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ORIGINAL ARTICLE

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Potentiation of the antitumour effect of cyclophosphamide in mice by thalidomide

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Abstract *Purpose:* Thalidomide has recently shown significant promise in the treatment of some types of cancer, and trials in combination with conventional chemotherapy are being undertaken. We wished to determine whether thalidomide potentiated the effect of cyclophosphamide, a commonly used cytotoxic drug, in a murine tumour model. *Methods:* C57Bl/6 mice implanted with subcutaneous Colon 38 tumours were treated with cyclophosphamide alone or together with thalidomide as a single intraperitoneal injection and tumour growth was measured. Concentrations of cyclophosphamide, 4-hydroxycyclophosphamide, 4-ketocyclophosphamide and 2-dechloroethylcyclophosphamide were determined in plasma, liver and tumour tissue using coupled high-performance liquid chromatography-mass spectrometry at different times after treatment. *Results:* Cyclophosphamide alone (220 mg/kg) induced growth delays of 11–13 days with no cures, whereas cyclophosphamide together with thalidomide (100 mg/kg) cured mice of their tumours. Thalidomide at lower doses (1–20 mg/kg) also potentiated the antitumour effect. Coadministration of thalidomide (100 mg/kg) dramatically decreased the clearance of cyclophosphamide and its metabolites from plasma and tissue, with corresponding increases in the area under the concentration-time curves. The magnitude of the effect was dependent on the dose of thalidomide over the range 1–20 mg/kg with no further effect at a dose of 100 mg/kg. *Conclusions:* Coadministration of thalidomide and cyclophosphamide gave markedly greater activity against Colon 38 tumour compared with either drug alone. Investigation of the reason for this effect revealed thalidomide to possess the novel property of dramatically decreasing the clearance of cyclophosphamide and its metabolites.

Keywords Thalidomide · Cyclophosphamide · Pharmacokinetics · Antitumour · Colon 38

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Introduction

Thalidomide, marketed initially for its sedatory effects [14] and later rediscovered for its effectiveness in controlling the acute symptoms associated with a number of inflammatory and autoimmune diseases [2], has recently shown promise in the treatment of cancer [8]. The clinical evaluation of thalidomide as an anticancer agent followed from the demonstration that thalidomide could inhibit angiogenesis [7]. Observations that bone marrow of patients with haematological cancers have increased vascularity [17] provided the rationale for applying thalidomide to the treatment of multiple myeloma, in which it has shown noteworthy activity [20]. Activity against renal carcinomas [14] and gliomas [10] has also been observed and thalidomide is also currently being tested as a treatment for prostate cancer [9]. Following these promising results as a single agent, combination trials of thalidomide with conventional chemotherapy are now being considered.

Previous studies by our group have shown that thalidomide improves the antitumour activity of the new anticancer agent DMXAA (5,6-dimethylxanthenone-4-acetic acid), an agent that has an indirect antitumour action, involving intratumoral cytokine production and the induction of tumour vascular collapse [1]. DMXAA alone induced a 67% cure rate in murine Colon 38

tumours, thalidomide alone had no effect, but the combination led to complete eradication of tumours [5]. Coadministration of thalidomide both elevated and sustained intratumoral concentrations of the cytokine tumour necrosis factor (TNF) [3]. However, coadministration of thalidomide also increased the area under the plasma concentration-time curve (AUC) of DMXAA twofold and the half-life ($T_{1/2}$) threefold [13]. The basis for the productive interaction between thalidomide and DMXAA could therefore involve pharmacokinetic interactions as well as immunomodulation.

Cyclophosphamide is commonly used in clinical cancer treatment and is also active in the murine Colon 38 tumour model. In the study reported here, we sought to determine whether thalidomide potentiates the toxicity and antitumour effects of cyclophosphamide, and also whether it modulates the pharmacokinetics of cyclophosphamide. We used coupled high-performance liquid chromatography-mass spectrometry (LC-MS) to determine the effects of thalidomide on the major metabolites of cyclophosphamide in plasma, liver, and tumour tissue.

