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Protective Effect of Liposome Encapsulation on Paclitaxel Developmental Toxicity in the Rat

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ABSTRACT Paclitaxel is an anticancer drug that has demonstrated severe embryotoxicity in chicks. This embryotoxicity is reduced by liposome encapsulation of the drug. The current study was designed to evaluate the potential of liposome encapsulation for reducing paclitaxel embryotoxicity in rats. Wistar rats were treated with paclitaxel on day 8 of pregnancy (plug = day 0) at doses of 0.67, 2.0, or 10.0 mg/kg intravenously. The same doses of paclitaxel encapsulated in liposomes were administered intravenously to other groups of animals. Control animals were given blank liposomes. Free paclitaxel produced maternal and embryotoxicity at 10.0 mg/kg with three of seven dams dying and resorption of all embryos in surviving dams. Liposome encapsulation at 10.0 mg/kg was not associated with maternal death and there were live fetuses on evaluation at term, although litter size was reduced and malformations occurred in surviving fetuses. At 2.0 mg/kg free paclitaxel, fetal weight was decreased and resorptions increased. Liposome encapsulation at 2.0 mg/kg produced litter results similar to those obtained in control animals given empty liposomes. Malformations were prominent at 2.0 mg/kg free paclitaxel and at 10.0 mg/kg paclitaxel in liposomes and included exencephaly/anencephaly, ventral wall defects, facial clefts, anophthalmia, diaphragmatic hernia, and defects of the kidney, cardiovascular system, and tail. Liposome encapsulation appeared to shift the developmental response to paclitaxel such that 10 mg/kg encapsulated drug produced effects similar to 2.0 mg/kg free drug. These results may have implications for drug delivery of therapeutic agents used during human pregnancy. *Teratology 56:305–310, 1997.*

Paclitaxel is a diterpenoid anticancer agent obtained from the Pacific yew tree *Taxus brevifolia*. The drug is marketed as Taxol® (Bristol-Myers Squibb Oncology, Princeton, NJ) for the treatment of breast and ovarian cancer. The anticancer activity of this agent is mediated, at least in part, by inhibition of microtubule disassembly (reviewed by Rowinsky et al., '90). Previous work in our laboratory demonstrated the embryotoxicity of paclitaxel in the chick characterized by a steep dose-response curve with prominent embryolethality at doses similar to those causing malformations (Scialli et al., '94, '95).

Clinical and experimental use of paclitaxel has been limited by the poor solubility of the compound and the toxicity of the ethanol/polyethoxylated castor oil vehicle (Cremaphor EL®) in which the commercial preparation is solubilized. The encapsulation of paclitaxel in liposomes has been evaluated as a means of decreasing the toxicity of the preparation without sacrificing antitumor activity (Bartoli et al., '90; Riondel et al., '92; Straubinger et al., '93; Sharma et al., '93, '96; Sharma and Straubinger, '94). In our work with the chick embryo, we identified a 1.3 order of magnitude shift in the dose-response curve for paclitaxel embryotoxicity with liposome encapsulation compared to free paclitaxel (Scialli et al., '95); dose-response curves for free and liposome-encapsulated drug were parallel, but liposome-encapsulation substantially decreased the embryotoxicity of the agent.

The current study was undertaken to evaluate the potential protective effect of liposome encapsulation for paclitaxel administered to pregnant rats. Because the purpose of the study was to demonstrate amelioration of toxicity, high-dose exposure was used to maximize the developmental effects of the commercially available paclitaxel in Cremaphor.

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MATERIALS AND METHODS

Animals

Wistar rats were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the Georgetown University animal care facility at a temperature of 70 ± 2°F and relative humidity of 30 to 60%. A 12-h light: dark cycle was used with lights on at 0600. Feed (Purina rat chow) and water were available ad libitum. Females were mated with proven breeders of the same strain or obtained time-mated from the supplier. Plug day was considered day 0 of pregnancy. The design included at least five dams per treatment group in order to give an 85% power of detecting a 20% litter incidence of abnormalities in paclitaxel-treated groups. Additional dams were planned in the groups exposed to paclitaxel in Cremaphor to compensate for anticipated maternal lethality.

