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ORIGINAL ARTICLE

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Modulation of thalidomide pharmacokinetics by cyclophosphamide or 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in mice: the role of tumour necrosis factor

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Abstract *Purpose*: There is considerable current interest in the use of thalidomide as a single agent or in combination with drugs such as cyclophosphamide in the treatment of multiple myeloma and other cancers. Our previous work has shown that thalidomide potentiates the antitumour activity of both cyclophosphamide and 5,6-dimethylxanthenone-4-acetic acid (DMXAA) against murine Colon 38 tumours. In both of these cases, thalidomide extends the half-life ($t_{1/2}$) of the other drug. We wished to determine whether cyclophosphamide and DMXAA altered the $t_{1/2}$ of thalidomide. Since both thalidomide and DMXAA modulate tumour necrosis factor (TNF), we also wished to determine the role of TNF in this interaction. *Methods*: Mice with Colon 38 tumours were treated with cyclophosphamide (220 mg/kg) and/or thalidomide (20 mg/kg) or DMXAA (25 mg/kg) and thalidomide (100 mg/kg), combinations that have previously demonstrated synergistic activity. Plasma and tumour tissue drug concentrations were analysed by high-performance liquid chromatography. To determine the role of TNF, similar experiments were performed using mice defective in the TNF gene ($TNF^{-/-}$) or the TNF receptor-1 gene ($TNFR1^{-/-}$). *Results*: Coadministration of cyclophosphamide increased the thalidomide $t_{1/2}$ by 3.9- and 3.6-fold, respectively, in plasma and tumour tissue, with a corresponding increase in the concentration-time curve (AUC). The corresponding values following coadministration of DMXAA were 3.0- and 4.6-fold, respectively. Coadministration of cyclophosphamide had similar effects on thalidomide $t_{1/2}$ in C57Bl/6, $TNF^{-/-}$ and $TNFR1^{-/-}$ mice, while coadministration of DMXAA did not alter the $t_{1/2}$ or AUC in $TNF^{-/-}$ and $TNFR1^{-/-}$ mice. *Conclusions*: Both cyclophosphamide and DMXAA have a pharmacokinetic interaction with thalidomide, increasing $t_{1/2}$ and AUC. TNF mediates the effect of DMXAA on thalidomide pharmacokinetics but not that of cyclophosphamide.

Keywords Thalidomide · Cyclophosphamide · DMXAA · Pharmacokinetics · TNF

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Introduction

There is a renewed interest in the use of thalidomide for the treatment of cancer [9, 21], particularly for multiple myeloma [23]. Clinical trials of thalidomide as a single agent have yielded promising results and combinations with cytotoxic drugs, including cyclophosphamide, are being investigated [10]. The mechanism of the antitumour action of thalidomide is not yet understood, but may be related to its ability either to inhibit angiogenesis [6] and/or to suppress inflammatory responses such as tumour necrosis factor (TNF) production [18]. Preclinical studies in this laboratory have shown that thalidomide, while having no demonstrable antitumour activity against the murine Colon 38 tumour when administered alone, strongly potentiates the activity of cyclophosphamide when administered in combination [8]. Investigation of the mechanism of potentiation has indicated that coadministration of thalidomide significantly increases the area under the plasma concentration-time curve (AUC) as well as the plasma half-life ($t_{1/2}$) of the parent drug. It also prolongs the appearance and increases the AUC of its active metabolites [8].

A pharmacological interaction has also been demonstrated between thalidomide and DMXAA, an

antivascular agent that has recently completed two phase I clinical trials. DMXAA acts both through a direct effect on the tumour endothelium [3] and indirectly through the antivascular effects of TNF and other cytokines [5]. Coadministration of thalidomide significantly increases the AUC and $t_{1/2}$ of DMXAA within the tumour [12] and increases and prolongs intratumoral TNF production [2].

The question as to whether cyclophosphamide or DMXAA modulate the pharmacokinetics of thalidomide is of potential importance for clinical studies, but has not previously been addressed in an experimental system. In the study reported here we demonstrated that cyclophosphamide or DMXAA administration modulates the plasma and tissue pharmacokinetics of thalidomide in mice.

