Metabolism of Thalidomide in Liver Microsomes of Mice, Rabbits and Humans

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Running title page

Running title: In vitro metabolism of thalidomide

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The abbreviations used are: Thal, thalidomide; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; 5-OH Thal, 5-hydroxythalidomide; 5’-OH Thal, 5’-hydroxythalidomide; TCA, trichloroacetic acid; NADPH, β-nicotinamide adenine dinucleotide phosphate reduced form.

Recommended section: Absorption, Distribution, Metabolism, & Excretion.
ABSTRACT

Thalidomide is increasingly important in clinical treatment, not only of various inflammatory conditions but also in multiple myeloma and other malignancies. Moreover, the metabolism of thalidomide varies considerably among different species, indicating a need to understand its mechanistic basis. Our previous in vivo studies showed the plasma half-life of thalidomide to be much shorter in mice than in humans, with rabbits showing intermediate values. We were unable to detect hydroxylated thalidomide metabolites in humans and suggested that interspecies differences in thalidomide hydroxylation might account for the differences in plasma half-life. We sought here to establish whether these species differences in the formation of hydroxylated thalidomide metabolites could be discerned from in vitro studies. Liver microsomes of mice, rabbits and human donors were incubated with thalidomide and analyzed using liquid chromatography-mass spectrometry (LC-MS). Hydrolysis products were detected for all three species and the rates of formation were similar to those for spontaneous hydrolysis, except in rabbits where phthaloylisoglutamine formation increased linearly with microsomal enzyme concentration. Multiple hydroxylation products were detected, including three dihydroxylated metabolites not observed in vivo. Thalidomide-5-O-glucuronide, detected in vivo, was absent in vitro. The amount of 5-hydroxythalidomide formed was high in mice, lower in rabbits and barely detectable in humans. We conclude that major inter-species differences in hepatic metabolism of thalidomide relate closely to the rate of in vivo metabolite formation. The very low rate of in vitro and in vivo hydroxylation in humans strongly suggests that thalidomide hydroxylation is not a requirement for clinical anticancer activity.
INTRODUCTION

Thalidomide (α-phthalimido glutarimide, Thal), despite its teratogenicity (McBride, 1961; Lenz, 1962), is attracting increasing clinical interest, initially as an anti-inflammatory agent (Sheskin, 1965; Zwingenberger and Wnendt, 1996; Calabrese and Fleischer, 2000) and more recently for the treatment of malignancies, particularly of multiple myeloma (Singhal et al., 1999; Eisen, 2000). Differences in metabolism among different species represent an important feature of its pharmacology. It has been suggested that Thal exerts its teratogenicity through an active metabolite, which is produced by human and rabbit liver microsomes preparation but not by mouse liver microsomes (Gordon et al., 1981). Following in vivo administration, Thal is both hydrolyzed chemically to a series of acid derivatives and hydroxylated by liver enzymes. In a previous study (Chung et al., Submitted) we showed that the plasma half-life of Thal in mice was much shorter than that in patients with multiple myeloma, with New Zealand White rabbits showing an intermediate half-life. We also showed that hydroxylated metabolites were not detected in multiple myeloma patients but were found in C57/Bl/6 mice and rabbits, suggesting that relative rates of Thal hydroxylation in different species could account for the differences in plasma pharmacokinetics, hence the differences in biological sensitivity. In this communication we have addressed the question of whether these species differences are reflected in the in vitro activity of liver microsomal enzymes.

There have been a number of other studies on the in vitro metabolism of Thal in different species. Ando and co-workers compared the ability of liver microsomes from five different species to form 5-hydroxythalidomide (5-OH Thal) and cis-5’-OH Thal and found that human
microsomes had the lowest activity (Ando et al., 2002a). The formation of 5,6-dihydroxythalidomide was also reported, with CYP2C19 identified as responsible for the hydroxylation of Thal in humans. Another study (Eriksson et al., 1998) has compared the formation of hydroxylated metabolites \textit{in vitro} using human S9 liver fractions and \textit{in vivo} in healthy human volunteers: both 5-OH Thal and \textit{cis}-5’-OH Thal were formed \textit{in vitro} but only \textit{cis}-5’-OH Thal was detected \textit{in vivo}. However, Teo and co-workers did not detect any metabolites after incubating Thal with human liver microsomes or in the plasma of patients with Hansen’s disease, although trace amounts of 5-OH Thal were detected in urine (Teo et al., 2000).

