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Thalidomide teratogenesis: Evidence for a toxic arene oxide metabolite

(epoxide hydrolase/metabolic activation/*in vitro* drug toxicity/cytochrome *P*-450)

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ABSTRACT **It was postulated that thalidomide causes birth defects by being metabolized to a toxic electrophilic intermediate. This hypothesis was tested by using an *in vitro* assay in which drug toxicity to human lymphocytes was assessed in the presence of a hepatic microsomal drug metabolizing system. Maternal hepatic microsomes from pregnant rabbits mediated the production of a metabolite that was toxic to lymphocytes. Toxicity was enhanced by inhibitors of epoxide hydrolase (EC 3.3.2.3) and abolished by adding the purified enzyme to the incubation medium. The metabolite thus appears to be an arene oxide, consistent with the previously reported isolation of phenolic metabolites of thalidomide from the urine of treated animals. Two teratogenic analogs of thalidomide (phthalimidophthalimide and phthalimidinoglutarimide) were also toxic in the system; two nonteratogenic analogs (phthalimide and hexahydrothalidomide) were not toxic, even in the presence of epoxide hydrolase inhibitors. The toxic metabolite of thalidomide was not produced by rat liver microsomes (the rat is not sensitive to thalidomide teratogenesis) but was produced by hepatic preparations from maternal rabbits, and rabbit, monkey, and human (all sensitive species) fetuses. A toxic arene oxide therefore may be involved in the teratogenicity of thalidomide.**

Thalidomide was identified as a human teratogen 20 years ago (1–3). Compared to other teratogens, thalidomide's selective toxicity in the embryo, particularly for the developing limbs, and its relative lack of toxicity in the adult is striking (4). Despite intensive investigation, however, the mechanism of the fetal toxicity of the drug remains unknown.

An interesting early observation was that rats were resistant to the teratogenic effects of thalidomide but rabbits and monkeys were sensitive (5–7). Differences in species susceptibility could result from differences in biotransformation of the compound. It was noted that rabbit liver homogenates enhanced the rate of disappearance of the drug from incubation mixtures whereas rat liver homogenates did not (8). Similarly, *in vivo*, more thalidomide metabolites were bound to liver macromolecules in the rabbit than in the rat, suggesting that a metabolite might interact covalently with macromolecules important in morphogenesis. Furthermore, after thalidomide treatment, 4- and 5-hydroxylated metabolites of thalidomide were recovered from the urine of rabbits but not from rats (9).

The presence of phenolic derivatives of thalidomide suggests that the drug might undergo oxidative metabolism via an arene oxide intermediate. Arene oxides have been implicated as mutagens, cytotoxins, and teratogens (10–12). Thus, birth defects caused by the anticonvulsant phenytoin may result from an arene oxide metabolite which covalently binds to critical structures in the developing fetus (12).

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Therefore, we have attempted to look for a possible toxic arene oxide metabolite of thalidomide. We have used an *in vitro* assay system in which human lymphocytes are the target of metabolites generated by hepatic microsomes from various species (13). The system was developed initially to study individual differences in toxicity of electrophilic metabolites in easily obtained human cells and has been useful in exploring the toxicity of several drugs that form arene oxides. The present study provides evidence that thalidomide can be metabolized to a toxic arene oxide, that structural analogs of thalidomide that are teratogenic form such epoxides but nonteratogenic analogs do not, that liver homogenates from rabbits mediate the reaction and those from rats do not, and that fetal liver homogenates from several sensitive species are active in producing the metabolite.

MATERIALS AND METHODS

Reagents. Thalidomide was obtained from Eli Lilly; EM-12 (phthalimidinoglutarimide) and phthalimidophthalimide were gifts from Chemie Grunenthal (Stolberg, Federal Republic of Germany); phthalimide and 1,2-epoxy-3,3,3-trichloropropane (TCPO) were obtained from Aldrich. Hexahydrothalidomide was synthesized by a modification of the procedure of Koch and Kotlan (14). DL-Glutamic acid and *cis*-1,2-cyclohexane dicarboxylic anhydride (Aldrich) were fused, and the product was refluxed with acetic anhydride. The intermediate was crystallized and then treated with urea. The product was dissolved in boiling water, decolorized with charcoal, and recrystallized from water. The identity of the final product was confirmed by mass spectroscopy at the National Science Foundation Mass Spectroscopy Facility, The Johns Hopkins University School of Medicine.

