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Abstract

Drug carriers such as liposomes provide a means to alter the biodisposition of drugs and to achieve concentration–time exposure profiles in tissue or tumor that are not readily accomplished with free drug. These changes in biodisposition can improve treatment efficacy. For hydrophobic drugs, incorporation in liposome carriers can increase drug solubility markedly. The taxanes paclitaxel (taxol) and docetaxel (Taxotére) are members of one of the most important new classes of oncology drugs. However, their poor solubility presents pharmaceutical challenges, and
emerging data suggest that specific tissue exposure profiles, such as low drug concentrations for extended times, can enhance beneficial antitumor mechanisms. Incorporation of the taxanes into liposomes eliminates not only the toxic effects of cosolvents required to administer these drugs clinically but also increases drug efficacy in animal tumor models, usually through a reduction in dose-limiting tissue toxicities. Although the taxanes are poorly water soluble, the preparation of physically stable taxane/liposome formulations requires the balancing of three factors: (1) the drug:lipid ratio, (2) the liposome composition, and (3) the duration of storage in aqueous media. Biophysical evaluation of formulation characteristics, principally using circular dichroism (CD) and differential scanning calorimetry (DSC), can provide the information necessary to develop stable taxane–liposome formulations. These techniques provide information on drug–drug and drug–lipid interactions that underlie the events that lead to taxane formulation instability. Owing to the unusually low solubility of the taxanes, special consideration is necessary to devise methods for resolving drug-containing liposomes from released or precipitated drug to obtain reliable estimates of drug incorporation and retention in liposomes.

Introduction

The taxanes represent one of the most important new classes of oncology drugs approved in the past two decades. An unprecedented drug development effort resulted from the observation that taxol (paclitaxel), the prototype of this class, showed activity in recurrent, platinum-resistant ovarian cancer (Suffness, 1993). Taxotére (docetaxel), a semisynthetic derivative, followed paclitaxel into the clinic, and both are now FDA approved. Through widespread clinical experience, the taxanes have progressed from drug of last resort to first-line therapy for a variety of cancers, such as refractory ovarian, breast, and non-small cell lung cancer (Adler et al., 1994; Cortes et al., 2003; Guastalla et al., 1994; Kubota et al., 1997; Murphy et al., 1993; Sledge et al., 2003).

The main mechanism of taxane action results from interaction with cellular microtubules (Schiff et al., 1979), which promotes their assembly and stabilization. However, the mechanisms of tumor growth control may vary with drug concentration (Derry et al., 1995; Gan et al., 1996; Jordan et al., 1993; Milross et al., 1996; Yen et al., 1996) or the concentration versus time pharmacokinetic profile (Bocci et al., 2002). At the highest (μM) concentrations, the taxanes induce cytoplasmic microtubule bundling and aster formation (De Brabander et al., 1981; Manfredi et al., 1982; Rowinsky et al., 1988; Schiff and Horwitz, 1981; Schiff et al., 1979). In the low nM range, regarded as more relevant clinically, paclitaxel blocks the cell cycle
at the G2/M interface by kinetic stabilization of microtubule dynamics. Cell death through the apoptosis pathway has been observed, with activation of a variety of signaling cascades (Fan, 1999; Huang et al., 2000; Jordan and Wilson, 1995; Jordan et al., 1993; Kawasaki et al., 2000; Lee et al., 1998; Ling et al., 1998; Manfredi et al., 1982; Ojima et al., 1999; Roy and Horwitz, 1985; Schiff et al., 1979; Tudor et al., 2000; Wang et al., 1998, 1999; Yeung et al., 1999; Yvon et al., 1999). Most recently, it has been observed that the taxanes possess significant antiangiogenic activity at low concentrations (mid pM range) or synergize with antiangiogenic agents (Bocci et al., 2002; Farinelle et al., 2000; Grant et al., 2003; Satoh et al., 1998; Wang et al., 2003). For paclitaxel, inhibition of vascular endothelial cell proliferation and migration has been observed at concentrations 10- to 100-fold lower than those inducing mitotic arrest in nonendothelial cells. Docetaxel appears even more active, with an estimated 10-fold greater potency than paclitaxel in in vitro antiangiogenic assays (Grant et al., 2003). This spectrum of concentration- or exposure (concentration × time)-dependent effects suggests that drug delivery approaches that control the exposure profile can alter therapy in beneficial ways.