We have taken advantage of progress in the chemical synthesis of Thal metabolites, together with the development of high performance liquid chromatography (HPLC) and LC-MS technologies, to determine the rate of 5-OH Thal formation in liver microsomes of mice, rabbits and humans, as well as to characterize all the major metabolites formed \textit{in vitro} in order to compare them with those formed \textit{in vivo}.

**MATERIALS AND METHODS**

**Materials.** Thal was kindly provided by Dr George Muller, Celgene Corporation (Warren, NJ). Phthaloylglutamic acid, TCA and NADPH were from Sigma-Aldrich (St. Louis, MO), acetonitrile from BDH Laboratory Supplies (Poole, UK) and glacial acetic acid from Panreac Quimica SA (Barcelona, Spain). Phthaloylisoglutamine, 4-hydroxyphthaloylisoglutamine, 5-hydroxyphthaloylisoglutamine, 5-OH Thal, phthaloylglutamine, 4-hydroxyphthaloylglutamine, 5-hydroxyphthaloylglutamine, 4-hydroxythalidomide, \textit{N}(o-carboxybenzoyl)glutamine, \textit{N}(o-carboxybenzoyl)isoglutamine, \textit{N}(o-carboxybenzoyl)glutamic acid imide and 5-hydroxy-\textit{N}(o-
carboxybenzoyl)glutamic acid imide were synthesized as previously described (Lu et al., 2003) and their structures were confirmed using 400 MHz $^1$H nuclear magnetic resonance spectroscopy and mass spectrometry. 5,6-Dihydroxythalidomide was prepared by reaction of 5,6-dimethoxyphthalic anhydride (Barfield et al., 1975) with glutamine in refluxing pyridine (16 h), followed by cyclisation of the product with $N,N$-carbonyldiimidazole and catalytic 4-($N,N$-dimethylamino)pyridine in refluxing p-dioxane (16 h) and finally demethylation with pyridinium hydrochloride melt at 210 °C (20 min). 5'-OH Thal was a generous gift from Professor Sven Bjorkman (Malmo University Hospital, Malmo, Sweden) and was a mixture of 5'-cis and 5'-trans diastereoisomers.

**Liver Microsome Preparation.** All studies with animals and humans conformed to institutional ethical guidelines. Human livers were obtained from two liver donors HL5 and HL18, and stored in the human liver bank, Department of Pharmacology and Clinical Pharmacology, Faculty of Medical and Health Sciences, University of Auckland. These livers have been genotyped for CYP2C19, HL18 is $CYP2C19$ *1/*2 and HL5 is $CYP2C19$ *1/*1. Pooled C57/Bl/6 mouse livers and livers from New Zealand White rabbit and human donors were rinsed in ice-cold phosphate buffer (pH 7.4) and blotted dry. Liver weights were recorded. Livers were then homogenized in 67 mM phosphate buffer containing 1.15% KCl (volume of buffer was 3 times weight of liver). The homogenate was then centrifuged at 10,000 x g for 20 min, and the supernatant was removed and centrifuged at 100,000 x g for 1 h. The supernatant was again removed and the remaining microsomal pellets were rinsed with phosphate buffer. The rinsed pellets were re-suspended in a small volume of phosphate buffer and stored at -80°C until
analysis. The protein content of microsomes of all three species was determined using bicinchoninic acid assay (Smith et al., 1985).

