Cancer Chemother Pharmacol (2001) 47: 541–544
DOI 10.1007/s002800100285

SHORT COMMUNICATION

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

A difference between the rat and mouse in the pharmacokinetic interaction of 5,6-dimethylxanthenone-4-acetic acid with thalidomide

Received: 7 December 2000 / Accepted: 16 February 2001 / Published online: 23 March 2001

Abstract *Purpose*: Coadministration of thalidomide, cyproheptadine or diclofenac has been shown to increase the area under the plasma concentration-time curve (AUC) of the novel antitumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in mice. The aim of this study was to further investigate these pharmacokinetic DMXAA-drug interactions in the rat model. *Methods*: The effects of coadministration of L-thalidomide, cyproheptadine or diclofenac on the pharmacokinetics of DMXAA were investigated in male Wistar Kyoto rats. The effects of L-thalidomide, cyproheptadine and diclofenac on microsomal metabolism and plasma protein binding of DMXAA were also investigated. *Results:* No significant alteration in the plasma concentration profile for DMXAA was observed following L-thalidomide pretreatment in rats. In contrast, when combined with diclofenac or cyproheptadine, the plasma AUC of DMXAA was significantly ($P < 0.05$) increased by 48% and 88% and the $T_{1/2}$ by 36% and 107%, respectively, compared to controls. Both diclofenac and cyproheptadine at 500 μ*M* caused a significant inhibition of DMXAA metabolism in rat liver microsomes. In contrast, L-thalidomide had no or little inhibitory effect on DMXAA metabolism in rat liver microsomes except for causing a 32% decrease in 6-methylhydroxylation at 500 μ*M*. None of the drugs had a significant effect on the plasma protein binding of DMXAA in the rat. *Conclusion:* These studies showed that coadministration of L-thalidomide did not alter the plasma DMXAA AUC in rats, in contrast to previous studies in mice, whereas diclofenac and cyproheptadine significantly reduced the plasma clearance of DMXAA in rats in a similar manner to their effect in mice. The cause of the species difference in the pharmacokinetic response to thalidomide by DMXAA is unknown, and indicates difficulties in predicting the outcome of such a combination in patients.

Keywords Cyproheptadine · Diclofenac · DMXAA · Thalidomide · Drug interaction

S. F. Zhou · M. D. Tingle · J. W. Paxton (✉)
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 either L-thalidomide (L-α-(*N*-phthalimido)glutarimide) or cyproheptadine with the novel antitumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) has been shown to potentiate the latter's antitumour effects in mice [7, 26], and also to increase the AUC for DMXAA [13, 26, 29]. Coadministration of L-thalidomide with DMXAA reduces the biliary excretion of DMXAA acyl glucuronide (DMXAA-G) in mice, suggesting that L-thalidomide may interfere with either DMXAA glucuronidation, or its transportation from the hepatocyte into the bile [13]. Further in vitro mouse studies have indicated that neither direct inhibition of metabolism by L-thalidomide (and/or its activated metabolites), nor plasma protein binding displacement interactions are responsible for DMXAA's altered pharmacokinetics [29]. In contrast, in vitro mouse studies have suggested that the reduced plasma clearance of DMXAA by diclofenac is due to direct competitive inhibition of DMXAA metabolism [29].

The aim of this study was to use the rat model to further investigate the pharmacokinetic interaction between L-thalidomide and DMXAA with a view to identifying the mechanism(s) involved. Diclofenac, which has potent inhibitory effects on the metabolism of DMXAA in vitro [16], was used as a positive control. Cyproheptadine was also included as it has demonstrated the ability

Table 1 Effect of diclofenac, cyproheptadine and L-thalidomide on the plasma pharmacokinetic parameters for DMXAA in rats. Values are means ± SD

Treatment	Dose (mg/kg)	$AUC_{(0–10h)}$ (μM·h)	C_{max} (μM)	$T_{1/2}$ (h)	AUC ratio[a]
DMXAA alone	30 (i.v.)	1505 ± 412	716 ± 280	1.55 ± 0.33	
+ Diclofenac	100 (i.p.)	2234 ± 144*	955 ± 253	2.01 ± 0.85	1.48
+ Cyproheptadine	30 (i.p.)	2823 ± 549*	973 ± 132	2.58 ± 0.64*	1.88
+ L-Thalidomide	150 (i.p.)	1511 ± 222	634 ± 162	1.85 ± 0.83	1.00

*$P < 0.05$ vs control, unpaired t-test
[a]$AUC_{(0–10h)}$(DMXAA + diclofenac, cyproheptadine, or L-thalidomide)/AUC(DMXAA alone)

to alter DMXAA's pharmacokinetics in the mouse [26]. The rat model was chosen as it offers several advantages: it allows easier intravenous (i.v.) administration of DMXAA, thus removing the potential for alterations in rate and extent of absorption of DMXAA from the intraperitoneal (i.p.) site (used in mouse studies); repeated blood sampling from a single animal is possible, thus reducing some of the variability observed with the mouse model; and a wider variety of more manageable in vitro models (e.g. rat hepatocytes and isolated perfused liver model) are available for subsequent studies.