The taxanes are complex diterpenoid natural products or semisynthetic derivatives (Fig. 1). They consist of a bulky, fused ring system and an extended side chain (at C13) that is required for activity. Paclitaxel, the prototype, is a natural product (Wani et al., 1971), and docetaxel is a semisynthetic analogue that differs from paclitaxel by substitutions at C10 and C13 (Bissery et al., 1991; Guérin-Voegelein et al., 1991; Ringel and Horwitz, 1991).

One common characteristic of the clinically used taxanes is poor aqueous solubility. Although the taxane molecular structure has several relatively hydrophilic domains (in the vicinity of C7–C10 and C1′–C2′), hydrophobic domains of the fused ring system and side chain (Balasubramanian et al., 1994; Guénard et al., 1993) contribute to the overall poor aqueous solubility. Estimates of paclitaxel solubility vary

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**Fig. 1.** Chemical structure of paclitaxel and docetaxel. Bu, butyl; Ac, acetyl; Bz, benzoyl.
widely, including \( \sim 35 \, \mu M \) (Ringel and Horwitz, 1991; Swindell et al., 1991), \( \sim 7 \, \mu M \) (Tarr and Yalkowsky, 1987), and \( \leq 0.77 \, \mu M \) (\( \sim 0.7 \, \mu g/ml \)) (Mathew et al., 1992). The discrepancy may reflect the measurement of solubility under equilibrium versus nonequilibrium conditions (Adams et al., 1993). A time-dependent decline in solubility was reported for paclitaxel (Sharma et al., 1995b); over 24 h, the measured solubility fell 10-fold from initial values to a final value of \( 0.4 \, \mu M \). Detailed studies of paclitaxel solid state (Liggins et al., 1997) confirmed the low equilibrium solubility of paclitaxel, and demonstrated that polymorphic crystalline forms exist (anhydrous and a dihydrate solvate), each with distinct dissolution properties.

The taxanes are poorly soluble, not only in water but also in the oils and surfactants commonly used in preparing emulsions or other formulations (Adams et al., 1993; Rose, 1992; Straubinger, 1995; Suffness, 1993). Therefore, paclitaxel is formulated for clinical use (Taxol, Bristol Myers Squibb, Inc., Princeton, NJ) at a concentration of 6 mg/ml in a mixture containing 50% (v/v) of the organic solvent ethanol plus the surfactant polyethoxylated castor oil (Cremophor EL) (USPDI, 2003a). Prior to administration, the solution is diluted with saline or dextrose to a concentration of 0.3–1.2 mg/ml, producing a microemulsion. The solution is administered through an in-line filter (U.S. Public Health Service, 1990). These procedures and guidelines are necessary to avoid precipitation upon dilution.

Formulation considerations for docetaxel have been described previously (Bissery et al., 1991; Bisset et al., 1993; Extra et al., 1993; Rhone Poulenc Rorer S.A.). For clinical use (Taxotere, Aventis, Inc., Bridgewater, NJ), it is also formulated and administered in a cosolvent system. The drug is packaged at 40 mg/ml in polysorbate-80 (USPDI, 2003b). Prior to use, it is diluted to 10 mg/ml with a solution containing 13% (v/v) ethanol in water. Before administration, the drug is further diluted in 250 ml saline or dextrose, achieving a final concentration of 0.3–0.9 mg/liter. The solution is used within 4 h.

The ethanol:Cremophor vehicle required to solubilize paclitaxel is toxic and has been observed to cause life-threatening anaphylactoid reactions (Donehower et al., 1987; Dye and Watkins, 1980; Friedland et al., 1992; Grem et al., 1987; Lorenz et al., 1977; Rowinsky et al., 1992; Weiss et al., 1990). Furthermore, Cremophor appears to modify the pharmacological activity of paclitaxel (Webster et al., 1993) and to contribute to nonlinear pharmacokinetics (Sparreboom et al., 1996). Toxicities associated with docetaxel administration partially overlap those of paclitaxel. However, some apparently unique adverse effects are associated with docetaxel administration. Delayed-onset pleural effusions and edema (Aapro et al., 1993; Behar et al., 1997; Burris et al., 1993; Fumoleau et al., 1993; Irvin et al.,
have led in some cases to the discontinuation of treatment. The polysorbate-80 vehicle has been suspected of contributing to this unique spectrum of side effects (Irvin et al., 1993). Most adverse effects of taxane administration are managed by premedication of patients with corticosteroids and antihistamines (Arbuck et al., 1993; Behar et al., 1997; Onetto et al., 1993; Rowinsky et al., 1991; Runowicz et al., 1993; Schrijvers et al., 1993; Weiss et al., 1990).