**Microsomal Incubation and Sample Preparation for HPLC and LC-MS.** In the preliminary control experiments, the degradation of 400 µM Thal was <10% in phosphate buffer at 37°C. The rate of Thal hydroxylation was linear with respect to microsomal protein concentration (0.4 – 2 mg/ml) and time (10 – 30 min) in mouse and rabbit hepatic microsomes and linear between 0.6 – 2 mg/ml and 40 – 60 min in the presence of human liver microsomes. Incubations were carried out in a shaking water bath at 37°C for 30 min for mouse and rabbit microsomes and 60 min for human microsomes. The reaction mixture consisted of 67 mM phosphate buffer (pH 7.4), 4 mM NADPH, Thal (6.25 – 600 µM) and 2 mg/ml microsomes in a final volume of 300 µl. Thal was dissolved in DMSO, then diluted in phosphate buffer (final concentration of DMSO was <0.2%). After a 5-min pre-incubation, the reaction was initiated by addition of hepatic microsomes. Boiled microsomes were added to the control incubations. The reaction was terminated by addition of 300 µl 10% TCA containing phenacetin as an internal standard and was then vortexed and centrifuged (3,000 x g) for 10 min to remove precipitated protein (Torano et al., 1999). The supernatants were then processed by solid phase extraction as described previously (Lu et al., 2003) and the dry residues were reconstituted in 300 µl of mobile phase. Reconstituted samples were analyzed for 5-OH Thal concentration (200 µl each) using HPLC and metabolite identification (100 µl each) determined by LC-MS immediately afterwards.
Detection and Identification of Metabolites Formed \textit{in vitro} using LC-MS. Samples (100 µl) from preliminary \textit{in vitro} incubations (using 2 mg/ml of liver microsomes, 400 µM Thal and 60 min incubation time) and the final \textit{in vitro} incubations (using 2 mg/ml of liver microsomes, 6.25 – 600 µM Thal, and incubation time of 30 min for rabbit and mouse, 60 min for human) were analyzed together with authentic standards using an Agilent 1100 series LC-MS system (Agilent Technonologies, Avondale, PA) as described previously (Lu et al., 2003) with two modifications. The proportions of solution A (80% ACN, 1% glacial acetic acid and 19% Milli Q water) and solution B (9.5% ACN, 1% glacial acetic acid and 89.5% Milli Q water) in the mobile phase were altered slightly to improve resolution; all samples were analyzed using diode array UV detection at 230 nm and mass spectral detection set on negative-ion scan mode with a molecular weight range of 70 to 1000 amu, negative single-ion monitoring mode, with the sensitivity of 1 pg, at the molecular weights 257, 273, 275, 276, 289, 291, 293, 294 and 449, and positive single-ion monitoring mode at the molecular weights 259, 275, 277, 278, 291, 293, 295, 296 and 451 (corresponding to each of the peaks) simultaneously. Chromatograms of each sample were compared with control samples and authentic standards, and molecular masses of metabolites were obtained by mass spectral detection. The relative abundances of peaks of interest in negative single-ion mass spectral detected chromatograms or UV detected chromatograms were obtained from integration using ChemStation Software (Agilent Technologies, Avondale, PA).

\textit{In vitro} 5-OH Thal Formation. Samples were analyzed for 5-OH Thal concentrations using HPLC. Solutions containing a range of concentrations of 5-OH Thal, together with phenacetin as internal standard, were processed alongside with samples. 5-OH Thal
concentrations were determined using the method developed from this laboratory with slight modifications of HPLC run time and mobile phase (Chung et al., 2004). In brief, duplicate aliquots (100 µl) of re-constituted samples were loaded onto a Waters Breeze chromatograph (Waters Associates, Milford, MA, USA), which consisted of a Model 717plus auto-sampler, Model 1525 binary pump and Model 2487 dual wavelength absorbance detector. Compounds of interest were separated using a 100 x 4.6 mm stainless steel Luna 5 µm Phenylhexyl column (Phenomenex, Torrance, CA, USA) as well as a combination of the following solutions: Solution A, which contained 100% acetonitrile and Solution B, which contained 10% acetonitrile and 1% acetic acid in Milli Q water. The elution program was 100% solution B at 0.5 ml/min over 0-10 min, addition of 0-10 % solution A in a linear gradient at 1 ml/min over 10-15 min, 90% solution B and 10% solution A at 1 ml/min over 15-23 min, subtraction of 10-0% solution A in a linear gradient at 0.5 ml/min over 23-27 min and 100% solution B at 0.5 ml/min over 27-30 min. Phenacetin, Thal and 5-OH Thal were detected at ultraviolet wavelengths of 220 and 248 nm. The retention times of 5-OH Thal and phenacetin were 20.1 and 22.8 min, respectively. Data acquisition and integration was achieved using Breeze™ Software (Milford, CA, USA). A calibration curve of 5-OH Thal in phosphate buffer was prepared fresh for each HPLC run. To construct the calibration curve, the peak-area ratios relative to the internal standard were plotted against 5-OH Thal concentrations and the best-fit straight line was obtained by linear regression analysis. The range of calibration curve of 5-OH Thal was 0.1-25 µM ($r^2=0.999$). The intra-assay accuracy and precision were acceptable with relative recoveries and coefficient of variation (CV) of 90-110% and 5-9% ($n = 3$), respectively. Similar results were achieved for inter-assay accuracy and precision with relative recoveries and CV’s of 96-104% and 2-4% ($n = 5$). Quality control liver microsomes with three nominal 5-OH Thal concentrations (0.2, 5, and 25 µM)
added 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 3% of the validated value respectively. Reconstituted samples (100 µl each in duplicate) were injected onto HPLC and 5-OH Thal concentrations were determined using calibration curve described above. The 5-OH Thal concentrations were used to calculate the rate of formation. Michaelis-Menten models were used to describe the in vitro enzyme kinetics, and the kinetic parameters were determined by Prism 3.0 program (Graphpad Software Co., CA).