Test agents

Paclitaxel in 15% Cremaphor (hereinafter identified as free paclitaxel) was purchased from the manufacturer (Taxol®; Bristol-Myers Squibb Oncology, Princeton, NJ). Liposomes were prepared from cardiolipin, phosphatidylcholine, and cholesterol (Avanti Polar Lipids, Birmingham AL; 3:9:5 weight ratio; 3:7.5:1 molar ratio) with a final lipid concentration of 3.9 mg/mL and a particle diameter of 0.1 to 0.2 µm. Particles were negatively charged. For the paclitaxel/liposome preparation, paclitaxel was incorporated in a 1:17 molar ratio in the following manner. Paclitaxel 5.8 µmol was dissolved in methanol and to this solution was added 5.7 µmol of cardiolipin in ethanol followed by 56.4 µmol of phosphatidylcholine and 38.8 µmol of cholesterol, both dissolved in chloroform. The mixture was stirred gently and evaporated to dryness under a flash evaporator. The final film was resuspended in 6 mL sterile saline or saline containing 5% trehalose. After a 30-min hydration time, the resulting suspension was sonicated (Heat Systems, Farmingdale, NY, model W-380) in a fixed temperature bath at 25°C for 15 min. Unincorporated paclitaxel was separated from liposome-entrapped paclitaxel by extensive dialysis over 20 h against 500 mL sterile saline with three changes in between.

The amount of paclitaxel encapsulated in liposomes was found to be >90% of the input amount. The concentration of paclitaxel in liposomes was determined by the HPLC method of Wiernik et al. ('87) as modified by our laboratory. Samples were analyzed using a Waters C_{18} ODS column (3.9 × 30 cm) and a Waters Associates 600 E liquid chromatograph device equipped with a UV-variable detector 484. Detection was set at 230 nm. The mobile phase was composed of CH_3CN:H_2O (45:55, v:v) at a flow rate of 1.5 mL/min, which produced a retention time of paclitaxel at 4.5 min. A portion of the liposome sample was dissolved in methanol and injected into the column. Paclitaxel concentrations were determined by comparison with a standard curve using fresh paclitaxel samples. Paclitaxel standard curves were found to be linear over a concentration range of 0.5 to 50 µM.

The liposome control group received blank liposomes at a concentration similar to the liposome component of the paclitaxel in liposome preparations. There was no difference in outcome based on liposome concentration and blank liposome groups were combined for analysis.

Treatment regimen and evaluation

Pregnant rats were injected through the tail vein with 1 mL aliquots of free paclitaxel, paclitaxel in liposomes, or blank liposomes. Previous work in our laboratory (Scialli et al., 1995) demonstrated no difference in the viability and developmental normalcy of chick embryos exposed to free liposomes compared to phosphate buffered saline, and a saline-injected control was not used. Injections were performed on the morning of day 8 of pregnancy based on preliminary experiments identifying this day as the most sensitive for the production of paclitaxel-induced malformations in the rat. Paclitaxel doses were 0.67, 2.0, and 10.0 mg/kg, based on preliminary studies and planned to bracket the typical therapeutic dose of 4.5 mg/kg as a single intravenous administration in a 70-kg individual. Treatment dose for paclitaxel in liposomes was expressed in terms of the amount of paclitaxel contained in the liposomes. The top dose of free paclitaxel was about 50% of the single intraperitoneal LD_{50} in adult rats, calculated on a surface area basis (206 mg/m^2; Rowinsky et al., '90).

Dams were weighed on days 0, 8, and 20 of pregnancy. On day 20, animals were killed by excess carbon dioxide and hysterotomies were performed. Resorption sites and live and dead fetuses were noted. Fetuses were weighed and external malformations were recorded following which fetuses were stored in Bouin's fluid until dissected. Fetuses were dissected for evaluation of internal malformations following the recommendations of Manson and Kang ('94). Dilatation of the urinary collecting system, which was encountered commonly among control fetuses, was not recorded as a malformation. Skeletal evaluations were not performed due to the small number of fetuses in several of the treated litters.

Statistical analysis was performed using the litter as the experimental unit. Maternal and fetal weight and litter incidence of resorptions and malformations were analyzed by analysis of variance with post-hoc Dunnett's test and Bonferroni's multiple comparison test for significant results (GraphPad Prism™, San Diego, CA). Chi-square testing was used for proportions of dams or litters affected with adverse outcome such as maternal death, resorption, and malformation. An overall *P* value of 0.05 was accepted as significant.