Because of the toxicities associated with the cosolvents required for taxane administration, a variety of alternative formulation strategies have been investigated. Here we discuss methods for the preparation and characterization of liposomes containing active taxanes, focused principally on paclitaxel.

Formulation Considerations

A growing body of literature reports the preparation and characterization of liposomes containing taxanes. The majority of studies describe paclitaxel-containing formulations; less information is available for docetaxel-containing liposomes (Immordino et al., 2003; Sharma et al., 1995a). One common conclusion supported by much of the literature is that physical stability of taxane–liposome formulations is determined by interaction among three factors: (1) the drug/lipid molar ratio, (2) the liposome composition, and (3) the duration of storage in aqueous media. Drug–drug interactions determine the propensity for drug intermolecular aggregation that may lead to precipitation. Drug–lipid interactions, affected by liposome membrane factors such as lipid miscibility, phase separation, and the membrane phase state, determine the degree to which the drug is accommodated in the bilayer. Chemical stability is also a concern, particularly deesterification of the C13 side chain. However, several approaches reduce or avoid chemical instability. Physical stability remains one of the most important issues in taxane formulation.

Drug/Lipid Ratio

Maximizing the ratio of drug to lipid reduces the amount of lipid that must be administered for a given drug dose. High lipid doses may raise concerns of toxicity and reduce the economic feasibility of pharmaceutical-scale production. In the absence of other considerations, optimal taxane liposomes contain the highest achievable drug/lipid molar ratio. However, because drug–lipid interactions are a major determinant of formulation physical stability, and these are concentration dependent, a tradeoff exists between taxane content and duration of stability in aqueous media (Sharma and Straubinger, 1994b). In many studies, liposomes containing a
maximum of $\sim 3$–$4$ mol% drug (with respect to phospholipid) possess stability of sufficient duration as to be clinically usable. In several notable exceptions, liposomes appear to accommodate much higher drug/lipid ratios. However, physical stability is often not characterized rigorously. Little information is available on the stability characteristics of docetaxel formulations, but liposomes containing 3 mol% (drug–lipid) or more of docetaxel have been prepared and are stable (Immordino et al., 2003; Sharma et al., 1997).

**Compositional Dependence of Physical Stability**

Most studies utilize liposomes in which the majority phospholipid is a zwitterionic neutral component such as phosphatidylcholine (PC), either derived from natural sources or produced synthetically to define acyl chain composition. However, liposomes of 100% PC aggregate (Sharma and Straubinger, 1994b; Straubinger et al., 1993) and therefore anionic (Sharma and Straubinger, 1994b) or cationic (Campbell et al., 2001) phospholipids (or other amphiphiles) are included to inhibit aggregation. The effect of cholesterol, which is included commonly in liposome formulations to increase stability, has not been determined definitively in terms of paclitaxel liposome stability. Phospholipids modified covalently on the headgroup with simple sugars, or extended-chain poly(ethylene glycols) (PEG) show prolonged circulation time in vivo (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Klibanov et al., 1991). However, some reports indicate that the inclusion of PEG-modified lipids decreases the physical stability of paclitaxel liposomes (Crosasso et al., 2000).

**Duration of Aqueous Stability**

One basic composition of taxane liposomes that is physically stable in aqueous solution on the time scale of weeks or months contains PC, a small fraction of anionic lipid to reduce particle aggregation, and 3–$3.5$ mol% paclitaxel (with respect to phospholipid) (Sharma and Straubinger, 1994b). Liposomes of this composition but containing 4–$5$ mol% paclitaxel may be stable on the scale of hours to a day, and 8 mol% paclitaxel liposomes may be physically stable for 15 min or less.

**Preparation and Characterization of Liposomes**

**Preparation of Liposomes**

For lipophilic drugs such as the taxanes, a simple method for incorporation into liposomes is to dissolve the drug in a volatile organic solvent and
mix it with a solution containing the desired phospholipids, also in organic solvent. The solvents used must be miscible. The organic solvent is then removed using a rotary evaporator, producing a thin lipid film. Hydration of the dried drug–lipid film results in spontaneous formation of multilamellar liposomes (MLVs) (Bangham et al., 1965), and the hydrophobic drug partitions into the bilayer membrane.