RESULTS

Metabolites Formed following Incubation of Thal with Hepatic Microsomes. Following incubation of Thal (400 µM) with rabbit and mouse liver microsomes (0.4 – 2 mg/ml) for 60 min in the presence of NADPH (4 mM), eleven peaks (1-11), in addition to Thal (Table 1; Fig. 1B, 1C) were resolved. In contrast, only seven peaks (1, 2, 5-7 & 10-11) were detected using human (HL18) liver microsomes (Fig. 1A). Four peaks (1, 2, 5 & 6) in the control incubations (with boiled microsomes) were identified by UV and mass spectral analysis and comparison with authentic standards as products of hydrolysis (Fig. 1 & 2). Four of the remaining seven peaks formed by mouse and rabbit liver microsomes were identified as the previously reported hydroxyl derivatives (Lu et al., 2003; Chung et al., 2004), namely 5-OH Thal and 5’-hydroxy-N-(o-carboxybenzoyl)glutamic acid imide plus cis- and trans- 5’-OH Thal (4, 7, 10 and 11; Fig. 1B,C). The remaining three peaks (3, 8 and 9) were products of further hydroxylation reactions as they had an identical molecular mass that was 16 a.m.u. higher than 5-OH Thal or 5’-OH Thal and were therefore assigned as dihydroxylated metabolites. None of these metabolites had
retention times and UV spectrum similar to authentic 5,6-dihydroxythalidomide. Peaks 8 & 9 could possibly correspond to cis-5,5’-dihydroxythalidomide and trans-5,5’-dihydroxythalidomide, although authentic compounds were not available for confirmation. Peak 3 was unidentified. Apart from the four hydrolysis products (1, 2, 5, and 6) observed in control incubations, human liver microsomes from donor HL18 catalyzed the formation of three hydroxylated metabolites, 5-OH Thal (11), cis-5’-OH Thal (7) and trans- 5’-OH Thal (10) (Fig. 1A). However, microsomes from HL5 failed to produce any detectable hydroxylation metabolites.

A comparison of the relative formation of hydroxylated metabolites by hepatic microsomes in the different species indicates that metabolic rate via hydroxylation is mouse > rabbit > human (Table 2). In particular, the relative abundance of each of the hydroxylated metabolite peaks produced by HL18 microsomes was much lower than the corresponding peak formed by mouse or rabbit liver microsomes (Table 2). Similarly, the relative amount of hydroxylated metabolites was higher in the presence of mouse liver microsomes compared with rabbit liver microsomes, with the exception of Peak 3.

The relative amount of hydrolysis products formed in microsomes from all three species did not differ significantly from the amount in control samples regardless of microsomal concentrations, with the exception of phthaloylisoglutamine (Peak 5) and N-(o-carboxybenzoyl)isoglutamine (Peak 2) in rabbit microsomal solutions (Fig. 1 and 2; Table 3). The formation of phthaloylisoglutamine exhibited a linear relationship with rabbit microsomal protein concentration ($r^2 = 0.983$; Fig. 3). Hence, it appears that the hydrolysis of Thal to
phthaloylisoglutamine in rabbit microsomes, at high substrate concentrations (400 µM), is an enzymatic process. Increased formation of N-(o-carboxybenzoyl)isoglutamine (Peak 2) may be a consequence of the hydrolysis of the phthalimide ring of phthaloylisoglutamine (Peak 5). Another finding in accordance with the above finding is that incubating rabbit and mouse microsome with Thal at 6.25 µM did not show difference in phthaloylisoglutamine formation, but when Thal concentrations were raised to 12.5 µM and above, rabbit liver microsome formed more phthaloylisoglutamine than mice.