RESULTS

Litter data are shown in Table 1. Maternal death occurred in three of seven dams treated with 10 mg/kg free paclitaxel within minutes of injection. One dam in

TABLE 1. Litter data[1]

Parameter	Free paclitaxel (mg paclitaxel/kg)			Paclitaxel in liposomes (mg paclitaxel/kg)			Liposome only
	10.0 (n = 7)	2.0 (n = 8)	0.67 (n = 6)	10.0 (n = 5)	2.0 (n = 5)	0.67 (n = 8)	(n = 8)
Maternal death [n (%)]*	3 (43)	0	1 (17)	0	0	0	0
Maternal weight gain (g, mean ± se)[3]	18.50 ± 13.25	26.14 ± 11.19	53.25 ± 30.14	89.25 ± 3.70	35.2 ± 19.58	84.60 ± 20.67	44.14 ± 17.49
Live fetuses/litter (n, mean ± se)	0^{a} **	7.0 ± 1.6^{bc} **	12.6 ± 2.9^{bde}	7.0 ± 2.3^{abf} **	15.6 ± 1.4^{dg}	12.2 ± 1.3^{cdf}	13.25 ± 1.0^{efg}
Fetal weight/litter (g, mean ± se)	n/a[2]	2.89 ± 0.20^{a}	3.64 ± 0.04^{ab}	3.84 ± 0.30^{b}	3.76 ± 0.14^{b}	4.35 ± 0.24^{b}	3.96 ± 0.20^{b}
Litters with resorptions [n (% of surviving dams])*	4 (100)	8 (100)	1 (20)	2 (40)	0	0	0
Resorptions/litter (n, mean ± se)	15.3 ± 1.8^{a}	5.6 ± 1.6^{b}	0.2 ± 0.2^{c}	1.0 ± 0.8^{c}	0^{c}	0^{c}	0^{c}
Litters with malformations [n (% of surviving dams)]*	n/a	6 (75)	0	2 (50)	0	0	1 (12.5)
Malformed fetuses/litter (mean percent ± se)	n/a	26.8 ± 11.4	0	27.1 ± 17.8	0	0	0.9 ± 0.2

[1]Within rows, means with different superscripts are statistically different by Bonferroni's multiple comparison test.
[2]n/a, not applicable (no fetuses).
[3]Day 20 exluding uterine contents minus day 8.
*Overall $P < 0.05$, chi-square.
**Significantly different from liposome group at $P < 0.05$ by Dunnett's test after significant analysis of variance.

the 0.67 mg/kg free paclitaxel group also died. Maternal weight gain excluding uterine contents was not different among surviving dams; however, weight gain among dams was highly variable. There were no surviving fetuses in the group treated with free paclitaxel at the top dose. Among litters with surviving fetuses, litter size was decreased significantly at 2.0 mg/kg free paclitaxel and 10 mg/kg paclitaxel in liposomes compared to liposome-only controls. The distribution of litter sizes is illustrated in Figure 1.

Fetal weight was decreased in the 2.0 mg/kg free paclitaxel group compared to the paclitaxel in liposome and blank liposome groups. Resorptions were seen in all dose groups treated with free paclitaxel and in the highest dose group treated with paclitaxel in liposomes. The number of resorptions per litter was increased in a dose-dependent manner among groups treated with free paclitaxel, with the lowest dose group at control levels. The number of resorptions per litter was zero or not statistically different from zero in groups treated with paclitaxel in liposomes.

Malformations occurred in the 2.0 mg/kg free paclitaxel and the 10.0 mg/kg paclitaxel in liposome groups as well as in one control fetus. There were no differences in the litter percent of malformed fetuses by analysis of variance. Table 2 shows the distribution of malformations among dose groups.

DISCUSSION

Free paclitaxel was toxic to the pregnant rat at 10.0 mg/kg as a single intravenous administration on day 8 of pregnancy. Three of seven treated animals died shortly after injection and all of the surviving dams had completely resorbed litters. Liposome encapsulation attenuated the toxicity, with survival of all treated dams and the presence of live fetuses at the 10.0 mg/kg dose, although litter size was reduced and malformations occurred at this dose. Free paclitaxel at 2.0 mg/kg decreased live litter size and fetal weight in the absence of evidence of maternal weight alteration, while paclitaxel in liposomes did not produce alterations in these parameters at any tested dose compared to control litters. There was substantial variability in maternal weight gain among animals within the same dose groups, suggesting that these doses may represent a steep portion of the dose-response curve for maternal toxicity.

The distribution of litter sizes shown in Figure 1 suggested dose-related toxicity for free paclitaxel with liposome-encapsulation, producing a shift in dose-response characteristics such that 10 mg/kg in liposomes was similar to 2 mg/kg free paclitaxel. The protection afforded by liposome encapsulation with regard to fetal weight and resorptions per litter may have been greater, with 10 mg/kg paclitaxel in liposomes having no detectable effect compared to control litters.