Materials and methods

Chemicals and reagents

DMXAA, 2,5-dimethylxanthenone-4-acetic acid (SN24350, as internal standard), and L-thalidomide were synthesized in the Auckland Cancer Society Research Centre [6, 19]. Authentic DMXAA-G and 6-OH-MXAA were isolated and purified from the bile and urine of rats treated with DMXAA, and their structure confirmed by mass spectrometry and [^{1}H]-nuclear magnetic resonance [12]. Diclofenac and cyproheptadine were purchased from Sigma-Aldrich (Auckland, NZ); NADPH and UDPGA were from Roche Diagnostics NZ (Auckland, NZ). All other reagents were of analytical or HPLC grade as appropriate.

Drug administration and sampling

Healthy male Wistar Kyoto rats (180–230 g, $n = 5$ for each group) were treated with DMXAA (30 mg/kg) alone, or pretreated with L-thalidomide (150 mg/kg), cyproheptadine (30 mg/kg) or diclofenac (100 mg/kg). A previous study has indicated that 30 mg/kg DMXAA exhibits significant anticancer activity in Colon 38 tumour-implanted mice [12]. DMXAA (dissolved in sterile water) was administered via the tail vein under light halothane anaesthesia. Pretreatment drugs were dissolved in dimethylsulphoxide (DMSO, 1 µl/g body weight) given by i.p. injection 15 min prior to DMXAA. Controls received DMSO at 1 µl/g body weight. All experiments were conducted in subdued light. Blood (200 µl) was collected in heparinized tubes by snipping the tail tip at 0.25, 1, 2, 4, 6, 8 and 10 h following DMXAA administration. Plasma was separated immediately by centrifugation (1000 g for 15 min) and stored at –20°C until assayed.

In vitro metabolism and protein binding studies

Liver microsomes were prepared from male Wistar Kyoto rats as described previously [20], and protein concentration determined by the bicinchoninic acid method [23]. These rats also provided plasma for protein binding studies. All animal procedures were approved by the Animals Ethics Committee of the University of Auckland. The effects of L-thalidomide, cyproheptadine and diclofenac on DMXAA glucuronidation and 6-methylhydroxylation in rat liver microsomes were investigated under optimized conditions as previously described [28, 29]. When significant inhibition (> 30% at 100 μM inhibitor concentration) was observed, further inhibition kinetic studies were undertaken to determine K_i values. The effects of L-thalidomide, diclofenac and cyproheptadine on the protein binding of DMXAA in rat plasma were investigated using ultrafiltration [30].

HPLC assay

The determination of DMXAA, DMXAA-G and 6-OH-MXAA in plasma and microsomes by HPLC with fluorescence detection has been described previously [12, 27, 29]. All HPLC methods had acceptable accuracy (85–115% of true values) and precision (intra- and interassay coefficients of variation < 15%). The elimination half-life ($T_{1/2}$) and area under the plasma concentration-time curve (AUC) were calculated as described previously [29].

Results

No significant alteration in the plasma concentration profile for DMXAA was observed following L-thalido-

Table 2 Inhibition of in vitro rat DMXAA metabolism by L-thalidomide, diclofenac and cyproheptadine. DMXAA at a concentration of K_m was incubated at 37°C with pooled rat liver microsomes in the presence of L-thalidomide, cyproheptadine, or diclofenac. Values are the means ± SD from three determinations

Metabolic pathway	Inhibitor concentration (μM)	Diclofenac		Cyproheptadine		L-Thalidomide	
		Activity remaining (%)	K_i (μM)[a]	Activity remaining (%)	K_i (μM)[a]	Activity remaining (%)	K_i (μM)[a]
Glucuronidation	100	31.6 ± 3.4*	55	69.0 ± 2.0*	154	98.6 ± 3.4	> 500
	500	10.0 ± 0.8*		40.8 ± 1.5*		90.1 ± 1.2	
Hydroxylation	100	43.2 ± 0*	66	82.8 ± 1.3	387	80.1 ± 13	> 500
	500	27.9 ± 2.0*		40.5 ± 2.5*		68.5 ± 6.4*	

*$P < 0.05$ vs incubations in the absence of inhibitor
[a]Assessed using the methods describe in Materials and methods assuming the inhibition was competitive

mide pretreatment in rats. In contrast, when pretreated with diclofenac or cyproheptadine, the plasma $AUC_{(0-10h)}$ of DMXAA was significantly ($P < 0.05$) increased by 48% and 88% and the $T_{1/2}$ by 36% and 107%, respectively, compared to controls (Table 1). In rat liver microsomes, diclofenac (100 μM) caused significant ($P < 0.05$) inhibition of glucuronidation (> 68%) and 6-methylhydroxylation (> 54%) of DMXAA, whereas cyproheptadine (100 μM) caused significant ($P < 0.05$) inhibition of glucuronidation (31%), but little inhibition (17%, $P > 0.05$) of 6-methylhydroxylation (Table 2). Increasing the concentration of cyproheptadine to 500 μM resulted in inhibition of 59% and 60% for glucuronidation and 6-methylhydroxylation, respectively. In contrast, L-thalidomide resulted in negligible inhibition of DMXAA metabolism in rat liver microsomes, except for a 32% decrease ($P < 0.05$) in 6-methylhydroxylation at 500 μM. The apparent K_i of L-thalidomide for DMXAA metabolism was estimated to be > 500 μM. Preincubation of microsomes with L-thalidomide, cyproheptadine or diclofenac (500 μM) did not enhance the inhibition of either DMXAA glucuronidation or 6-methylhydroxylation. In plasma proteins, the unbound fraction of DMXAA (f_u $2.38 \pm 0.52\%$) was not altered significantly ($P > 0.05$) by diclofenac ($2.55 \pm 0.35\%$), L-thalidomide ($2.85 \pm 0.78\%$) or cyproheptadine ($2.47 \pm 0.68\%$), all at 500 μM.