The relative percentages of metabolite formation were obtained by comparing the peak areas of metabolites detected by UV with the sum of peak areas of all the metabolites and thalidomide (Fig. 2). A comparison of the relative total formation of hydrolysis products and hydroxylated metabolites in microsomes from the three species indicates that rabbit has the highest overall metabolic clearance of Thal (25.7%) mainly due to enzymatic hydrolysis (Table 4). In human and mouse liver microsomes, the formation of hydrolysis products was lower (7.7-7.9%) and due solely to non-enzymatic hydrolysis (Table 4). In contrast, metabolic clearance of Thal via hydroxylation was negligible in human liver microsomes and highest in mouse microsomes (6.1%).

**Rate of 5-OH Thal Formation.** Previous studies have indicated that the formation of 5-OH Thal may be catalyzed by CYP2C19 (Ando et al., 2002a). In order to determine the relative rates of metabolism of Thal to 5-OH Thal in the three species the enzyme kinetics of 5-OH Thal formation were determined. HPLC with UV detection resolved the hydroxylated metabolites (*cis-* and *trans-* 5’-OH Thal, 5-OH Thal) and internal standard (phenacetin) from Thal with
retention times of 18.3, 19.6, 20.1, 22.8 and 23.6 min, respectively. The formation of 5-OH Thal increased linearly with mouse and rabbit microsomal protein concentrations up to 2 mg/ml ($r^2 = 0.984$ and 0.985, respectively), whereas, the formation of 5-OH Thal by human liver microsomes was close to the limit of detection. Microsomes from donor HL18 (CYP2C19 *1/*2) formed measurable amounts of 5-OH Thal (quantitative limit of 0.1 µM) at microsomal concentrations higher than 0.6 mg/ml, whereas 5-OH Thal formed by microsomes from donor HL5 (CYP2C19 *1/*1) was below the limit of detection. The formation of 5-OH Thal also increased with incubation time in the presence of 2 mg/ml mouse and rabbit liver microsomes and was linear up to 30 min ($r^2 = 0.998$ and 0.999 for mouse and rabbit respectively), whereas 5-OH Thal formation by human liver microsomes (HL18) required an incubation time of greater than 40 min. Microsomes from mouse and rabbit formed measurable 5-OH Thal at concentrations of Thal above 6.25 µM. The formation of 5-OH Thal followed Michaelis-Menten kinetics (Fig. 4) and the kinetic parameters, $V_{\text{max}}$ (maximum velocity of reaction) and $K_M$ (Michaelis-Menten constant), were 45.2 pmol/min/mg and 208.3 µM for mouse microsomes, and 11.91 pmol/min/mg and 88.02 µM for rabbit microsomes, respectively. Previous reports have indicated that the metabolism of Thal to 5-OH and cis 5’-OH Thal may be important routes of metabolic clearance catalyzed by CYP2C19 in vitro (Ando et al., 2002a), but the kinetic parameters of Thal to 5-OH Thal in microsomes were not calculated due to the non-linear formation of 5-OH Thal. We used conditions optimized for 5-OH Thal formation (higher microsomal protein concentration and shorter incubation time) to calculate the kinetic parameters because it appeared to be the major route of metabolism detected in vivo (Lu et al., 2003; Chung et al., Submitted). However, as products of further hydroxylation of cis-, trans-5’-OH Thal and 5-OH Thal were detected in the present in vitro study, kinetic parameters of formation of the primary
hydroxylated metabolites of Thal (5-OH and cis 5’-OH) are compromised by the rate(s) of the second hydroxylation step, hence the kinetic parameters, in particular $K_M$ can only be described as *apparent*. The apparent intrinsic clearance ($CL_{int}$) ($CL_{int} = V_{max}/K_M$) of Thal to 5-OH Thal in mouse and rabbit microsomes was 0.217 and 0.135 ml/min/g, respectively. Therefore, the apparent *in vitro* intrinsic clearance was 1.6 fold greater in mouse than rabbit microsomes. In contrast, the $V_{max}$, $K_M$ and $CL_{int}$ from Thal to 5-OH Thal in HL18 microsomes were 0.334 pmol/min/mg, 85.8 µM and 0.004 ml/min/g, respectively. However, given that the data were calculated only from 3 concentration points (200, 400 and 600 µM), where detectable 5-OH Thal was formed, the results are only an estimate of the kinetics of formation of 5-OH Thal in human microsomes. A comparison of the apparent $CL_{int}$ of Thal to 5-OH Thal in the different species and *in vivo* plasma clearance of Thal ($Cl/F$) determined previously (unpublished data) are shown in Table 5. The relative rates of metabolism of Thal to 5-OH Thal in the three species *in vitro* correlates with the total *in vivo* clearance of Thal. Mouse has the highest rate of *in vivo* clearance (0.03 L/min/kg) and the highest rate of *in vitro* clearance to 5-OH Thal, whereas the negligible *in vitro* clearance of Thal to 5-OH Thal occurs in human liver microsomes corresponds to the very low *in vivo* clearance of Thal (0.0023 L/min/kg).