Preparation of Liver Fractions. Except as noted below, hepatic subcellular fractions were prepared from animals that had been pretreated with Aroclor 1254 (Monsanto Chemicals, St. Louis, MO; 500 mg/kg intraperitoneally 5 days prior to tissue preparation). Livers were homogenized in 0.15 M KCl, and 9000 × *g* supernatant (S-9) and microsomal fractions were prepared and stored at −80°C. Term pregnant maternal and fetal New Zealand White rabbit (Bunnyville, Littletown, PA) liver S-9 fractions and maternal rabbit and term pregnant maternal rat (Sprague–Dawley, Charles River Breeding Laboratories) microsomes were studied. A nonpretreated 63 day *Macaca fascicularis* fetus was obtained from a breeding colony at The Johns Hopkins Medical Institutions, and a hepatic homogenate was prepared in 0.15 M KCl. The homogenate was spun at 500 × *g*, and the supernatant fraction was stored at −80°C. Human fetal liver (18–20 weeks' gestation) was obtained through the cooperation of the Department of Gynecology and Obstetrics when the mother required a total abdominal hysterectomy for

Abbreviation: TCPO, 1,2-epoxy-3,3,3-trichloropropane.

medical indications. An S-9 fraction was prepared from the liver and was stored at −80°C.

Preparation of Cells and Experimental Procedure. The *in vitro* cytotoxicity assay used has been reported in detail (13). Mononuclear cells were prepared from heparinized whole blood from normal volunteers on Ficoll-Paque (Pharmacia). Cells were suspended in a Hepes-buffered medium (15 mM Hepes, pH 7.4/125 mM NaCl/6 mM KCl/1.2 mM $MgSO_4$/1 mM NaH_2PO_4/1 mM $CaCl_2$/10 mM glucose) to yield 10^6 cells per assay tube. A mixture containing 0.5 mg of microsomal protein (1.0 mg of S-9 protein), 10^6 cells, 0.6 mM $NADPH^+$, 2.4 mM glucose 6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase were incubated with varying concentrations of the test compound for 2 hr at 37°C. Thalidomide and other drugs were added in dimethyl sulfoxide (final concentration, 5%). After the 2-hr incubation, cells were collected by centrifugation and resuspended in Hepes-buffered medium containing 5 mg of albumin per ml. Incubations were continued at 37°C for 16 hr, and aliquots were taken for assessment of toxicity by trypan blue dye exclusion. Dye exclusion was previously found to correlate well with other measures of cell damage including lactic dehydrogenase release and loss of ability to respond to concanavalin A by blastogenesis (13).

In order to assess the role of a possible arene oxide metabolite of the drugs in cell damage, cell aliquots were pretreated with TCPO, an uncompetitive epoxide hydrolase inhibitor (15) at a final concentration of 5 μg/ml. The tissue preparations and drugs were then added, and toxicity assays were performed as above. The dose of the inhibitor chosen was the maximum associated with no increase in cell toxicity in the absence of the test drug. In other experiments, purified epoxide hydrolase [prepared from rat liver according to Lu *et al.* (16); 500 units/mg of protein with benzo[*a*]pyrene-4,5-oxide as substrate, 665 units/mg with styrene oxide] was added to the reaction mixture and the experiments were performed as usual.

RESULTS

Fig. 1 presents the dose-dependent lymphocyte toxicity caused by thalidomide in the presence of term pregnant rabbit maternal hepatic microsomes and an NADPH-generating system. Thalidomide metabolites produced toxicity at concentrations above 0.625 μg/ml. Omission of microsomes, NADPH, or both eliminated toxicity (data not shown). TCPO, an uncompetitive epoxide hydrolase inhibitor, significantly enhanced the cytotoxicity of thalidomide ($P < 0.05$, analysis of variance). Cyclohexene oxide (3 μg/ml), a noncompetitive epoxide hydrolase inhibitor (15), similarly increased thalidomide toxicity (data not shown).