The production of cytokines, and particularly of TNF, is known to modulate the pharmacokinetics of a number of drugs, in many cases by modulating the activity of drug metabolism or transport [14, 17]. Both thalidomide and DMXAA have well-documented immunomodulatory actions, and in addition cyclophosphamide is known to suppress the production of inflammatory cytokines such as TNF [11]. Such activity may be relevant to the treatment of multiple myeloma and other types of cancer. We therefore compared the effects of cyclophosphamide and DMXAA on the pharmacokinetics of thalidomide in mice that are unable to mount normal TNF responses. We used mice defective in the TNF gene ($TNF^{-/-}$) as well as those defective in the TNF receptor-1 gene ($TNFR1^{-/-}$) and found these mice to distinguish the pharmacokinetic interactions between thalidomide and cyclophosphamide from those between thalidomide and DMXAA.

Materials and methods

Chemotherapeutic drugs and reagents

DMXAA was synthesized in this laboratory [20], and dissolved in saline for intraperitoneal (i.p.) injections at 25 or 50 mg/kg in a volume of 10 µl/g body weight. Thalidomide was obtained from Celgene Corporation (New Jersey, N.J.) through the courtesy of Dr George Muller, and was dissolved in dimethyl sulphoxide and injected i.p. at 20 or 100 mg/kg in a volume of 2.5 µl/g body weight. Cyclophosphamide (Sigma-Aldrich Company, St. Louis, Mo.) was dissolved in saline and administered i.p. at 220 mg/kg in a volume of 10 µl/g body weight. All other chemicals and solvents were of analytical grade and were purchased from Merck, Darmstadt, Germany.

Mice and tumour implantation

Wild-type C57Bl/6, and $TNF^{-/-}$ and $TNFR1^{-/-}$ mice on the C57Bl/6 background were obtained from the Animal Resources Unit, Faculty of Medical and Health Sciences, University of Auckland, and were used for experiments between 8 and 12 weeks of age. All experiments were approved by the Animal Ethics Committee and conformed to the Guidelines for Animal Welfare set out by the United Kingdom Co-ordinating Committee on Cancer Research. The Colon 38 tumour was implanted subcutaneously into the left flank of anaesthetized (sodium pentobarbitone, 82 mg/kg) mice, and used approximately 10–14 days after implantation when the tumours were approximately 6–8 mm in diameter.

Collection of samples

To determine of the effects of cyclophosphamide on thalidomide pharmacokinetics, C57Bl/6 mice with implanted Colon 38 tumours (three to six per group) were injected i.p. with thalidomide (20 mg/kg) alone or in combination with cyclophosphamide (220 mg/kg). Cyclophosphamide was administered in a separate solution directly after thalidomide. After 5, 15, 30, 60 or 120 min, mice were anaesthetised and bled through the ocular sinus into heparinized tubes during halothane anaesthesia. Mice were then killed by cervical dislocation, and brain, liver and tumour tissue were removed. To determine the effects of DMXAA on thalidomide pharmacokinetics, mice were injected with thalidomide (100 mg/kg) alone or in combination with DMXAA (25 mg/kg), and blood and tissues were removed 1, 2, 4, 8, 12 and 16 h after treatment. Blood was separated by centrifugation (3000 *g* for 10 min), and the plasma collected. Tumour, brain and liver tissues were blotted to remove excess fluid, weighed and homogenized in ice-cold Milli-Q water and stored along with the plasma samples at −80°C until assayed in duplicate.

To investigate the effect of TNF on thalidomide pharmacokinetics, C57Bl/6, $TNF^{-/-}$ and $TNFR1^{-/-}$ mice without tumours were used. Plasma was removed 0.5, 1, 2, 4 and 8 h after treatment with thalidomide (20 mg/kg) with or without cyclophosphamide (220 mg/kg), or 1, 2, 4, 8, 12 and 24 h after treatment with thalidomide (100 mg/kg) with or without DMXAA (25, 25 and 50 mg/kg in C57Bl/6, $TNF^{-/-}$ and $TNFR1^{-/-}$ mice, respectively).