**DISCUSSION**

Numerous hydrolysis and metabolic products have been detected following the *in vitro* incubation of Thal with microsomes prepared from mouse, rabbit and human liver. Some of the metabolites have been previously identified from *in vivo* studies (Lu et al., 2003; Chung et al., Submitted) and include 5-OH Thal, *cis*- and *trans*- 5’-OH Thal and 5’-hydroxy-N-(o-
carboxybenzoyl)glutamic acid imide. In addition, three metabolites (3, 8 and 9), with masses consistent with dihydroxylation have been detected. These were not observed in vivo, and it is possible that their sources, mono-hydroxylated metabolites, are rapidly cleared by glucuronidation, as in the case of 5-OH Thal (Lu et al., 2003). In vitro formation of 5-OH Thal and cis-5’-OH Thal, as well as of 5,6-dihydroxythalidomide and several unidentified metabolites, has been reported (Ando et al., 2002a). However, in our study none of metabolites (3, 8 and 9) corresponded to authentic 5,6-dihydroxythalidomide. Thal-5-O-glucuronide, which has been detected in our in vivo study was also not detected possibly due to the lack of uridine diphosphate glucuronide as a co-factor in the in vitro experiment setting.

Non-enzymatic hydrolysis of Thal was observed in these studies confirming previous results from other groups in a number of species including rats, humans and rabbits (Faigle, 1962; Keberle et al., 1965; Schumacher et al., 1965; Williams et al., 1965). In addition, we have found evidence of significant enzymatic hydrolysis of Thal to phthaloylisoglutamine in rabbit hepatic microsomes. This confirms an earlier report (Schumacher et al., 1968) indicating that the rate of hydrolysis of Thal was higher in rabbit liver than in rat liver, although the specific hydrolysis pathway from Thal to phthaloylisoglutamine was not identified. Since no differences in the relative amounts of phthaloylisoglutamine in the plasma of mice and rabbits following administration of Thal (2 mg/kg p.o.) were observed (Chung et al., Submitted), enzymatic hydrolysis would be expected to occur only at the high Thal concentrations used in in vitro experiments (≥ 12.5 µM) and not at plasma concentrations (Cmax < 2.2 µM) that are observed in vivo (Chung et al., Submitted). Thalidomide is mainly excreted in the form of metabolites as shown by that less than 1% of the administered dose is excreted unchanged in urine in many
species (Smith et al., 1965). Total metabolic clearance of Thal \textit{in vitro} via both hydrolysis (enzymatic and non-enzymatic) and hydroxylation was greatest in the rabbit and negligible in human liver microsomes. However, since enzymatic hydrolysis of Thal in the rabbit may not occur at \textit{in vivo} concentrations, clearance via hydroxylation may be more important.