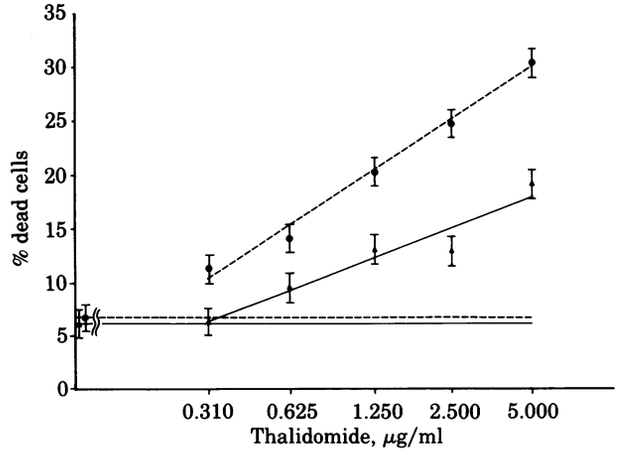

FIG. 1. Toxicity of thalidomide to human lymphocytes in the presence of maternal rabbit hepatic microsomes from Aroclor-treated term pregnant animals. Incubations were performed in the presence (●---●) or absence (▲——▲) of TCPO. Cell death was assessed by trypan blue dye exclusion. Each point represents the mean ± SEM for three determinations. Horizontal lines with ± 1 SEM indicated at the left are for TCPO-treated (---) and control (——) lymphocytes in the presence of microsomes without thalidomide. TCPO significantly increased the toxicity of thalidomide metabolites ($P < 0.05$, analysis of variance).

Table 1. Effect of epoxide hydrolase on toxicity of thalidomide to human lymphocytes in the presence of term pregnant maternal rabbit hepatic microsomes

Epoxide hydrolase, unit/ml*	% dead cells†	
	No thalidomide	Thalidomide at 5 μg/ml
0.0	6.6 ± 0.3	17.9 ± 1.4‡
1.0	7.4 ± 0.2	6.4 ± 0.6
0.5	7.2 ± 0.5	6.8 ± 0.4
0.25	7.1 ± 1.2	6.6 ± 0.2
0.125	6.6 ± 0.7	17.2 ± 1.3‡
0.5§	6.2 ± 0.9	20.2 ± 0.4¶

* Epoxide hydrolase was prepared according to Lu *et al.* (16); 1 unit of enzyme activity catalyzes formation of 1 nmol of styrene glycol from styrene oxide.
† Values represent mean ± SEM for three determinations as assessed by trypan blue dye exclusion.
‡ For difference from control without thalidomide, $P < 0.005$ (Student's *t* test).
§ Enzyme preparation had been heat inactivated at 100°C for 6 min.
¶ For difference from control, $P < 0.001$ (Student's *t* test).

Thalidomide toxicity could be prevented by adding purified epoxide hydrolase to the incubation mixture (Table 1). The en-

Table 2. Toxicity of four thalidomide analogs to human lymphocytes in the presence of term pregnant maternal rabbit hepatic microsomes with or without TCPO

Compound	Conc., μg/ml	% dead cells*	
		No TCPO	TCPO at 5 μg/ml
Control		7.7 ± 0.9	8.7 ± 0.8
Phthalimidophthalimide	1.25	17.0 ± 2.0	26.5 ± 1.8
	5.00	28.7 ± 2.4	32.6 ± 0.9
Phthalimidinoglutarimide (EM-12)	1.25	11.9 ± 0.9	18.0 ± 0.9
	5.00	20.1 ± 1.0	27.6 ± 0.4
Phthalimide	1.25	7.4 ± 0.8	8.4 ± 1.2
	5.00	7.4 ± 1.1	8.1 ± 0.7
Hexahydrothalidomide	1.25	7.3 ± 0.5	8.6 ± 2.9
	5.00	7.4 ± 0.7	8.4 ± 1.3
	10.00	7.4 ± 1.0	9.4 ± 0.4
	25.00	7.9 ± 0.5	10.8 ± 0.4
	50.00	8.8 ± 0.6	10.0 ± 0.4