Analysis of thalidomide

A specific assay was developed and validated using automated solid-phase extraction and high-performance liquid chromatography (HPLC) to measure thalidomide concentrations. Aliquots of plasma or tissue homogenate (100 µl) were vortexed with an internal standard, phenacetin (50 µl of a 120 µ*M* stock) and 10% trichloroacetic acid (100 µl). After centrifugation (3000 *g*, 10 min), the supernatant (200 µl) was diluted with Milli-Q water (800 µl). The samples were loaded onto a preconditioned 1 ml/100 mg (1 ml acetonitrile and 1 ml Milli-Q water) C18 Bond Elut column (Varian, Harbor City, Calif.). This was performed using an automated solid-phase extraction (ASPEC XL4, Gilson Medical, Middleton, Wis.). The columns were washed with 1 ml Milli-Q water and 50% acetonitrile and the compounds of interest eluted using 50% acetonitrile in Milli-Q water. The eluates were evaporated to dryness using a centrifugal evaporator (Jouan, St. Nazaire, France). The residues were reconstituted in 100 µl 0.1 *M* hydrochloric acid and aliquots (50 µl) of the resulting solutions automatically injected into a Waters Breeze chromatograph (Waters Associates, Milford, Mass.) which consisted of a Model 717plus autosampler, Model 1525 binary pump and Model 2487 dual wavelength absorbance detector. Compounds were separated using a 100×4.6 mm stainless steel Luna 5 µm Phenylhexyl column (Phenomenex, Torrance, Calif.) and a mobile phase (Milli-Q water/acetonitrile 10:1.3 v/v) that was pumped at a flow rate of 2 ml/min. Thalidomide and phenacetin were detected at ultraviolet wavelengths of 220 and 248 nm, respectively. The retention times of phenacetin and thalidomide and were 13 and 11 min, respectively. Data acquisition and integration was achieved using Breeze software (Waters, Milford, Mass.). A calibration curve of thalidomide (0.6–100 µ*M*) in human plasma was prepared fresh for each HPLC run. To construct the calibration curve, the peak-area ratios to the internal standard were plotted against thalidomide concentration and the best-fit straight line was obtained by linear regression analysis. The correlation coefficient of the calibration curves had r^2 values

of greater than 0.9995. The thalidomide/phenacetin peak ratio was determined for all samples and the concentration of thalidomide in each sample was determined from the calibration curve.

The intraassay accuracy and precision were acceptable with relative recoveries and coefficient of variation (CV) of 90–110% and 5–9% ($n=8$), respectively. Similar results were achieved for interassay accuracy and precision with relative recoveries and CVs of 96–104% and 2–4% ($n=15$). Quality control plasma and tissue homogenates with added three nominal thalidomide concentrations (0.6, 40 and 80 μM) were stored at −80°C. These were included in each analysis and were found to be stable over a period of 14 days and within 10% of the validated value ($n=15$).

Pharmacokinetic calculations

Pharmacokinetic parameters were determined using WinNonLin version 4.0.1 (WinNonlin Professional Software, Mountain View, Calif.). The optimal system was selected by visual assessment of the predicted curves determined by the Akaike Information Criterion and Schwartz Criterion. A non-compartmental method was found to be the best model for thalidomide pharmacokinetic calculations. The elimination rate constant (λ) was determined from the terminal linear portion of the concentration versus time curve. The elimination $t_{1/2}$ was calculated from the slope of the terminal phase of the log-linear concentration-time curve. The area under the plasma concentration versus time curve (AUC_{0-t}) from time zero to the last quantifiable concentration (C_t) was calculated by the trapezoidal rule. AUC extrapolated to infinity was calculated as $(AUC_{0-t})+C_t/\lambda$. Concentration was expressed as micromoles per litre or per kilogram of the mean ± SEM. Statistical significance was determined by Student's t-test with $P<0.05$ considered as significant.

Results

Effect of cyclophosphamide on thalidomide pharmacokinetics

Cyclophosphamide significantly ($P<0.05$) increased the maximum concentration (C_{max}) of thalidomide in plasma, brain and tumour, but not in liver (Fig. 1, Table 1). The $t_{1/2}$ of thalidomide was also significantly ($P<0.05$) increased in plasma, liver, brain and tumour by coadministered cyclophosphamide. The $t_{1/2}$ was increased 3.9- and 3.6-fold, respectively, in plasma and tumour and correspondingly the AUC was increased 3-fold in both plasma and tumour. The AUC and $t_{1/2}$ in brain and liver tissues increased 2- to 3-fold with coadministered cyclophosphamide.