Materials and methods

Materials

Racemic thalidomide (synthesized at Celgene Corporation) [15] was dissolved in dimethyl sulphoxide and administered intraperitoneally (i.p.) in a volume of 2.5 µl/g body weight. Cyclophosphamide (Sigma-Aldrich Company) was dissolved in saline and administered i.p. in a volume of 10 µl/g body weight. Ifosfamide was kindly supplied by Dr. U. Niemeyer (Asta Medica, Frankfurt, Germany). Other chemicals or solvents used were of analytical grade.

Tumour growth delays

C57Bl/6 male mice (8–12 weeks old) bred in the Animal Resources Unit, Faculty of Medical and Health Sciences, University of Auckland, were used in all experiments, with observance of all institutional animal ethical guidelines. Colon 38 tumour fragments (1 mm^3) were implanted in the left flank of anaesthetized (pentobarbitone, 81 mg/kg) mice. Growth delay experiments were initiated when the tumours reached approximately 4 mm in diameter. Mice were treated with drugs and the tumours measured thrice weekly. Tumour volume was calculated as $0.52 \times a^2 \times b$, where *a* and *b* were the minor and major tumour axes. The arithmetic means and standard error of the means were calculated for each time-point, counting cured animals as having zero tumour volume, and expressed as fractions of the pretreatment tumour volume. Growth delay was determined as the difference in the number of days required for the untreated and treated tumours to reach four times the pretreatment volume.

Pharmacokinetic studies

Tumour-bearing mice (three to six per group) were treated with cyclophosphamide (220 mg/kg) alone or coadministered with thalidomide (100 mg/kg). After 5, 15, 30, 45, 60, 90 and 120 min, mice were anaesthetized with halothane and blood was collected from the ocular sinus into heparinized tubes and the plasma was separated by centrifugation (10,000 *g* for 10 min). Tumours were excised, blotted to remove any fluid, weighed and homogenized in 28 m*M* phosphate buffer (1 ml, pH 4.0). Tumour homogenates and plasma were stored at –20°C until analysis.

Having established the concentration-time profiles for cyclophosphamide (220 mg/kg), with and without thalidomide (100 mg/kg), blood collection 1 h after administration was selected as the appropriate sampling time for further studies on the effects of lower doses (1–80 mg/kg) of thalidomide on cyclophosphamide (220 mg/kg).

Assay of cyclophosphamide, 4-ketocyclophosphamide and 2-dechloroethylcyclophosphamide

A specific assay was developed using automated solid-phase extraction and LC-MS. Plasma (100 µl) was mixed with 28 m*M* phosphate buffer pH 7.4 (400 µl) in a glass tube containing the internal standard solution (50 µl, 77 µ*M* ifosfamide) and loaded onto preconditioned (1 ml acetonitrile and 1 ml Milli-Q water) C18 Bond Elut columns (1 ml/100 mg; Varian, Harbor City, Calif.). The columns were then washed with Milli-Q water and the compounds of interest eluted using acetonitrile. Activation of the columns, whole sample preparations and manipulations were all carried out automatically by an ASPEC XL4 system (Gilson Medical, Middleton, Wis.). The eluates were evaporated to dryness, the residues reconstituted in mobile phase (Milli-Q water/acetonitrile, 87:23 v/v) and analysed by LC-MS using an Agilant 1100 Series LC/MSD system (Agilant Technologies, Avondale, Pa.), an atmospheric pressure chemical ionization interface and single ion monitoring mode at m/z 199, 261, 275. The fragmenter and capillary voltages of the interface were set at 100 and 3500 V, respectively. Other interface settings were: drying gas flow rate 5 l/min, corona current 4 µA, vaporizing temperature 400°C and nebulizing pressure 276 kPa. Chromatographic separation was achieved with a LUNA C8 5 µm 100×4.6 mm stainless steel column (Phenomenex, Torrance, Calif.) with a mobile phase flow rate of 0.5 ml/min. The retention times of cyclophosphamide and the internal standard were 12.0 and 13.5 min, respectively. Those of 4-ketocyclophosphamide and 2-dechloroethylcyclophosphamide were 5.3 and 9.5 min. Tumour and liver homogenates (200 µl) were similarly processed except that before solid-phase extraction, proteins were precipitated by the addition of 750 µl acetonitrile containing 50 µl internal standard followed by centrifugation. The resulting supernatants were diluted in Milli-Q water (9 ml) and extracted as described above.