Although hydroxylated metabolites were measurable in human microsomal incubations with donor liver HL18, no metabolism of Thal was observed with another human liver donor HL5. This is in agreement with previous reports (Ando et al., 2002a) where of two human livers, one produced metabolites at or below the limit of quantification and the other source had very low rate of metabolite formation. Ando et al., (2002a) indicated that this was related to the relative expression of CYP2C19, however, pooled human liver microsomes were also very poor metabolizers of Thal. Of the two livers used in this study, one HL5 was homozygous wild type (*1/*1) for CYP2C19 and the other liver HL18 was a heterozygote for the mutant allele (*1/*2). Although, \textit{in vitro} studies with purified CYP2C19 indicated that this enzyme is involved in the hydroxylation of Thal, more recent \textit{in vivo} studies (Ando et al., 2002b) have indicated that some patients with an extensive metabolizer genotype do not hydroxylate Thal to any significant extent. The role of CYP2C19 in the metabolism of Thal requires further study, but importantly, regardless of the CYP isozyme(s) involved, the overall rate of metabolism of Thal is negligible in human liver in comparison to mouse and rabbit.

In conclusion, the results support previous reports of low \textit{in vitro} metabolic clearance of Thal in humans (Ando et al., 2002a) and suggest that there is minimal involvement of the hepatic CYP system in Thal metabolism. The data support \textit{in vivo} data showing that hydroxylated
metabolites are not detectable in patients with multiple myeloma (Lu et al., 2003; Chung et al., Submitted) or with Hansen’s disease (Teo et al., 2000). Hydroxylated metabolites can be detected in vitro but only at concentrations of Thal (200-600 µM) that are well above the highest plasma concentrations of Thal (50 µM) that have reported in human studies (Figg et al., 1999). These results suggest that Thal is unlikely to interact with other drugs extensively metabolized by human CYP system (Trapnell et al., 1998; Scheffler et al., 1999; Teo et al., 2000), which makes it a good candidate for combined chemotherapy. The data also strongly suggest that hydroxylated metabolites are unlikely to be involved in the mechanism of action of Thal in humans, and that the parent compound and/or its hydrolysis product(s) are involved in its action.
REFERENCES


**Legends for Figures**

Fig. 1. LC-MS chromatograms of Thal metabolites following incubation (60 min; 37°C) of Thal (400 µM) with liver microsomes (solid lines) of (A) humans, (B) rabbits and (C) mice, or with boiled liver microsomes (dotted lines). Metabolites were detected by single ion monitoring mass spectrometry as described in methods.

Fig. 2. HPLC chromatograms with UV detection of Thal metabolites following incubation (60 min; 37°C) of Thal (400 µM) with liver microsomes (solid lines) of (A) humans, (B) rabbits and (C) mice, or with boiled liver microsomes (dotted lines).

Fig. 3. Enzymatic hydrolysis of phthaloylisoglutamine by rabbit liver microsomal protein in the presence of NADPH (4mM).

Fig. 4. Formation of 5-OH Thal in rabbit and mouse liver microsomes following incubation with Thal.
Rabbit Microsomal Protein Levels (mg/ml)

Relative Abundance of Phthaloylisoglutamine
(Normalized against the relative abundance in control)

0.0 0.5 1.0 1.5 2.0 2.5

0 2 4 6 8 10

Rabbit Microsomal Protein Levels (mg/ml)
Thal Concentration (µM) vs. V (pmol/min/mg)

Mouse plot-fitted line

Rabbit plot-fitted line

Mouse

Rabbit

0 5 10 15 20 25 30

0 100 200 300 400 500 600
<table>
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</tr>
</tbody>
</table>

* hydrolysis metabolite, # metabolite formed via hydroxylation, a Molecular Weight, b Proposed metabolite.
Table 2. Comparison of relative levels of hydroxylated metabolites formed following a 60 min incubation of Thal with liver microsomal protein (2 mg/ml). Metabolites were determined by mass spectral detection using single ion monitoring. The response of each metabolite peak produced by mouse liver microsomes was normalized to 1.