External and visceral malformations could not be assessed in the 10 mg/kg free paclitaxel group because there were no surviving fetuses, but several malformations were found in a large proportion of litters exposed to 2.0 mg/kg free paclitaxel. Malformations were also

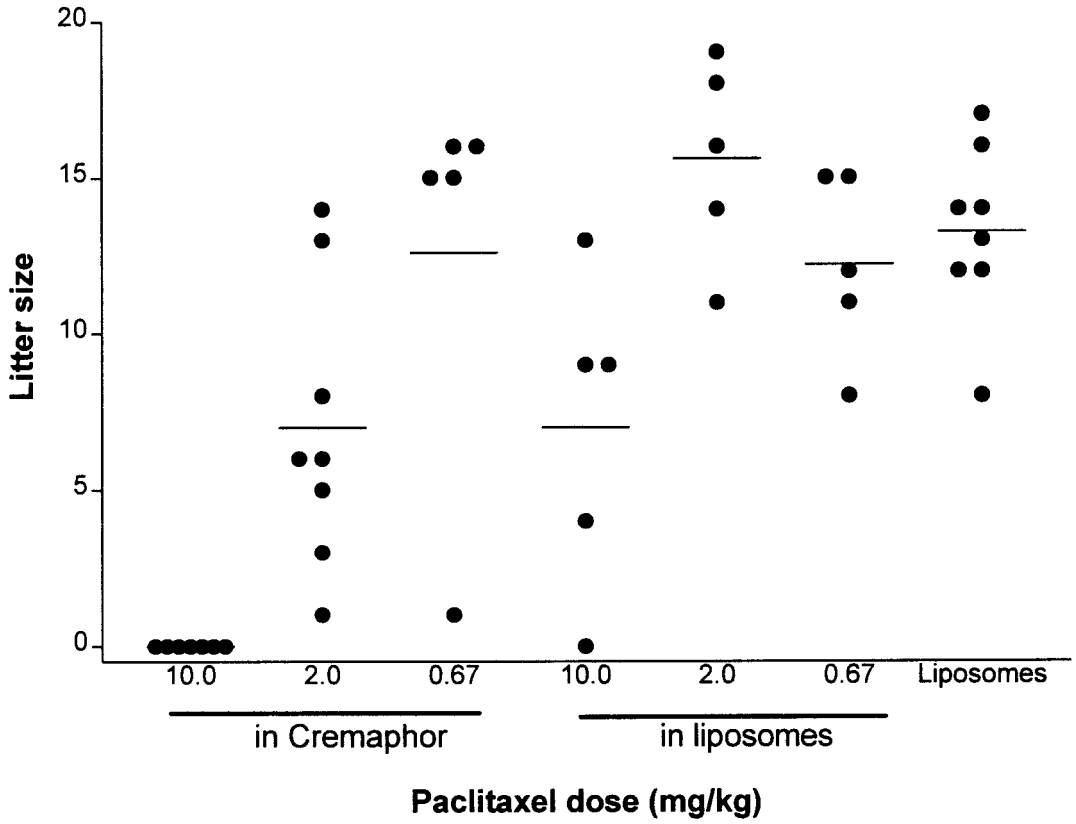

Fig. 1. Distribution of litter sizes by dose group. Bars = means.

TABLE 2. Malformation data

	Free paclitaxel (mg paclitaxel/kg)			Paclitaxel in liposomes (mg paclitaxel/kg)			
Malformation	10.0	2.0	0.67	10.0	2.0	0.67	Liposome only
Number of fetuses	0	56	63	35	78	61	106
Number of litters	4	8	5	5	5	5	8
Affected fetuses (affected litters in parentheses)							
Exencephaly/anencephaly	n/a[1]	3 (2)	0	1 (1)	0	0	0
Ventral wall defect	n/a	2 (2)	0	2 (2)	0	0	0
Facial clefts	n/a	2 (2)	0	0	0	0	0
Anophthalmia	n/a	4 (4)	0	1 (1)	0	0	0
Diaphragmatic hernia	n/a	2 (2)	0	1 (1)	0	0	0
Kidney defect	n/a	2 (2)[2]	0	3 (2)[3]	0	0	1 (1)[4]
Cardiovascular defect	n/a	3 (3)[5]	0	1 (1)[6]	0	0	0
Short or absent tail	n/a	1 (1)	0	5 (2)	0	0	0

[1]n/a, not applicable.
[2]Two anephric fetuses.
[3]One fetus with a horseshoe kidney and two fetuses with ectopic kidneys.
[4]One fetus with unilateral renal agenesis.
[5]One fetus with an atrial septal defect, one fetus with pulmonic stenosis, and one fetus with a hypoplastic left atrium.
[6]One fetus with a ventricular septal defect and overriding aorta.

seen in half the litters exposed to 10 mg/kg paclitaxel in liposomes but in none of the other paclitaxel in liposome groups. There was a single malformed fetus with unilateral renal agenesis in the blank liposome control group.