The calibration samples were prepared by adding cyclophosphamide, 4-ketocyclophosphamide and 2-dechloroethylcyclophosphamide to mouse plasma, liver or tumour homogenates over the concentration range 3.58–358 µ*M* for cyclophosphamide and 1.25–100 µ*M* for 4-ketocyclophosphamide and 2.5–100 µ*M* for 2-dechloroethylcyclophosphamide. The peak-area ratios to the internal standard were plotted against concentration for each of these compounds and the best fit straight line obtained by linear regression analysis. The intraassay accuracy and precision were acceptable with relative recoveries and coefficients of variation (CV) of 99–114% and 0.5–8.2% ($n = 9$), respectively. Similar results were achieved for interassay accuracy and precision with relative recoveries of 98–108% and CVs of 0.5–6% ($n = 9$). Quality control samples for these compounds were prepared using mouse plasma, liver or tumour homogenates, stored at –80°C and included in each analytical run. The concentrations of cyclophosphamide, 4-ketocyclophosphamide and 2-dechloroethylcyclophosphamide were found to be within 10% of the validated value when standards were stored at this temperature for 3 weeks.

Assay of 4-hydroxycyclophosphamide

Plasma, liver or tumour homogenates (50 µl) were mixed with semicarbazide (500 m*M*) in phosphate buffer (pH 7.4, 28 m*M*, 100 µl) in a glass tube containing the internal standard solution (50 µl, 62.5 µ*M* 4-hydroxyifosphamide), and incubated at 60°C for 60 min. After centrifugation (10,000 *g*, 10 min), supernatants were removed and diluted with 800 µl Milli-Q water and loaded onto

preconditioned (1 ml acetonitrile and 1 ml Milli-Q water) C18 Bond Elute columns (1 ml/100 mg, Varian, Harbor City, Calif.). The columns were then washed with Milli-Q water and compounds of interest eluted using acetonitrile. Activation of the ASPEC columns, whole sample preparation and manipulations were all carried out as above, except that ammonium acetate pH 6.0/acetonitrile (77:23 v/v) was used as the mobile phase for reconstitution of the eluates, and that single ion monitoring was carried out at m/z 334. Chromatographic separation was achieved using a LC-MS system fitted with a Zorbax Eclipse XDB-C8 5 μm 150×4.6 mm stainless steel column (Agilant Technologies, Avondale, Pa.) and a mobile phase flow rate of 0.5 ml/min. The retention times of 4-hydroxycyclophosphamide and the internal standard were 6.8 and 6.1 min, respectively. Intraassay accuracy and precision were achieved with relative recoveries and CVs of 92–112% and 0.2–8.5% ($n=9$), respectively. As 4-hydroxycyclophosphamide is unstable in biological matrix, it was not possible to do the interassay precision and accuracy as above. However, to get a measure of the variability between assays, fresh quality samples were prepared and analysed with every assay.

Pharmacokinetic analysis

The AUC was calculated as a function of time using the log trapezoidal rule. The elimination half-life ($t_{1/2}$) was calculated as $\ln 2/k_{el}$. The determination of statistical significance was based on the unpaired Student's *t*-test, with $P<0.05$ being considered significant.

