10 μ*M* for glucuronidation, and 52 and 93 μ*M* for 6-methylhydroxylation in male mouse and human microsomes, respectively (Table 1). Similar inhibition results for diclofenac were also observed in female mice. Dixon plots indicated competitive inhibition of both the glucuronidation and 6-methylhydroxylation pathways by diclofenac (Fig. 2). In contrast, L-thalidomide at 100 μ*M* had no significant effects on glucuronidation or 6-methylhydroxylation, except for a decrease of about 25% ($P < 0.05$) in 6-methylhydroxylation in both male and female mice. Increasing L-thalidomide to 500 μ*M* resulted in further significant decreases in 6-methylhydroxylation in both male and female mice (30–60%) and human microsomes (30%) (Table 1). The apparent K_i of L-thalidomide for DMXAA metabolism was estimated to be > 500 μ*M* in both species, except in female mice ($K_i = 330$ μ*M*). Preincubation of microsomes with L-thalidomide or diclofenac (500 μM) did not enhance its inhibitory effects on DMXAA metabolism in either species.

Effects of L-thalidomide and diclofenac on plasma pharmacokinetics of DMXAA

A significant alteration in the plasma concentration-time profile for DMXAA was observed when coadministered with L-thalidomide in male mice. There was a 23% increase in the plasma DMXAA $AUC_{(0–8h)}$ (2204 vs 1790 μ*M* · h) and a twofold increase in $T_{1/2}$ (6.43 ± 1.19 h vs 2.80 ± 0.19 h, $P < 0.05$) in L-thalidomide-treated male mice compared with controls, but C_{max} was not significantly altered (Table 2). These results are similar to those previously reported for the interaction of L-thalidomide with DMXAA in female mice using the same protocol, with the exception that the increase in DMXAA AUC (80%) with L-thalidomide was more pronounced in the previous study [16].

In both male and female mice, coadministration of diclofenac caused a 23% and 31% increase in plasma DMXAA AUC compared to controls (male 2212 vs 1790 μ*M* · h; female 2201 vs 1680 μ*M* · h), with a two- to threefold increase in $T_{1/2}$ compared to controls (male 8.20 ± 1.19 vs 2.80 ± 0.19 h; female 7.89 ± 0.88 vs 2.69 ± 0.30 h; $P < 0.05$). The C_{max} of DMXAA was not significantly changed in either male or female mice pretreated with diclofenac.

Table 1 Inhibition of DMXAA metabolism in vitro by L-thalidomide and diclofenac. DMXAA at a concentration of K_m was incubated at 37 °C with liver microsomes from mice (pooled) and three sources of human livers (HL12, HL13 and HL14) in the presence of L-thalidomide. Values are the means ± SD from three determinations

Metabolic pathway	Species	Inhibitor concentration (μM)	Diclofenac		L-Thalidomide	
			Enzyme activity remaining (%)	K_i[a] (μ*M*)	Enzyme activity remaining (%)	K_i[a] (μ*M*)
Glucuronidation	Mouse					
	M	100	27.7 ± 2.7*	54	105.7 ± 12.1	> 500
		500	13.6 ± 0.2*		75.7 ± 4.1*	
	F	100	28.8 ± 4.2*	50	93.8 ± 4.4	> 500
		500	12.1 ± 0.4*		95.7 ± 0.1	
	Human ($n = 3$)	100	14.4 ± 0.9*	10	87.6 ± 5.4	> 500
		500	8.4 ± 0.9*		83.0 ± 4.7	
6-Methylhydroxylation	Mouse					
	M	100	35.4 ± 4.8*	52	76.4 ± 9.4*	> 500
		500	19.9 ± 0.9*		69.5 ± 17.2*	
	F	100	38.2 ± 6.2*	55	74.1 ± 8.2*	330
		500	22.3 ± 1.2*		40.0 ± 7.8*	
	Human ($n = 3$)	100	45.9 ± 0.9*	93	84.2 ± 14.7	> 500
		500	33.9 ± 1.1*		68.1 ± 4.3*	