The current study was not designed to evaluate the full range of developmental effects of paclitaxel. A rat Segment II study reported by the manufacturer showed that pregnant rats given up to 0.6 mg/kg/d paclitaxel showed no maternal or embryotoxicity except a de-

crease in pup hair growth at the top dose (Lochry et al., '95). When rats were given up to 1 mg/kg/d in a Segment III design, maternal toxicity (decreased food consumption and a decrease in body weight and the weight of selected organs) occurred at the top dose accompanied by delayed hair and incisor growth, delayed testicular descent, and decreased weight of the pups. The reported maternal and developmental no-observed effect levels (NOELs) were 0.3 mg/kg/d. It is not possible to directly compare our results with those of Lochry et al. ('95) because their report, presented in abstract, did not specify details such as rat strain or administration vehicle for the paclitaxel.

Because our intention was to evaluate the moderation of paclitaxel developmental toxicity by liposome encapsulation, the doses used were selected to produce effects ranging from no detectable toxicity to severe embryo and maternal toxicity for free drug. Sample size was reduced from the typical 20 dams per dose group used for developmental toxicity studies to the minimum number necessary to show the anticipated amelioration of toxicity (Gad and Chengelis, '88).

The protective effect of liposome encapsulation in the pregnant rat is consistent with our previous findings in the chick (Scialli et al., '95). In that model, the dose-response curve for paclitaxel in liposomes was parallel to that for free paclitaxel but was shifted 1.3 orders of magnitude to the right. The current results are consistent with a similar effect in the rat; however, given the small number of dose points, we cannot evaluate whether the dose-response curves for free and liposome-encapsulated paclitaxel are parallel.

Liposome encapsulation of paclitaxel has been evaluated in intact tumor-bearing rodents and tumor cells in culture (Bartoli et al., '90; Rafaeloff et al., '92; Riondel et al., '92; Straubinger et al., '93; Sharma et al., '93, '96; Sharma and Straubinger, '94; Alkan-Onyuksel et al., '94). Intact animals tolerate larger doses of paclitaxel when encapsulated compared to free drug, without loss of antitumor effectiveness. Effectiveness in vitro is also uncompromised by liposome encapsulation. It is possible that selective uptake by tumor cells of liposome-encapsulated material accounts for maintenance or even enhancement of antitumor effectiveness in addition to reduced host toxicity.

Placental transfer of chemicals across the perfused in vitro human term placenta has been shown to be reduced by liposome encapsulation for valproic acid (Barzago et al., '96), chloramphenicol (Onur et al., '92), and carboxyfluorescein (Bajoria and Contractor, '93). Valproic acid transfer was reduced by about 30% while transfer of the other agents was reduced by 80% or more.

The inhibition of valproic acid transfer raises the question of whether liposome encapsulation of this agent will permit use during human pregnancy without the increase in congenital malformations associated with use of current free valproic acid therapy. It should be noted that liposome encapsulation increased the antiseizure activity of valproic acid in amygdaloid-kindled rats (Mori and Ohta, '92). An evaluation of the potential protective effect of liposome encapsulation of valproic acid during pregnancy in the mouse is currently underway in our laboratory.

There is evidence in intact rats and rabbits, however, that liposome encapsulation may enhance placental transport of some drugs. Animals injected in the latter half of pregnancy with liposome-encapsulated drugs showed considerable placental uptake of the liposomes, with subsequent intracellular sequestration and degradation, releasing free drug for transport to the fetus (Nilden Tüzel-Kox et al., '95). In rats, placental transfer of methotrexate, penicillin, and riboflavin increased four- to nearly ninefold with liposome encapsulation compared to the injection of free drug. In rabbits, penicillin transfer decreased by 50% with infusion of liposome-encapsulated compared to free drug. It is possible that intracellular sequestration and degradation of liposomes within the placenta permits greater maternal biotransformation of the drugs contained within these particles. Whether liposome encapsulation of a chemical increases or decreases placental transfer is likely to be dependent on the chemical, species, and perhaps on the stage of gestation.

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