Results

Effect of thalidomide on the antitumour activity of cyclophosphamide

Cyclophosphamide, administered at its maximum tolerated single dose (275 mg/kg i.p.) in C57Bl/6 mice, produced a growth delay in the Colon 38 tumour of 13 days. The effect of coadministration of thalidomide (100 mg/kg), which has been shown previously to have no effect on the growth of Colon 38 tumours [3, 5], was measured. Evidence of toxicity was observed with this combination with some deaths occurring after 9–10 days. A lower dose of cyclophosphamide (220 mg/kg), which also induced a growth delay of 13 days, was therefore employed, and coadministration of thalidomide (100 mg/kg) induced complete regression of the Colon 38 tumour in all mice with no regrowth over the following 3 months of monitoring (Fig. 1). The dose-dependence of potentiation of the antitumour effects of cyclophosphamide was also determined (Fig. 2). In this experiment cyclophosphamide (220 mg/kg) alone gave a growth delay of 11 days (20% cure rate), coadministration of thalidomide at 1 mg/kg increased the growth delay to 21 days (20% cure rate) and coadministration of thalidomide at 20 mg/kg increased the growth delay to 27 days (60% cure rate).

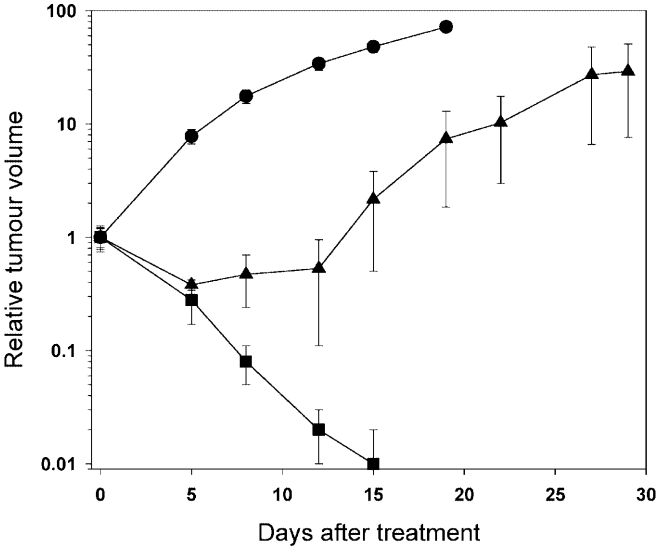

Fig. 1. Antitumour activity of thalidomide and cyclophosphamide in combination. Growth of Colon 38 tumours in mice (six per group): untreated (*circles*), or treated with cyclophosphamide (220 mg/kg) only (*triangles*), or with cyclophosphamide together with thalidomide (100 mg/kg) (*squares*)

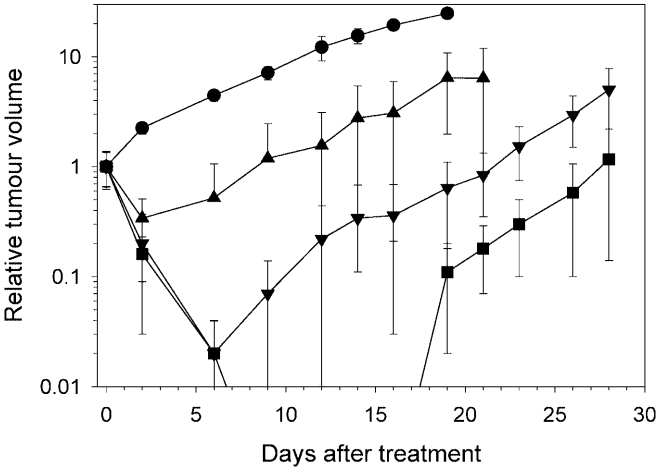

Fig. 2. Dose dependence of antitumour activity of cyclophosphamide and thalidomide. Tumour-bearing mice were untreated (*circles*), or treated with cyclophosphamide (220 mg/kg) only (*triangles pointing up*), or together with thalidomide at 1 mg/kg (*triangles pointing down*) or 20 mg/kg (*squares*)