*$P < 0.05$, vs incubations in the absence of L-thalidomide or diclofenac

[a] Apparent K_i values were determined using the methods describe in Materials and methods assuming the inhibition was competitive

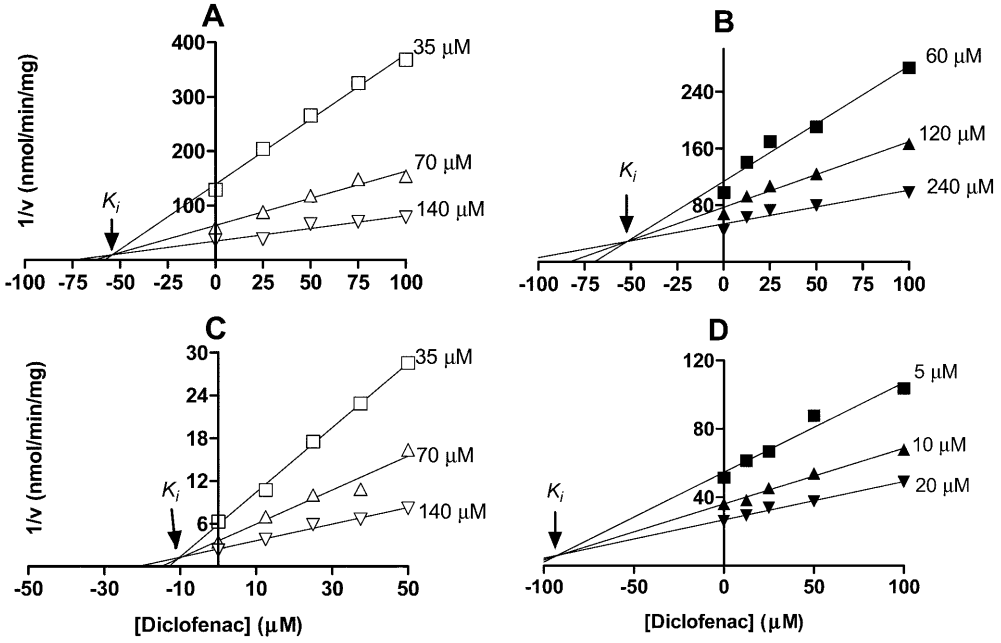

Fig. 2A–D Dixon plots for the inhibition of in vitro DMXAA glucuronidation (*open symbols*) and 6-methylhydroxylation (*close symbols*) by diclofenac in the male mouse (**A**, **B**) and human liver microsomes (HL13) (**C**, **D**)

Table 2 Effect of diclofenac and L-thalidomide on the plasma pharmacokinetic parameters of DMXAA in mice. Values are means ± SD (three mice for each time-point)

Mouse gender	Treatment	Dose (mg/kg)	Route	AUC_{0-8h} ($\mu M \cdot h$)	C_{max} (μM)	$T_{1/2}$ (h)	AUC ratio[a]
Male	DMXAA alone	25	i.p.	1790	571 ± 37	2.80 ± 0.19	
	+ Diclofenac	100	i.p.	2212	363 ± 123	8.20 ± 1.19	1.24
	+ L-Thalidomide	100	i.p.	2204	404 ± 123	6.43 ± 1.19	1.23
Female	DMXAA alone	25	i.p.	1680	372 ± 27	2.69 ± 0.30	1.31
	+ Diclofenac	100	i.p.	2201	373 ± 65	7.89 ± 0.88	
	DMXAA alone[b]	25	i.p.	880[c]	312 ± 27	2.3 ± 0.3	
	+ L-Thalidomide[b]	100	i.p.	1542[c]	360 ± 22	6.8 ± 0.9	1.8

[a] AUC ratio = AUC (DMXAA + diclofenac or L-thalidomide)/AUC (DMXAA alone)
[b] Data from previous study [16]
[c] The DMXAA AUC was 0–6 h, and a batch of DMXAA synthesized earlier and a different source of mice were used, which may have contributed to the discrepancy in AUC between the two studies