Effect of DMXAA on thalidomide pharmacokinetics

Coadministered DMXAA had no effect on thalidomide C_{max}, but significantly ($P<0.05$) increased thalidomide $t_{1/2}$ in plasma, brain, liver and tumour (Fig. 2, Table 1). Thalidomide $t_{1/2}$ in tumour and plasma were increased 4.6- and 3-fold, respectively, and the AUC was increased 2.4- and 1.6-fold in tumour and plasma, respectively. The AUC and $t_{1/2}$ in brain and liver tissues were also increased approximately 2-fold by DMXAA. To rule out the possibility that the pharmacokinetic alterations were due to changes in drug absorption when the two drugs were both administered i.p., we compared plasma thalidomide concentrations at the 8-h time-point in mice to which the two drugs were given i.p. with the concentrations in mice to which thalidomide was given i.p, but DMXAA was given intravenously. There was no significant difference in plasma thalidomide concentrations in mice to which both drugs were given i.p. (75 ± 10 μM) and in mice to which DMXAA was given intravenously

Fig. 1 Thalidomide concentrations at different times after treatment in plasma, brain, liver and Colon 38 tumours from mice treated with thalidomide at 20 mg/kg alone (○) or coadministered with cyclophosphamide at 220 mg/kg (●). Each point represents the mean ± SEM from three to six mice

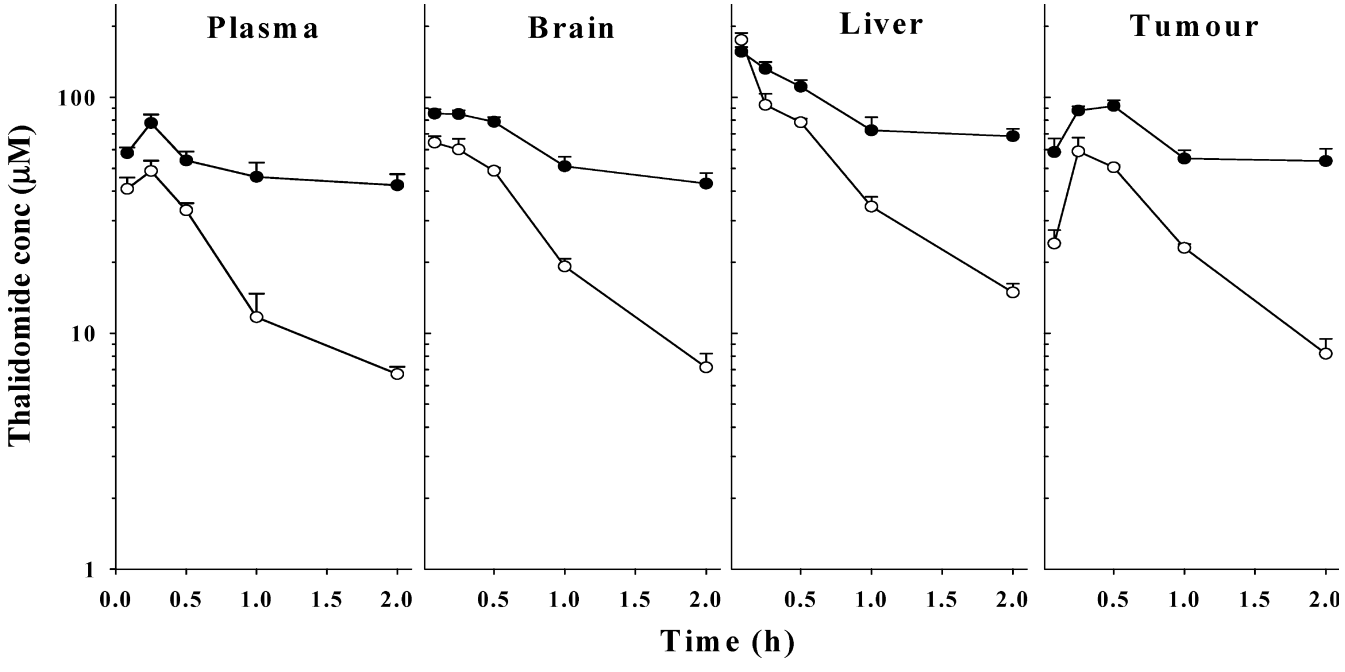


Table 1 Comparison of the effects of cyclophosphamide and DMXAA on thalidomide pharmacokinetics in Colon 38-bearing mice