Effect of thalidomide on the pharmacokinetics cyclophosphamide and its metabolites

Concentration-time profiles in plasma (Fig. 3), liver (Fig. 4) and tumour tissue (Fig. 5) were determined for cyclophosphamide, 4-hydroxycyclophosphamide and 4-ketocyclophosphamide either with or without coadministration of thalidomide. Plasma concentrations of 2-dechloroethylcyclophosphamide, which was present at a much lower concentration than the other two metabolites, were also determined (Fig. 3). Coadministration of thalidomide caused a dramatic increase in the AUC values for cyclophosphamide and its metabolites (Table 1) and a corresponding decrease in the plasma clearance of cyclophosphamide (as defined by the dose divided by the AUC) from 2.1 to 0.6 $l \cdot h^{-1} \cdot kg^{-1}$. There was a smaller effect on Cmax values (Table 1).

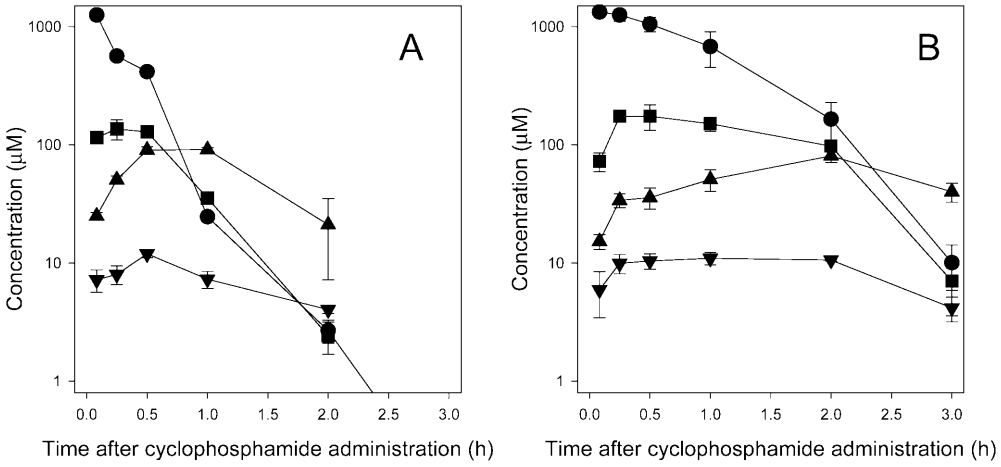

Fig. 3A, B. Effect of thalidomide on the plasma pharmacokinetics of cyclophosphamide and its metabolites. Tumour-bearing mice were treated with cyclophosphamide (220 mg/kg) alone (**A**), or with thalidomide (100 mg/kg) (**B**). Concentrations of cyclophosphamide (*circles*), 4-hydroxycyclophosphamide (*squares*), 4-ketocyclophosphamide (*triangles pointing up*) and 2-dechloroethylcyclophosphamide (*triangles pointing down*) were determined at different times. Individual points represent means ± SEM of three mice per group