* Values represent mean ± SEM for three determinations. Statistical analysis: TCPO did not affect control toxicity; phthalimide and hexahydrothalidomide did not differ from control in the presence or absence of TCPO; for phthalimidophthalimide and EM-12, 1.25 μg/ml increased toxicity over control and 5 μg/ml further increased toxicity over 1.25 μg/ml ($P < 0.01$; Student's *t* test); TCPO enhanced the toxicity of both compounds over control and non-TCPO samples ($P < 0.05$).

Table 3. Toxicity of thalidomide to human lymphocytes in the presence of Aroclor-pretreated term pregnant maternal rabbit or rat microsomes, with or without TCPO

Species	TCPO, μg/ml	% dead cells*	
		No thalidomide	Thalidomide at 5 μg/ml
Rabbit	0	7.1 ± 0.7	17.1 ± 0.8
	5	6.8 ± 0.6	25.5 ± 0.1
Rat	0	6.5 ± 0.3	8.2 ± 1.3
	5	7.1 ± 0.8	8.6 ± 0.8

* Values represent mean ± SEM for three determinations. TCPO did not affect baseline toxicity. In the presence of rabbit microsomes, thalidomide caused lymphocyte cell death ($P < 0.005$) which was enhanced by TCPO ($P < 0.005$; Student's t test). No increase in toxicity over baseline was noted in rat microsomal incubations.

zyme was effective in preventing toxicity at concentrations as low as 0.25 unit/ml. Heat inactivation of the enzyme preparation at 100°C for 6 min abolished the protective effect.

Two teratogenic analogs of thalidomide, phthalimido-phthalimide (17, 18) and EM-12 (19), both produced dose-dependent lymphocyte toxicity in the presence of the full microsomal system, and toxicity was enhanced by TCPO (Table 2). Purified epoxide hydrolase protected against toxicity caused by the analogs (data not shown). In contrast, two nonteratogenic analogs, phthalimide (20, 21) and hexahydrothalidomide (20, 21), did not cause toxicity in the presence or absence of TCPO. In the case of hexahydrothalidomide, there was no toxicity at concentrations 80 times the minimal thalidomide concentration associated with toxicity in the system.

Microsomes prepared from a sensitive (term pregnant Aroclor-pretreated rabbit) and insensitive (term pregnant Aroclor pretreated rat) species were then compared (Table 3). Rat microsomes failed to produce toxicity in the presence or absence of TCPO; toxicity with rabbit microsomes and its enhancement by TCPO were apparent.

Preliminary results with available fetal hepatic tissues from sensitive species are shown in Table 4. S-9 prepared from term Aroclor-pretreated fetal rabbit liver was somewhat less active than S-9 prepared from maternal liver; toxicity was enhanced by TCPO. Similarly, both 18 to 20-week human fetal liver S-9 and 63 day monkey 500 × g supernatant fractions appeared able to produce a toxic metabolite and its toxicity was enhanced by TCPO.

Table 4. Toxicity of thalidomide in the presence of hepatic preparations from term pregnant rabbit fetus, 63-day *Macaca fascicularis* fetus, and 18- to 20-week human fetus

Tissues*	TCPO, μg/ml	% dead cells†		
		No thalidomide	With thalidomide	
			At 5 μg/ml	At 10 μg/ml
Maternal rabbit	0	6.8 ± 0.6	21.8 ± 1.3	—
	5	8.1 ± 0.9	30.0 ± 1.8	—
Fetal rabbit	0	5.7 ± 1.1	9.5 ± 0.9	14.0 ± 2.1
	5	6.9 ± 0.8	20.5 ± 2.0	25.4 ± 2.9
Fetal human	0	4.9 ± 0.3	11.0 ± 1.0	17.8 ± 1.0
	5	7.0 ± 0.7	20.4 ± 1.7	23.4 ± 1.7
Fetal monkey	0	10.8 ± 0.5	24.5 ± 1.5	33.1 ± 2.5
	5	10.0 ± 1.2	31.1 ± 2.3	36.6 ± 1.3