Tissue	Treatment	Dose (mg/kg)	C_{max} (μmol/l or μmol/kg)[a]	$AUC_{(0-\infty)}$ (μmol·h/l)	$t_{1/2}$ (h)	$t_{1/2}$ ratio	AUC ratio
Plasma	Thalidomide	20	49 ± 5	29	0.6 ± 0.1	3.9	2.9
	Thalidomide + cyclophosphamide	20 + 220	78 ± 7*	85	2.4 ± 0.6*		
Liver	Thalidomide	20	175 ± 12	93	0.6 ± 0.1	2.9	1.8
	Thalidomide + cyclophosphamide	20 + 220	156 ± 7	170	1.6 ± 0.4*		
Brain	Thalidomide	20	64 ± 4	52	0.6 ± 0.1	3.0	2.2
	Thalidomide + cyclophosphamide	20 + 220	85 ± 4*	114	1.8 ± 0.3*		
Tumour	Thalidomide	20	59 ± 8	46	0.7 ± 0.1	3.6	2.8
	Thalidomide + cyclophosphamide	20 + 220	92 ± 5*	125	2.5 ± 0.6*		
Plasma	Thalidomide	100	135 ± 9	649	1.3 ± 0.02	3.0	1.6
	Thalidomide + DMXAA	100 + 25	135 ± 12	1004	3.9 ± 0.4*		
Liver	Thalidomide	100	83 ± 12	353	1.5 ± 0.2	2.1	1.7
	Thalidomide + DMXAA	100 + 25	117 ± 31	585	3.2 ± 0.4*		
Brain	Thalidomide	100	140 ± 50	586	2.0 ± 0.4	2.5	1.5
	Thalidomide + DMXAA	100 + 25	137 ± 6	851	5.0 ± 0.8*		
Tumour	Thalidomide	100	179 ± 54	618	2.1 ± 0.5	4.6	2.4
	Thalidomide + DMXAA	100 + 25	160 ± 18	1468	9.7 ± 2*		

*$P < 0.05$ vs thalidomide alone
[a]Means ± SEM from at least three mice per group

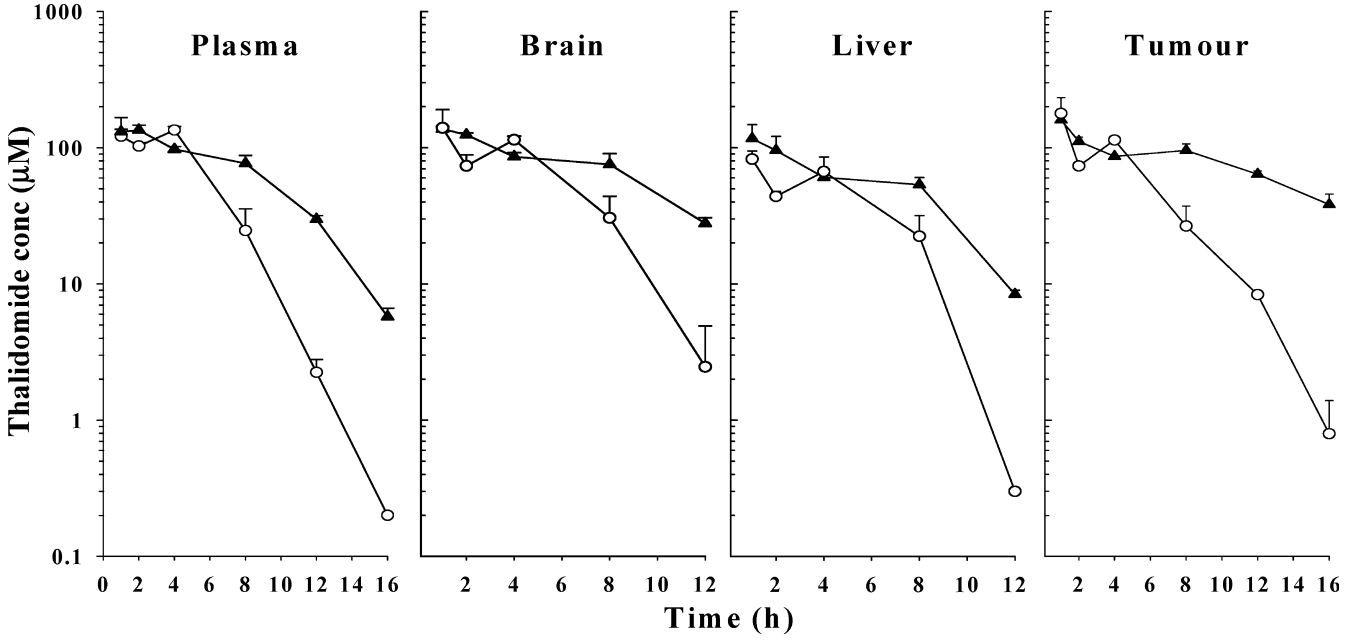


Fig. 2 Thalidomide concentrations in plasma, brain, liver and Colon 38 tumours of mice at different times after treatment with thalidomide alone at 100 mg/kg (○) or coadministered with DMXAA at 25 mg/kg (▲). Each point represents the mean ± SEM from three to six mice