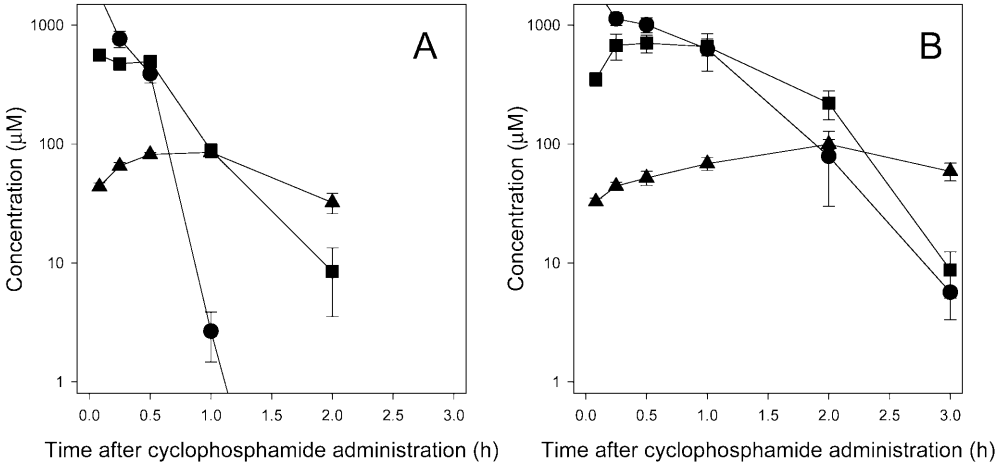

Fig. 4A, B. Effect of thalidomide on the liver pharmacokinetics of cyclophosphamide and its metabolites. Tumour-bearing mice were treated with cyclophosphamide (220 mg/kg) alone (**A**), or with thalidomide (100 mg/kg) (**B**). Concentrations of cyclophosphamide (*circles*), 4-hydroxycyclophosphamide (*squares*), 4-ketocyclophosphamide (*triangles pointing up*) and 2-dechloroethylcyclophosphamide (*triangles pointing down*) were determined at different times. Individual points represent means ± SEM of three mice per group

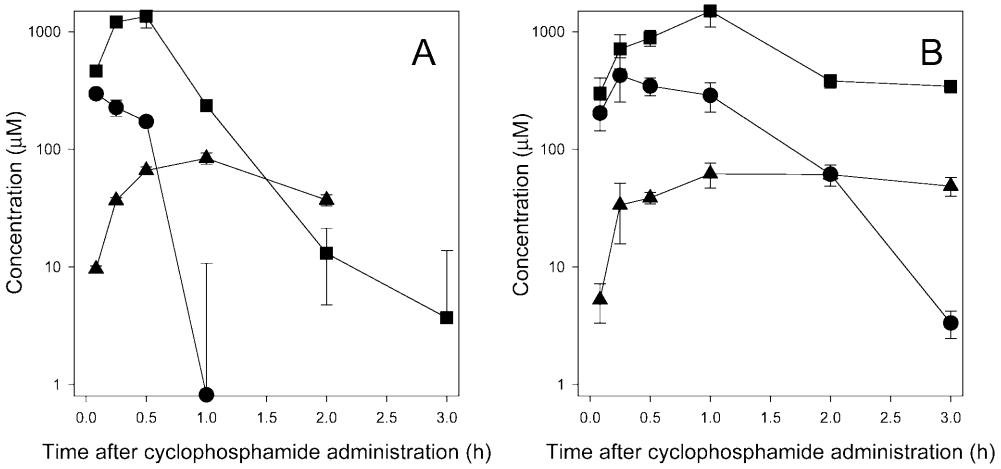

Fig. 5A, B. Effect of thalidomide on the tumour pharmacokinetics of cyclophosphamide and its metabolites. Tumour-bearing mice were treated with cyclophosphamide (220 mg/kg) alone (**A**), or with thalidomide (100 mg/kg) (**B**). Concentrations of cyclophosphamide (*circles*), 4-hydroxycyclophosphamide (*squares*), 4-ketocyclophosphamide (*triangles pointing up*) and 2-dechloroethylcyclophosphamide (*triangles pointing down*) were determined at different times. Individual points represent means ± SEM of three mice per group

Cyclophosphamide (220 mg/kg) was also administered in combination with different doses of thalidomide and plasma cyclophosphamide concentrations were measured after 1 h (Fig. 6). Plasma concentrations increased with increasing thalidomide doses from 1 to 20 mg/kg, reflecting an increased AUC, but did not increase with the concentration of cyclophosphamide at doses higher than 20 mg/kg.

