Novel Taxol Formulations: Preparation and Characterization of Taxol-Containing Liposomes

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Taxol is a promising anticancer agent under investigation for therapy of ovarian, breast, colon, and head and neck cancer. One problem associated with the administration of taxol is its low solubility in most pharmaceutically-acceptable solvents; the formulation used clinically contains Cremophor EL® (polyethoxylated castor oil) and ethanol as excipients, which cause serious adverse effects. To eliminate this vehicle and possibly improve the antitumor efficacy of taxol, we have formulated taxol in liposomes of various compositions. Liposome formulations containing taxol and phospholipid in the molar ratio 1:33 were prepared from phosphatidylglycerol (PG) and phosphatidylcholine (PC) (1:9 molar ratio), and were physically and chemically stable for more than 2 months at 4°C, or for 1 month at 20°C. A method of producing taxol-liposomes by lyophilization has been developed, by which large batches can be prepared reproducibly in a 'pharmaceutically rational' manner. Taxol-liposomes retained the growth-inhibitory activity of the free drug in vitro against a variety of tumor cell lines. In mice, taxol-liposomes were well-tolerated when given in bolus doses by both iv and ip routes. The Maximum Tolerated Dose (MTD) was >200 mg/kg; it exceeded that of free taxol, which had a MTD of 30 mg/kg by iv or 50 mg/kg by ip administration. Free taxol administered in the Cremophor vehicle was toxic at doses >30 mg/kg, as was the equivalent volume of vehicle without drug. Taxol-liposomes may prove to be useful not only for eliminating the toxic effects attributed to the Cremophor vehicle, but also for providing opportunities to widen the taxol therapeutic index through alterations in route and schedule of administration.

KEY WORDS: taxol; liposomes; lyophilization.

Introduction

Taxol, a diterpenoid derived principally from the bark (1) of the Western Yew, Taxus brevifolia, is under investigation as an anticancer agent (2,3) in human clinical trials; target tumors include a variety of human cancers, including ovarian, breast, colon, non-small cell lung, and head and neck cancer. Taxol is the first of a new class of antineoplastic drugs (3); it causes stabilization of microtubules (4), thus interfering with cellular progress through mitosis (5), and arresting cell replication. In phase II trials with patients treated previously with high-dose chemotherapy, the response rate in advanced and refractory ovarian cancer was 30% (6). The overall response rate in phase II trials in previously-treated patients with metastatic breast cancer was 56% (7). Recently, the U.S. Food and Drug Administration (FDA) approved taxol for use against ovarian cancer. One problem associated with the use of taxol is its poor solubility in water and in most pharmaceutically-acceptable solvents, which necessitates administration in a lipid vehicle. Presently, the vehicle used clinically is polyethoxylated castor oil (Cremophor EL®) containing 50% absolute ethanol. The amount of Cremophor necessary to deliver the required doses of taxol is significantly higher than that administered with any other marketed drug. This vehicle has been observed to cause serious, life-threatening anaphylactoid reactions in animals (8) and humans (3,9), and is physically incompatible with some intravenous (iv) infusion sets, as shown by the leaching of plasticizers (10). Since hypersensitivity reactions occur more frequently with shorter infusion schedules, most phase II and III trials in the United States have used 24-hour schedules (3). Premedication with corticosteroids (dexamethasone), antihistaminics (diphenhydramine), and histamine H₂ receptor antagonists (cimetidine or ranitidine) has reduced the intensity and incidence of adverse reactions associated with taxol-Cremophor administration (8,11); however, they are not completely eliminated (12). Clinically, pharmacological intervention is less desirable than a safer, better-tolerated formulation; with multiple agents in general, and with the co-medication agents used with taxol specifically, there exists considerable potential for drug interactions that can alter the pharmacokinetics and pharmacodynamics, and thereby the toxicity or efficacy, of taxol.

The primary goal of our work is to eliminate the Cremophor vehicle by reformulation of the drug in a better-tolerated vehicle. Reformulation also provides the possibility of improving the efficacy of taxol-based anticancer therapy. Liposomes represent a versatile drug carrier technology with considerable potential for improved solubilization of lipophilic drugs [reviewed in 13–15], and various formulations are in clinical trials or under investigation for treatment of a number of neoplastic and infectious diseases. The widening variety of liposome-encapsulated drugs entering clinical trials reflects an emerging understanding of the safety, utility, and methodology required to produce the quantities of this experimental drug carrier for human therapeutic trials. Liposomes consist of one or more aqueous compartments contained within lipid membrane bilayers. Because liposomes contain a hydrophilic domain, a hydrophobic domain, and an interfacial region, they may accommodate therapeutic agents having diverse physical characteristics. It is plausible to assume that well over 10 gm of phospholipid may be administered safely to humans; 8–20 gm doses of liposomes have been reported in different studies (16,17).

Several previous examples suggest the utility and potential therapeutic gains from reformulation in liposomes of other drugs currently given in the Cremophor EL vehicle. Cyclosporin has been administered to humans in Cremophor EL, and acute toxic side effects have been observed (18). A liposomal formulation showed immunosuppressive activity equal to that of the Cremophor-based formulation, but with reduced renal and vehicle toxicity. In addition, greater compatibility with intravenous administration equipment was reported for the liposome-cyclosporin formulation (19). Therefore, there was a strong rationale for reformulating taxol in
lipo-sones. The taxol-liposome formulations were prepared and evaluated for stability and in vitro and in vivo activity.