(84 ± 18 μ*M*). Both were significantly higher than the concentrations in mice given thalidomide alone (11 ± 2 μ*M*), indicating that thalidomide concentrations were altered by DMXAA coadministered by either route.

Role of TNF in the thalidomide pharmacokinetic interactions

To investigate whether endogenous TNF played a role in the observed pharmacokinetic interactions, experiments were carried out in $TNF^{-/-}$ mice, which do not produce functional TNF, and in $TNFR1^{-/-}$ mice, which are defective in their ability to respond to the cytokine. The C_{max} and $t_{1/2}$ values in $TNF^{-/-}$ and $TNFR1^{-/-}$ mice for thalidomide administered alone were found to be comparable to those for wild-type C57Bl/6 mice (Table 2). Coadministration of cyclophosphamide caused 2.9- and 2.6-fold increases in thalidomide AUC in $TNF^{-/-}$ and $TNFR1^{-/-}$ mice, respectively (Table 2, Fig. 3B, C), similar to the effects observed in wild-type mice (Fig. 3A). Preliminary experiments also showed that coadministration of thalidomide increased the AUC of cyclophosphamide in $TNF^{-/-}$ mice to a similar extent to that reported [8] in C57Bl/6 mice (results not shown).

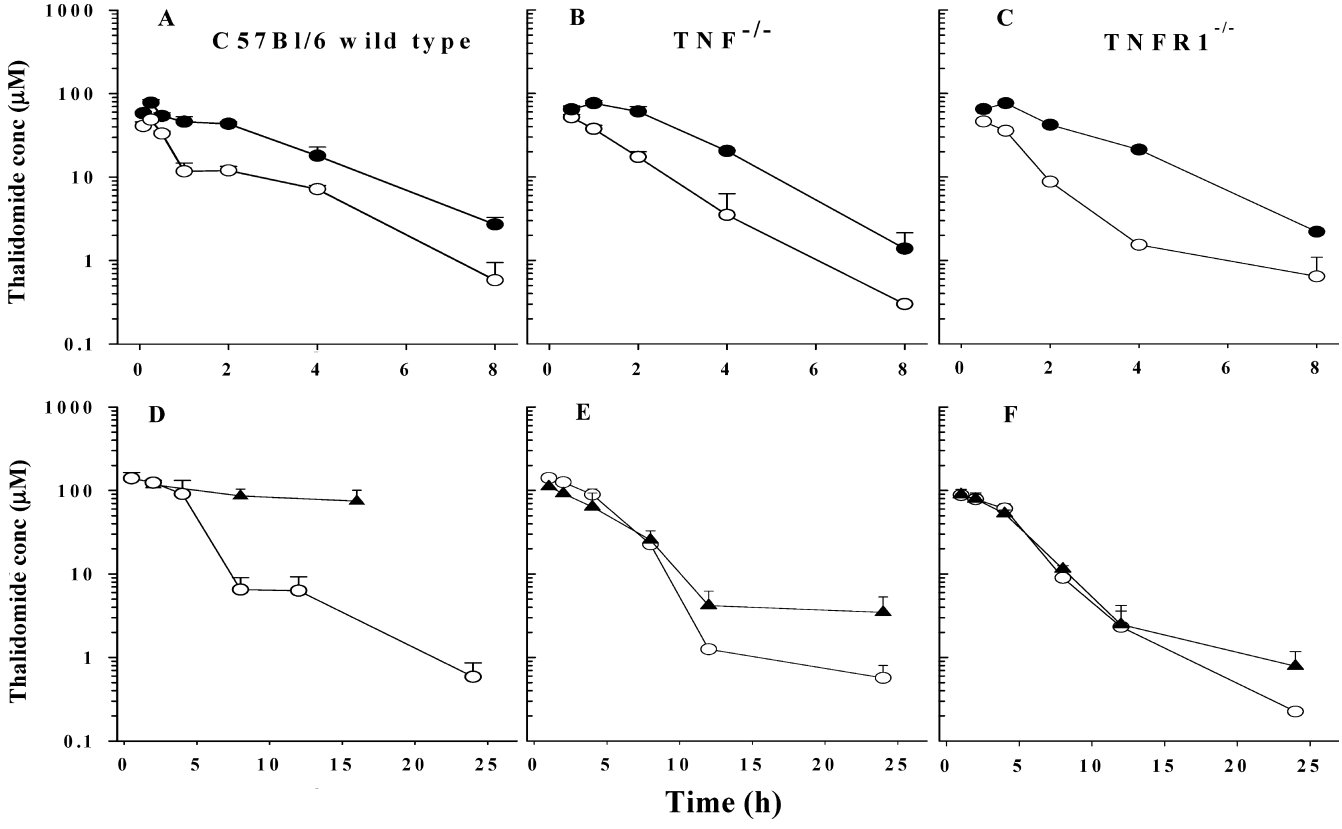
Table 2 Comparison of the effect of cyclophosphamide and DMXAA on plasma thalidomide pharmacokinetics in wild-type, $TNF^{-/-}$ and $TNFR1^{-/-}$ mice