Taxanes can be incorporated at small scale using this simple MLV method (Bartoli et al., 1990; Riondel et al., 1992; Sharma et al., 1993), but our unpublished experiments suggested problems in scaling up to the larger preparations required for preclinical or animal studies. One hypothesis for the observed problems was that the drug concentrates in the organic solvent during rotary evaporation and undergoes self-aggregation, as was observed in detailed physical studies (Balasubramanian et al., 1994). This self-aggregation of drug arises from specific intermolecular interactions among taxane molecules, in which one face of the molecule interacts with the opposite face of another, leading to a “stacked” structure capable of infinite propagation (Balasubramanian et al., 1994). The conformation of this intermolecular aggregate in organic solvent (chloroform) is shown in Fig. 2. The conformation of the aggregate in organic and aqueous solvents differs somewhat, in that head-to-tail interactions, reminiscent of interactions observed in the taxane crystal structure (Mastropaolo et al., 1995),

![Concentration- and environment-dependent aggregation of taxol](image)

**Fig. 2.** Paclitaxel–paclitaxel interactions in nonpolar media. NMR and CD spectroscopy indicate that paclitaxel intermolecular interactions can occur at sub-mM concentrations in organic solvents such as chloroform (Balasubramanian et al., 1994). Dashed lines indicate intermolecular hydrogen bonds inferred from NMR spectroscopy. The stacked structure may propagate from both faces and thus grow indefinitely. Conformation of the stack in aqueous environments can include head/tail interactions as well (our unpublished observations). (Reprinted with permission from Balasubramanian et al., 1994.)
are observed in water. We hypothesize that this “stacking” can nucleate destabilization of formulations and precipitation of the drug, and procedures or conditions that reduce this type of drug–drug interaction enhance formulation stability.

To avoid achieving drug concentrations that could permit intermolecular stacking when the drug–lipid mixture is initially dried from organic solvents, a freeze-drying method (Perez-Soler et al., 1990) was adapted for the preparation of taxane-containing liposomes (Sharma and Straubinger, 1994b; Sharma et al., 1993). Because the method results in a dried drug–lipid mixture, greater chemical or physical stability during long-term storage is an additional benefit.

The freeze-drying method includes several of the steps described above for the preparation of MLV. Typically, taxane is dissolved in methanol at 10–30 mM and mixed with phospholipids dissolved in chloroform at 10–150 mM. The critical nature of the drug/lipid ratio was discussed above. The drug–lipid mixture is dried on a rotary evaporator, and the thin film is redissolved at 50 mM in tert-butanol. The solution is flash frozen in liquid nitrogen, freeze dried, and stored until use.

Immediately prior to use, the freeze-dried drug–lipid powder is reconstituted with an isotonic aqueous solution such as normal saline (0.9% w/v) or nonionic solutions such as glucose, sucrose, or mannitol. The aqueous reconstitution medium is added to produce a final phospholipid concentration of 50–150 mM, depending on the application. Higher concentrations may become too viscous to administer through a small-gauge needle. Final drug concentrations are typically 1.6–38 mM. Following addition of the reconstitution medium, the solution is mixed vigorously by vortexing to ensure the suspension and hydration of the dried lipid.

Size Definition of Liposomes

After aqueous reconstitution, the reduction of liposome size may be desirable for reasons of subsequent sterilization by filtration or extending the circulation time in vivo. Size reduction may also simplify the analysis of physical stability during the development of taxane formulations (Sharma and Straubinger, 1994b) (below). Two methods for reducing the size distribution of liposomes include (1) sonication or (2) extrusion through polycarbonate filters having well-defined pores (Olson et al., 1979; Szoka et al., 1980).

Sonication. Following hydration of the dried drug–lipid mixture, the resulting liposomes are sonicated under nitrogen for 30 min at room temperature, using a high-energy bath-type sonicator (Laboratory Supplies, Inc., Hicksville, NY). The milky MLV suspension clears as particle size
decreases, and extended sonication produces a nearly clear suspension of liposomes of ~25–35 nm diameter.