Discussion

Cyclophosphamide is a prodrug that is activated by hepatic hydroxylation to 4-hydroxycyclophosphamide, which then undergoes spontaneous degradation to release the phosphoramide mustard as the ultimate alkylating antitumour species. Two major inactive

Table 1. AUC and Cmax values for cyclophosphamide and its metabolites. Effect of coadministration of thalidomide (100 mg/kg) with cyclophosphamide (220 mg/kg)

Compound	Source	Cmax (μ*M*)		Cmax ratio	AUC (μmol·h/l or kg)		AU Cratio
		Cyclophos-phamide	Cyclophos-phamide/ thalidomide		Cyclophos-phamide	Cyclophos-phamide/ thalidomide	
Cyclophosphamide	Plasma	1257	1332	1.1	397	1402	3.5
	Liver	1804	2003	1.1	471	1283	2.7
	Tumour	297	426	1.4	81	449	5.5
4-Hydroxycyclo-phosphamide	Plasma	136	174	1.3	107	287	2.7
	Liver	979	1212	1.2	704	1962	2.8
	Tumour	1351	1494	1.1	875	2109	2.4
4-Ketocyclophosphamide	Plasma	91	81	0.9	118	159	1.3
	Liver	85	100	1.2	125	195	1.6
	Tumour	94	62	0.7	112	151	1.3
2-Dechloroethylcyclo-phosphamide	Plasma	12	11	0.9	15	27	1.8

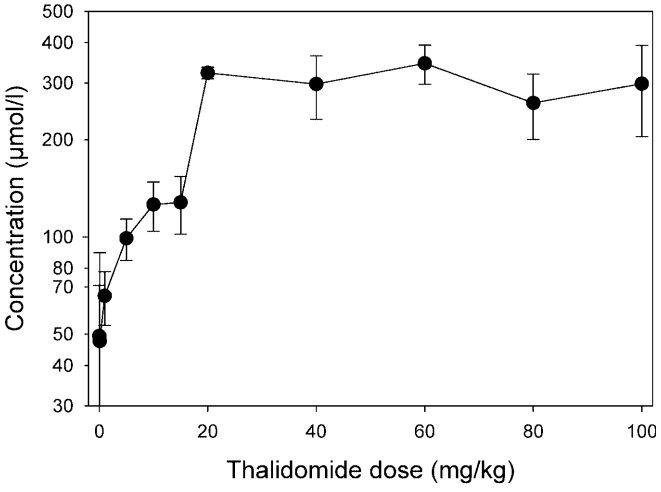

Fig. 6. Dose dependence of cyclophosphamide plasma kinetics on thalidomide dose. Tumour-bearing mice were treated with cyclophosphamide (220 mg/kg) with different doses of thalidomide, and cyclophosphamide concentrations in plasma 1 h after treatment were measured. Individual points represent means ± SEM of three mice per group

metabolites are also produced, 4-ketocyclophosphamide, derived from 4-hydroxycyclophosphamide, and 2-dechloroethylcyclophosphamide, derived from cyclophosphamide [6]. Various isoenzymes from the CYP family appear to hold the balance between activation and detoxification of cyclophosphamide [22]. This study was the first to use LC-MS to examine in detail the pharmacokinetics of cyclophosphamide and its metabolites in plasma, liver tissue and tumour tissue of mice. Comparable plasma pharmacokinetic studies have been reported in rats [11] and in humans [16]. It is clear from these studies that the half-lives of cyclophosphamide and 4-hydroxycyclophosphamide are shorter in mice than in rats, and considerably shorter than in humans.