Discussion

The coadministration of diclofenac or cyproheptadine significantly reduced the plasma clearance of DMXAA in rats as in mice. It appears likely that competitive inhibition of DMXAA-metabolizing enzymes is the major mechanism responsible for their effects, as plasma protein binding interactions were excluded. Diclofenac is mainly glucuronidated at the carboxylic acid side chain by UGT1A9/2B7 [4] and cyproheptadine *N*-glucuronidated by UGT1A3/1A4 [10, 11]. In addition, diclofenac is hydroxylated by multiple CYP isoforms including CYP2C9, CYP3A4 and CYP1A2 [2]. Cyproheptadine may be a substrate of CYP1A2, as the latter has been found to catalyse metabolic pathways associated with *O*- or *N*-demethylation and/or *N*-hydroxylation [15]. Thus, the competitive inhibitory effects on DMXAA glucuronidation (UGT1A9/UGT2B7-catalysed) [16] and 6-methylhydroxylation (CYP1A2-catalysed) [28] might have been expected, resulting in reduced DMXAA clearance in mice and rats. However, other mechanisms, such as inhibition of transportation and excretion, cannot be excluded.

In contrast to previous mouse studies, coadministration of L-thalidomide in rats did not alter the pharmacokinetics of DMXAA. No significant inhibition of DMXAA metabolism by L-thalidomide was observed in either rat or mouse liver microsomes [29], and yet there was a reduced plasma clearance in the mouse in vivo. It is perhaps not surprising that L-thalidomide did not show any direct competitive inhibition of DMXAA's metabolism, as the major degradation pathway for thalidomide is hydrolysis [21], and CYP appears to play only a minor role in its disposition [3, 25]. Possible explanations for this species-dependent thalidomide-DMXAA interaction in rodents include: (1) species differences in the metabolism and disposition of L-thalidomide (significant variation in the absorption, distribution and metabolism of thalidomide has been observed in mice and rats [21, 22]); and (2) faster thalidomide metabolism and disposition in rats than in mice (it could be speculated that more L-thalidomide is required for similar inhibition in rats than in mice. However, this was taken into consideration, as the L-thalidomide dose in rats was 150 mg/kg compared to 100 mg/kg in the previous mouse studies [13, 29]).

The pharmacological effects of thalidomide appear to be dependent on the animal species [9]. For example, rodents are less sensitive than rabbits to the teratogenic effects of thalidomide [8], and the Galago, a prosimian primate, is relatively resistant to the teratogenic effects of thalidomide [5]. In addition, thalidomide has been shown to inhibit angiogenesis in the rat and human aorta endothelial cell models when coincubated with human or rabbit liver microsomes, but not with rat liver microsomes [1]. Also, the regulation of TNF-α production by thalidomide has been reported to be bidirectional, depending on the cell type, the mode of stimulation, and the concentration of drug used [14]. Cytokines (such as TNF-α) and nitric oxide have been associated with the modulation of drug metabolism [17, 24], and may provide an explanation for the species difference in DMXAA pharmacokinetic response to L-thalidomide. L-Thalidomide was used in this study, as it has a greater effect than both D-thalidomide and racemic thalidomide on DMXAA pharmacokinetics in mice [13]. However, it should be noted that L-thalidomide racemizes in vivo [18].

Urinary disposition data from patients in a phase I trial have indicated that DMXAA is mainly eliminated by glucuronidation, and to a lesser extent by 6-methylhydroxylation, with up to 60% of the total dose excreted as DMXAA-G, 5.5% as 6-OH-MXAA, and 4.5% as the glucuronide of 6-OH-MXAA [31]. Although in vitro studies with human liver microsomes have indicated that L-thalidomide has little inhibitory effect on DMXAA metabolism [29], it is not possible to predict the influence of thalidomide on DMXAA pharmacokinetics in humans, as demonstrated by the in vivo differences in the DMXAA-thalidomide interactions between rat and mouse. Presumably, identification of the mechanism responsible for the reduced DMXAA clearance by thalidomide in the mouse would help in predicting the outcome of such a combination in patients, and work is in progress in our laboratory to do this.

Acknowledgements The authors appreciate the support of the Maurice and Phyllis Paykel Trust and the University of Auckland Research Fund. S.F. Zhou is a holder of an Auckland Medical Research Foundation Senior Scholarship.

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