* Maternal rabbit, fetal rabbit, and human = hepatic 9000 × g supernatant fractions, fetal monkey = hepatic 500 × g supernatant fraction. Rabbit preparations were from Aroclor-pretreated animals.
† Values represent mean ± SEM for three determinations. Thalidomide produced toxicity in the presence of all tissue preparations and toxicity was enhanced by TCPO ($P < 0.05$; Student's t test for each tissue).

DISCUSSION

Despite intensive efforts at elucidation of the mechanism of the fetal toxicity of thalidomide in the 20 years since the identification of the compound as a human teratogen, the mechanism remains obscure. The findings in the present study of the cytotoxicity of thalidomide in the *in vitro* lymphocyte assay system provide a basis for biochemical dissection of the pathways of such toxicity and its relationship to teratogenicity. The drug itself was not toxic to cells; toxicity required both a source of hepatic drug metabolizing enzymes and an NADPH-generating system. In this system, Lymphocytes do not produce significant quantities of electrophilic drug metabolites (13). These data, along with the enhancement of toxicity by two epoxide hydrolase inhibitors (TCPO and cyclohexene oxide) with different mechanisms of action (15), and the prevention of toxicity by the addition of purified epoxide hydrolase strongly argue that toxicity is related to metabolism of the compound by mixed function monooxygenases to an intermediate arene oxide.

Arene oxides of benzo[*a*]pyrene and phenytoin are believed to be responsible for the teratogenic effects of the drugs (11, 12). Benzo[*a*]pyrene-7,8-diol 9,10-epoxide has been specifically invoked as an ultimate teratogenic metabolite (22). An analogy may be drawn between our present results and studies on phenytoin teratogenesis. Phenytoin is toxic to lymphocytes in this system, and its toxicity is enhanced by TCPO (unpublished data). *In vivo*, the teratogenicity of phenytoin and its covalent binding to fetal macromolécules are increased by TCPO (12). The link between the cytotoxicity observed in these studies and thalidomide teratogenesis is strengthened by the data on the toxicity of thalidomide analogs and on species and fetal metabolism of the compound. Two teratogenic analogs of thalidomide, phthalimidophthalimide and EM-12, both exhibited cytotoxicity in the lymphocyte system with metabolic activation, and their toxicities were enhanced by TCPO. Toxicity was also abolished by epoxide hydrolase. Conversely, two nonteratogenic analogs, phthalimide and hexahydrothalidomide, were not toxic in the presence or absence of TCPO. Thus, there is no evidence that phthalimide is metabolized to a toxic arene oxide. Hexahydrothalidomide was synthesized and tested specifically because it lacks an unsaturated ring where an arene oxide could be formed.

The rat is not sensitive to thalidomide teratogenesis (5, 6). Rat microsomes did not produce a toxic metabolite of thalidomide in these experiments. The lack of formation of an arene oxide is consistent with previous findings that urine from thalidomide treated rabbits but not rats contained a phenolic metabolite and that only rabbit microsomes increased the rate of disappearance of the parent drug from *in vitro* incubation mixtures (8, 9). Our preliminary results with fetal tissues from sensitive species confirm the capability for formation of a toxic metabolite in the fetus even in the absence of Aroclor pretreatment. This is consistent with previous reports of cytochrome *P*-450 activity in first-trimester human liver (23) and in 8-day cultured mouse embryos (24).

Many questions remain about specific sites of formation of the thalidomide metabolites and target macromolecules which might explain the specific malformations resulting from the drug. The demonstration, in these studies, of such toxic me-

tabolites and initial structural and species correlation with teratogenicity, however, provide the basis for future exploration.

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