Coadministration of thalidomide (100 mg/kg) dramatically changed the plasma and tissue pharmacokinetics of cyclophosphamide (Figs. 3, 4 and 5), resulting in an increase in AUC (Table 1) and a corresponding decrease in clearance. The thalidomide dose-response curve (Fig. 6) was consistent with the hypothesis that even at a dose of 20 mg/kg thalidomide has a marked effect. Calculations from the data in Figs. 3, 4 and 5 indicate that if the sum of the plasma concentrations of cyclophosphamide and its metabolites is plotted versus time following administration of the drug alone, a good fit is found to a single exponential, corresponding to a half-life of 22 min, with the rate of drug metabolism being approximately 20% of the rate of elimination. If the same calculation is made following coadministration of thalidomide, there is almost no elimination over the first hour. During this time, 28% of the parent drug is metabolized, indicating that coadministration of thalidomide has little effect on cyclophosphamide metabolism. At times later than 1 h, elimination increases, and after 3 h the half-life of the sum of cyclophosphamide and its metabolites is approximately 25 min.

It is noteworthy that the tissue (liver and tumour) concentrations of 4-hydroxycyclophosphamide were higher than the plasma concentrations (Figs. 4 and 5). This could reflect the production of this metabolite within tumour tissue as well as in liver tissue, but our preliminary results have shown no evidence that tumour tissue can hydroxylate cyclophosphamide. Alternatively, the result may reflect cellular uptake of the metabolite, providing a differential between intracellular and extracellular concentrations. If active uptake occurs it may vary for different cell types, which is of considerable relevance to the action of cyclophosphamide on individual tumours.

The mechanistic basis as to why thalidomide profoundly decreases the clearance of both cyclophosphamide and its metabolites has not yet been established. Thalidomide has little or no effect on cyclophosphamide metabolism in isolated enzyme systems [23], suggesting that its effect is indirect and perhaps related to its known antiinflammatory properties. Thalidomide is effective in

a number of clinical inflammatory conditions as a result of these properties [2]. However, in both clinical and experimental studies its effects have been shown to be markedly different from those of other steroidal and nonsteroidal antiinflammatory agents. Such effects may be related to the ability of thalidomide to inhibit or prevent the induction of TNF in response to stimulation by endotoxin or drugs such as DMXAA [4, 19]. This in turn has been suggested to arise from the ability of thalidomide to prevent the activation of the NF-κB transcription factor by inhibition of the enzyme IκB kinase-β [12]. TNF has been shown to upregulate expression of a multidrug transporter in the liver through NF-κB signalling [18]. Although this transport protein is not likely to be involved in cyclophosphamide excretion, other drug transport mechanisms might also be under the control of TNF or other cytokines. We are currently testing the hypothesis that TNF plays a role in this unexpected action of thalidomide.

The present studies provide evidence for a strong pharmacokinetic interaction between thalidomide and cyclophosphamide that might explain the potentiation of antitumour activity (Figs. 1 and 2). One might expect that the large increase in plasma AUC of the active metabolite 4-hydroxycyclophosphamide (Table 1) would be accompanied by a corresponding increase in host toxicity, but only a small change in the maximum tolerated dose was evident. It is noteworthy that the Cmax of the active metabolite was increased more modestly (Table 1) and this might better correlate with host toxicity. The maintenance of plasma concentrations of 4-hydroxycyclophosphamide has been reported by others to cause an improvement in antitumour activity [21], and such maintenance may allow better diffusion of the active metabolite to tumour tissue, which is poorly vascularized with respect to normal tissue.

In conclusion, the results demonstrate a novel property of thalidomide, that of dramatically altering the clearance of another, coadministered drug. The effects of thalidomide in mice were observed at doses below 20 mg/kg, which are in the range (200–800 mg/day orally) used for the thalidomide therapy of multiple myeloma in humans [20]. This suggests that thalidomide might be tested for its effects on cyclophosphamide activity and pharmacokinetics in a clinical situation. It also suggests that the effects of thalidomide on clearance of other drugs should be investigated. Previous results have already demonstrated thalidomide to inhibit the plasma clearance of the drug DMXAA [13]. Modulation of the pharmacokinetics of other drugs represents a new area by which thalidomide might exert its biological effects.

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