Materials and Methods

Materials. Crystalline taxol, Cremophor EL containing 50% absolute ethanol (Diluent 12) and taxol dissolved in Diluent 12 (30 mg/5 ml) were obtained from the National Cancer Institute (Bethesda, MD). Cremophor EL was also obtained as a gift from BASF Corporation. Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL) or Princeton Lipids (Princeton, NJ) and stored in chloroform under argon at −70°C. All organic solvents used were reagent or high performance liquid chromatography (HPLC) grade. Female BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN).

Preparation and Characterization of Taxol-liposomes: Taxol-liposomes were prepared by hydration of a lyophilized powder containing taxol and phospholipids, using a method adapted from (20). The following mixtures of phosphatidylcholine (PC) and phosphatidylglycerol (PG) were used: 10:0, 9:1, 7:3, 5:5, 3:7 or 0:10 molar ratio. Briefly, taxol was dissolved in chloroform and mixed with phospholipids in a round bottom flask, and the chloroform was evaporated in a rotary evaporator at 40°C. The taxol-lipid film was then dissolved in tert-butanol to achieve a lipid:taxol molar ratio of 33:1 and a lipid concentration of 100 mM. Two to 10 ml aliquots of the butanol solution were placed in sterile tubes, shell-frozen in liquid nitrogen, and lyophilized for 24 h. The lyophilized powder was hydrated with buffer (NaCl/Tes/EDTA: 140 mM/10 mM/0.1 mM) to produce suspensions of multimellar vesicles (MLV). To obtain smaller vesicles (SUV), the liposome suspension was sonicated under argon in a bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY) for 30 minutes at 20°C. Liposomes were analyzed for taxol by reversed-phase HPLC (21) and phospholipid content by Bartlett assay (22).

Physical Stability of Taxol-liposome Formulations: Physical stability of taxol-liposome formulations was determined by several methods. First, small liposomes (SUV) were subjected to centrifugation at 15,000 × g for 15 min, under which conditions the liposomes remain suspended and taxol precipitates are sedimented. Second, liposomes were passed through 0.1 μm pore polycarbonate filters, through which liposomes can pass, but which retain taxol precipitates. Third, suspensions were examined at high magnification (1200×), using Differential Interference Contrast microscopy (DIC), to observe aggregation of liposomes or crystallization of taxol. Fourth, negative-stain transmission electron microscopy was used to evaluate the suspensions. Taxol-liposome formulations subjected to the first two separation methods were re-analyzed for taxol and phospholipid content. A change in either was interpreted as an indication of instability.

Chemical Stability of Taxol in Liposome Formulations: The chemical stability of taxol in liposomes was assessed using reverse-phase HPLC to determine total taxol content of formulations at different time points during storage at 4° or 20°C. Detailed methods for HPLC analysis are given elsewhere (21). Briefly, HPLC analysis was performed with an isocratic solvent system (70% methanol, 30% water) at a flow-rate of 2 ml/min, and taxol was detected by absorbance at 227 nm. N-octyl benzamide was used as an internal standard. A Hitachi HPLC system was used, consisting of a Model L6200A pump, L-4250 spectrophotometric detector, AS-2000 autosampler and D-2500 Integrator. A Waters μ Bondapak C18 column (3.9 × 300 mm) and a C18 guard column were used for analysis.

Cell Growth Inhibition Experiments: Cells were plated at a density of 2 × 10⁴/ml in 24-well plates (Costar) and allowed to adhere overnight. Wells in triplicate were exposed to various concentrations of taxol, either added as liposomes, as a 100× concentrated stock of free drug in dimethylsulfoxide (DMSO), or adsorbed to serum proteins in the absence of organic solvent. Cells were counted after 72 h, and the IC₅₀ (concentration resulting in 50% growth inhibition) for each concentration-effect curve was calculated graphically.

Toxicity of Prototype Taxol-Liposomes: The Maximum Tolerated Dose (MTD) for taxol-liposome formulations was determined for intraperitoneal (ip) and intravenous (iv) routes of administration in healthy Balb/c female mice. Surveys experiments to define the MTD were performed with two animals per group. Doses were escalated in 2-fold increments, starting at 5 mg/kg. Drug effects were determined by close observation of weight changes; the maximal dose of taxol causing more than 10% weight loss within one week of treatment was defined as the MTD. Animals showing weight loss exceeding 20% were sacrificed, as changes of this magnitude usually indicate lethal toxicity (E. Mayhew, Roswell Park Cancer Inst., unpublished observations). After completing the survey MTD experiments, the approximate MTD was refined further using 3 groups of 8 mice.

Results

Over 300 sets of formulations were examined, representing a systematic variation of liposome properties including diameter, charge, membrane fluidity, length of lipid acyl chain, surface hydration, and inclusion of specific dopants. A wide variety of natural and synthetic lipids and phospholipids were screened for their ability to accommodate taxol. Preliminary results suggested that PC bilayers incorporate higher concentrations of taxol than can any other lipid investigated. Liposomes composed solely of PC encapsulating ≤3 mol% taxol, with respect to phospholipid, were stable for >2 weeks (data not shown). However, PC-taxol liposomes were found to be highly aggregated when examined by Differential Interference Contrast (DIC) Microscopy (Fig 2A). Incorporation of a negatively charged phospholipid such as PG reduced aggregation (Fig 2B–2F). Thus, liposomes composed of PC and PG in various ratios were examined further.

In addition, different methods for preparing and post-processing liposomes were tested (23): hydration of dry lipid films, reverse-phase evaporation, freeze-thaw, sonication, and extrusion. During the developmental process, hydration of dry drug-lipid films, optionally followed by sonication to limit particle diameter, was found to be a feasible method for preparation. In scaling up to larger quantities, physical stability of formulations was variable (data not shown). Our hypothesis is that the drug precipitation occurred when the drug concentration in organic solvent exceeded its solubility during the production of the dried lipid film. As an alterna-