Plasma protein binding studies

Since pharmacokinetic parameters can be significantly influenced by plasma protein binding [9], we investigated whether L-thalidomide or diclofenac displaced DMXAA from its binding sites on plasma proteins. In male and female mouse plasma, the addition of 500 μM L-thalidomide did not significantly alter the unbound fraction of DMXAA at 500 μM (male 4.5 ± 0.4% vs 5.9 ± 0.8%; female 4.2 ± 0.2% vs 5.4 ± 0.6%, n = 3, $P > 0.05$, unpaired *t*-test). Diclofenac (500 μM) had no effect on the in vitro plasma protein binding of DMXAA (500 μM) in male and female mice. L-Thalidomide and diclofenac (50 or 500 μM) had no significant effects on the unbound fraction for DMXAA (500 μM) ($P > 0.05$) in human plasma in vitro.

Quantitative prediction of DMXAA-drug interaction in vivo

The predicted percent inhibition and percent increase in AUC of DMXAA that might have been caused by coadministration of L-thalidomide or diclofenac based on our in vitro studies are shown in Table 3. The predicted increase in AUC for diclofenac was approximately 20% for both male and female mice. With L-thalidomide, it was not possible to calculate an accurate increase in DMXAA AUC, but the data suggest a small increase (< 5%). The in vitro data from human microsomes suggest that neither diclofenac nor L-thalidomide would cause a significant change in the DMXAA plasma profile in patients.

Discussion

Kestell et al. [16] have reported an 80% increase in plasma DMXAA AUC with coadministration of L-thalidomide (100 mg/kg) in both healthy and tumour-bearing female mice, providing a possible explanation for the enhanced antitumour activity observed with the combination of DMXAA and L-thalidomide [6]. However, the possibility that there is a pharmacodynamic rather than a pharmacokinetic basis for the potentiation

Table 3 Predicted pharmacokinetic DMXAA-drug interactions. Metabolic inhibition (R, %) and degree of increases in AUC (R_c, %) due to drug interaction were predicted using the method described in Materials and methods assuming that competitive inhibition occurred

		K_i (μM)	$[I]_{total}$ (μM)	$[S]_{total}$ (μM)	R (%)	R_c (%)	Total R_c (%)
DMXAA + diclofenac	Male mouse		11–1680[a]	540[c]			19
	Glucuronidation	54			20.9	14.1	
	Hydroxylation	65			22.6	4.9	
	Female mouse		11–1680[a]	540[c]			20
	Glucuronidation	54			22.2	15.1	
	Hydroxylation	65			20.6	4.7	
	Human		1–15[b]	2000[d]			1.3
	Glucuronidation	10			0.5	1.3	
	Hydroxylation	93			0.1	0.0	
DMXAA + L-thalidomide	Male mouse			540[c]			< 4.7
	Glucuronidation	> 500			< 6.4	< 4.0	
	Hydroxylation	> 500			< 6.8	< 0.7	
	Female mouse		100–200[e]	540[c]			< 5
	Glucuronidation	> 500			< 6.4	< 4.0	
	Hydroxylation	330			< 9.3	< 1.0	
	Human		4–50[f]	2000[d]			< 3.8
	Glucuronidation	> 500			< 1.4	< 3.3	
	Hydroxylation	> 500			< 0.3	< 0.5	

[a] Reference 13
[b] References 8 and 34
[c] Reference 15
[d] Reference 12
[e] Reference 20; Kestell et al., unpublished data
[f] References 4 and 27

of DMXAA's antitumour activity by L-thalidomide cannot be excluded. A smaller but still significant increase (23%) in the plasma DMXAA AUC was observed with L-thalidomide in healthy male mice in these studies. The reasons for the apparent gender differences are not clear. No obvious gender differences in DMXAA pharmacokinetics with or without diclofenac were observed in our study.