Mouse strain	Treatment	Dose (mg/kg)	C_{max} (μmol/l or μmol/kg)[a]	$AUC_{(0-\infty)}$ (μmol·h/l)	$t_{1/2}$ (h)	$t_{1/2}$ ratio	AUC ratio
C57Bl/6	Thalidomide	20	49 ± 5	62	1.4 ± 0.3	1.2	2.8
	Thalidomide + cyclophosphamide	20 + 220	78 ± 7*	176	1.7 ± 0.2		
$TNF^{-/-}$	Thalidomide	20	52 ± 4	71	1.0 ± 0.03	1.3	2.9
	Thalidomide + cyclophosphamide	20 + 220	77 ± 6*	206	1.3 ± 0.2		
$TNFR1^{-/-}$	Thalidomide	20	46 ± 3	71	1.0 ± 0.15	1.4	2.6
	Thalidomide + cyclophosphamide	20 + 220	76 ± 6*	187	1.4 ± 0.2		
C57BL/6	Thalidomide	100	141 ± 24	659	2.0 ± 0.6	10.5[b]	2.1[b]
	Thalidomide + DMXAA	100 + 25	117 ± 10	1371[b]	21 ± 15[b]		
$TNF^{-/-}$	Thalidomide	100	141 ± 8	581	1.7 ± 0.3	1.4	0.84
	Thalidomide + DMXAA	100 + 25	111 ± 1	488	2.4 ± 0.3		
$TNFR1^{-/-}$	Thalidomide	100	88 ± 15	350	2.0 ± 0.2	1	1
	Thalidomide + DMXAA	100 + 50	89 ± 2	366	2.0 ± 0.2		

*$P < 0.05$ vs thalidomide alone
[a]Means ± SEM from at least three mice per group
[b]Data analysed over the interval 0–16 h

To study DMXAA-thalidomide interactions, we used the same drug doses as those in wild-type mice with the exception of $TNFR1^{-/-}$ mice in which we used 50 mg/kg DMXAA because these mice have a higher tolerance to the drug [26]. As in the case of the tumour-bearing mice, coadministration of DMXAA increased both the $t_{1/2}$ and AUC of thalidomide (Table 2). However, in contrast to the situation with cyclophosphamide, DMXAA coadministration did not significantly alter thalidomide plasma pharmacokinetics in $TNF^{-/-}$ and $TNFR1^{-/-}$ mice as compared to wild-type mice (Fig. 3).