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Rabbit</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown dihydroxylated metabolite (3)*</td>
<td>1</td>
<td>4.59</td>
<td>0</td>
</tr>
<tr>
<td>5’-OH CG (4)</td>
<td>1</td>
<td>0.26</td>
<td>0</td>
</tr>
<tr>
<td>cis-5’-OH Thal (7)</td>
<td>1</td>
<td>0.34</td>
<td>0.07</td>
</tr>
<tr>
<td>cis-5,5’-OH Thal (8)</td>
<td>1</td>
<td>0.68</td>
<td>0</td>
</tr>
<tr>
<td>trans-5,5’-OH Thal (9)</td>
<td>1</td>
<td>0.13</td>
<td>0</td>
</tr>
<tr>
<td>trans-5’-OH Thal (10)</td>
<td>1</td>
<td>0.27</td>
<td>0.09</td>
</tr>
<tr>
<td>5-OH Thal (11)</td>
<td>1</td>
<td>0.37</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Number in brackets represents peak number in chromatogram. 1 5'-hydroxy-N-(o-carboxybenzoyl)glutamic acid imide, 2 cis-5,5'-dihydroxythalidomide, 3 trans-5,5'-dihydroxythalidomide.
Table 3. Comparison of relative levels of hydrolysis products formed following a 60 min incubation of Thal with liver microsomal protein (2 mg/ml). Hydrolysis products were determined by mass spectral detection using single ion monitoring. The response of each peak produced by mouse liver microsomes was normalized to 1.

<table>
<thead>
<tr>
<th></th>
<th>CG (1)*</th>
<th>CiG¹ (2)</th>
<th>PiG (5)</th>
<th>PG (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control²</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.90-1.2</td>
<td>1</td>
<td>0.98-1.08</td>
<td>0.96-1.09</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.95-1.13</td>
<td>8.9-17.8</td>
<td>2.65-7.92</td>
<td>0.87-1.03</td>
</tr>
<tr>
<td>Human</td>
<td>0.90-1.01</td>
<td>1</td>
<td>0.88-1.1</td>
<td>0.84-1.05</td>
</tr>
</tbody>
</table>

* Number in brackets represents peak number in chromatogram.

¹ N-(o-Carboxybenzoyl)isoglutamine.

² Thalidomide solutions incubated with boiled liver microsomes of mouse, rabbit and human.
Table 4. Comparison of the total products of hydrolysis and hydroxylation formed following a 60 min incubation of Thal with liver microsomal protein (2 mg/ml).

<table>
<thead>
<tr>
<th>Species</th>
<th>All metabolites of hydrolysis (%)*</th>
<th>All metabolites of hydroxylation (%)*</th>
<th>5-OH Th (%)*</th>
<th>Total metabolites (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>7.7</td>
<td>6.1</td>
<td>2.5</td>
<td>13.8</td>
</tr>
<tr>
<td>Rabbit</td>
<td>22.7</td>
<td>3.0</td>
<td>1.1</td>
<td>25.7</td>
</tr>
<tr>
<td>Human</td>
<td>7.9</td>
<td>-</td>
<td>-</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* The percentage was obtained by comparing the sum of the UV responses of all hydrolysis or/and hydroxylation peaks with the sum of the UV responses of all metabolite and parent peaks, including hydrolysis, hydroxylation and thalidomide peaks.
Table 5. Inter-species comparison of *apparent in vitro* kinetic parameters ($V_{\text{max}}$, hepatic intrinsic clearance) and *in vivo* plasma clearance.

<table>
<thead>
<tr>
<th>Species</th>
<th>$\text{Apparent } V_{\text{max}}$ (pmol/min/mg)</th>
<th>$\text{Apparent CL}_{\text{int}}$ to 5-OH Th (ml/min/g)</th>
<th>$\text{Cl/F}$ at oral dose of 2 mg/kg$^a$ (L/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>32.1-36.4</td>
<td>0.217</td>
<td>0.03</td>
</tr>
<tr>
<td>Rabbit</td>
<td>10.4-10.6</td>
<td>0.135</td>
<td>0.0167</td>
</tr>
<tr>
<td>Human</td>
<td>$\approx$ 0.334</td>
<td>$\approx$ 0.004</td>
<td>0.0023$^b$</td>
</tr>
</tbody>
</table>

$^a$ Unpublished data.

$^b$ Thal dose for multiple myeloma patients was 200 mg/kg, or 1.9-3.8 mg/kg according to patients’ body weight.