At concentrations that may be obtainable in vivo, L-thalidomide did not inhibit DMXAA glucuronidation or 6-methylhydroxylation in vitro in mouse or human microsomes. High concentration of L-thalidomide (500 μM) resulted in a 30–60% inhibition of the less-important 6-methylhydroxylation pathway, but it is doubtful whether these concentrations would be achieved in the liver in vivo. Maximum plasma thalidomide concentrations in mice following a dose of 100 mg/kg i.p. are approximately 150 ± 50 μM, declining with a $T_{1/2}$ of approximately 2 h (Kestell, unpublished data). Thalidomide exhibits 50–60% plasma protein binding [32], and thus the maximum free concentrations in the liver might be expected to be in the range 50–100 μM during the 8-h study of the pharmacokinetics of DMXAA. Our in vitro studies indicate that it is highly unlikely that the alteration of DMXAA's kinetics in the mouse is due to a direct inhibitory effect (either competitively or non-competitively) by L-thalidomide on DMXAA metabolism.

Diclofenac had a similar impact on DMXAA's pharmacokinetics in vivo in mice as L-thalidomide. In contrast, however, diclofenac caused > 70% and > 60% inhibition of DMXAA glucuronidation and 6-methylhydroxylation, respectively, in mouse liver microsomes at 100 μM. Diclofenac's impressive inhibitory actions appear to have a smaller impact in vivo, which may be due to low free concentrations of diclofenac in plasma and liver (99% plasma protein binding), and its relatively short $T_{1/2}$ in mice (approximately 20 min over the initial 1 h after the administration phase) [13].

Preincubation with L-thalidomide did not inhibit DMXAA metabolism, excluding the possibility of inhibition by activated metabolites of thalidomide or its hydrolysis products. In addition, L-thalidomide did not displace DMXAA from plasma protein binding sites. Thus the longer $T_{1/2}$ could not be explained by a larger apparent volume of distribution due to protein binding changes. Possible mechanisms for the pharmacokinetic DMXAA-thalidomide interaction include modulation of carrier-mediated transportation processes for DMXAA and/or its metabolites, alteration in the enterohepatic recirculation of DMXAA-G, or perhaps an altered rate of absorption of DMXAA from the i.p. site. Another possibility is that L-thalidomide modulates DMXAA-metabolizing enzyme activities by indirect mechanisms through regulation of cytokines and nitric oxide (NO) [1, 17]. DMXAA induces NO [30, 31] and tumour necrosis factor [7, 22, 23], and there is considerable evidence that NO and cytokines modulate drug-metabolizing enzymes, including P450s and UGTs at the mRNA, protein translation, and catalytic activity levels [19, 29]. More recently, in vivo mouse studies have indicated that coadministration of thalidomide with DMXAA reduces biliary DMXAA-G concentrations, suggesting that thalidomide possibly interferes with either DMXAA glucuronidation, or its transportation from hepatocytes into the bile [16].

It is important to be able at early stages of drug development to predict from in vitro data in vivo pharmacokinetic changes in order to avoid possible toxic drug-drug interactions [10, 11]. The predictions from our in vitro data and the equations of Ito et al. [11] indicate a 20% increase in DMXAA's AUC following coadministration of diclofenac in mice, which correlates well with the 24–31% observed in vivo. No increase in the AUC for DMXAA is predicted, however, in humans based on our in vitro studies in human liver microsomes.

The in vivo pharmacological interactions observed for L-thalidomide with DMXAA in mice could not be predicted from the in vitro data, illustrating the problems when using simple prediction models. Our prediction model had two assumptions: that both drugs are metabolized only in the liver, with possible competitive metabolic inhibition, and that there are only two metabolic pathways for DMXAA. The model cannot take into account inhibition by indirect mechanism through alterations in cytokines and/or NO concentrations or other complicating factors arising from drug disposition processes such as absorption, renal metabolism, and active transportation that may also play a role in drug interactions leading to inappropriate estimations of the pharmacokinetic changes [11].

This study demonstrated that a model based on the direct inhibition of metabolism appears to be appropriate for the prediction of diclofenac-DMXAA pharmacokinetic interactions in mice and humans in vivo, but is inappropriate for the prediction of L-thalidomide-DMXAA pharmacokinetic interactions.

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