Fig. 3A–F Plasma thalidomide concentrations at different times after treatment with thalidomide alone at 20 mg/kg (○) or coadministered with cyclophosphamide at 220 mg/kg (●) in C57Bl/6 (**A**), $TNF^{-/-}$ (**B**), or $TNFR1^{-/-}$ mice (**C**), or after treatment with thalidomide alone at 100 mg/kg (○) or coadministered with DMXAA (▲) at 25 mg/kg in C57Bl/6 (**D**) and $TNF^{-/-}$ mice (**E**) or at 50 mg/kg in $TNFR1^{-/-}$ mice (**F**). Each point represents the mean ± SEM from three to six mice



Discussion

Of the anticancer agents that we have screened for interaction with thalidomide, cyclophosphamide and DMXAA have produced the greatest synergistic effects. Thalidomide has no demonstrable antitumour effect against Colon 38 when administered alone [2, 4], but in combination with these drugs causes regression of tumours in 100% of the mice [4, 8]. Concomitantly with this increased antitumour activity, we found that the $t_{1/2}$ and AUC of DMXAA, cyclophosphamide and cyclophosphamide metabolites are significantly increased when thalidomide is coadministered [8, 12]. Here we demonstrated the converse result, that the $t_{1/2}$ and AUC of thalidomide in mice were increased by coadministered cyclophosphamide and DMXAA. A similar increase in AUC (approximately 2.8-fold) was seen in both tumour-bearing and non-tumour-bearing mice, although the AUC of thalidomide, when administered alone, was lower in tumour-bearing mice than in non-tumour-bearing mice (Tables 1 and 2). It is also of interest that there was a dose dependence: the AUC values following a dose of 20 mg/kg were divergent (29 and 62 μmol·h/l in tumour-bearing and non-tumour-bearing mice, respectively) but were similar (600 μmol·h/l in both cases) at a higher dose (100 mg/kg). A possible reason for these effects is that the presence of the tumour changes the rates of thalidomide hydroxylation and subsequent removal.

There are several possible explanations as to why the $t_{1/2}$ and AUC of thalidomide were increased by cyclophosphamide. One is that they compete for the same metabolizing enzymes. Members of the hepatic CYP450 isoenzyme family catalyse not only the conversion of cyclophosphamide to its active 4-hydroxycyclophosphamide metabolite [25], but also the formation of 5- and 5′-hydroxythalidomide [1]. However, in vitro drug inhibition studies have shown no direct interaction between cyclophosphamide and thalidomide [19]. Thalidomide is transformed in mice to a number of products and hydroxylation is only one of the pathways [15]. A second possible mechanism is that the interaction involves competition for drug transport proteins, although there have been no reports of common transport pathways for the elimination of cyclophosphamide and thalidomide metabolites. A third possible explanation for the interaction between cyclophosphamide and thalidomide is that it reflects an effect of cyclophosphamide on the function of the vascular endothelium. Cyclophosphamide metabolites might damage the hepatic sinusoidal endothelium, leading to microcirculation dysfunction [7, 16].

We showed here that, in contrast to cyclophosphamide, DMXAA was unable to increase thalidomide $t_{1/2}$ and AUC unless TNF function was present (Fig. 3, Table 2). This result is surprising because the induction of plasma TNF in response to DMXAA is inhibited by the coadministration of thalidomide [4]. However, tissue TNF concentrations are not reduced to the same extent, and liver concentrations, which may be important for the elimination of thalidomide, may even be increased 4 h after coadministration of thalidomide [2]. The similarity of thalidomide pharmacokinetic effects in wild-type, $TNF^{-/-}$ and $TNFR1^{-/-}$ mice, together with the observation that the effects of DMXAA on thalidomide pharmacokinetics (Fig. 3) occurred over a time where DMXAA induces TNF [2, 27], supports the hypothesis that DMXAA-induced TNF rather than endogenous TNF is responsible for the effect. One possible mechanism is that TNF induction inhibits thalidomide metabolism. TNF is known to affect both CYP450- and uridine diphosphate glucuronyl-transferase-dependent metabolic pathways [17], which are major pathways for both DMXAA [24] and thalidomide in mice [15]. TNF also decreases the activity of α_1-acid glycoprotein [13, 14], a protein reported to be involved in the hepatic transport of thalidomide [22]. In vitro drug inhibition studies have failed to demonstrate a direct interaction between DMXAA and thalidomide [28]. A further possibility is that the production of TNF affects the function of the liver vascular endothelium, reducing uptake and metabolism of thalidomide.

In conclusion, the results raise the question as to whether the pharmacokinetics of thalidomide in humans might be modified by combination therapy. The plasma half-life of thalidomide in humans is longer than that in mice, presumably as a consequence of reduced production of metabolites [28]. Nevertheless, if cyclophosphamide itself, or a metabolic product, modifies the elimination of thalidomide it might decrease its elimination in humans. The effect of TNF on thalidomide elimination in mice might also be considered in a clinical context.

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