Extrusion. Extrusion of liposomes through successively smaller defined-pore polycarbonate filters (Olson et al., 1979; Szoka et al., 1980) can be used to reduce liposome diameter, as can passage through a restricted orifice at high pressure using an emulsifier. These techniques are reproducible and reduce free radical formation that can damage lipids. Under certain conditions, the operation can be performed while maintaining sterility. Devices from Avestin, Inc (Ottawa, Canada) and Northern Lipids Inc. (Vancouver, Canada) permit both small-scale (0.5 ml) and larger scale extrusion or emulsification.

Characterization of Liposomes

Although useful liposome-based taxane formulations have been achieved, the development of optimal formulations remains the subject of active investigation. Formulations must be characterized for drug content and for physical and chemical stability. Routinely, the concentration of both drug and lipid should be quantified and the drug/lipid ratio determined. This information should be obtained before and after any series of processing steps to detect changes in chemical and physical stability; instability in either would be manifested as an alteration of the drug/lipid ratio. The taxanes are easily quantified by high-performance liquid chromatography (HPLC) assay (Sharma et al., 1994a) or by spectrophotometry. For the latter, concentrated liposome solutions are dissolved by serial dilution in methanol. Extinction coefficients at specific wavelengths in standard solvents are published; for paclitaxel, the molar extinction coefficient in methanol is 28,500 at 227 nm (U.S. Public Health Service, 1990). Phospholipid can be determined by a variety of methods; one simple method is inorganic phosphorus analysis following acid hydrolysis (Bartlett, 1959; Düızgünes, 2003).

Determination of Physical Stability by Measurement of Drug Retention

Optical Microscopy. The destabilization of paclitaxel-containing formulations results in the formation of crystalline drug or drug-rich–lipid-poor complexes, often leaving drug-poor–lipid-rich components that resemble liposomes (Sharma and Straubinger, 1994b; Straubinger et al., 1993). This type of destabilization can be observed readily by optical microscopy using phase contrast or differential interference contrast (DIC) optics (Sharma and Straubinger, 1994b; Straubinger et al., 1993). Samples are obtained at timed intervals from hydrated taxane–lipid
formulations, and multiple fields are scanned for the presence of characteristic needlelike or crystalline precipitates. The time at which the first such structures are observed is recorded, and the precipitate-free interval is regarded as a relative, qualitative indication of stability.

Procedures that can separate precipitated drug from liposomes, in conjunction with methods to determine drug and phospholipid concentration, can be used to quantify drug retention in liposomes (Sharma and Straubinger, 1994b).

**Dialysis.** Dialysis of drug-containing liposomes has been used frequently to investigate stability of liposome formulations; detection of drug in the dialysate, or a reduction in the drug/phospholipid ratio of the dialyzed material, indicates leakage or efflux of the drug from the carrier. In some studies, dialysis has been used to analyze the stability of paclitaxel-containing liposomes. However, the aqueous solubility of the drug is \( \sim 0.4 \mu M \) (Sharma et al., 1995b); if the paclitaxel concentration of a typical taxane liposome formulation were in the range of 10–150 mM, equilibration of 1 ml formulation with 1 liter of dialysate could remove only 4.0–0.27% (respectively) of the drug in each cycle of dialysis. If precipitated drug is not removed from the formulation within the period of dialysis, then the stability of formulations would be overestimated. Thus, dialysis may represent a poor method for determining the physical stability of taxane liposome formulations.

**Centrifugation/Filtration.** Centrifugation methods are capable of separating dense drug precipitates from liposomes, which are less dense. Similarly, filtration methods are capable of separating large drug precipitates from liposomes that are smaller. A combination of these simple methods has been used as a rapid screen of large numbers of drug–lipid compositions (Sharma and Straubinger, 1994b). For both of these methods, the size of liposomes is reduced after the preparation of MLV, as described above.

**Centrifugation.** MLVs sediment when centrifuged for 15 min at approximately 15,000g to 20,000g, but small unilamellar vesicles (SUVs) require much higher forces for longer times (Düzgüneş et al., 1983). Therefore, SUVs are centrifuged for 15 min at 20,000g (20\(^\circ\)) immediately after preparation in order to remove any residual MLVs (Düzgüneş, 2003). At intervals thereafter, the formulation is recentrifuged for 15 min at 15,000g (20\(^\circ\)). Both drug and phospholipid are quantified. A reduction in the drug/lipid ratio, without a reduction in the phospholipid concentration in the supernatant, is interpreted as destabilization of the formulation, accompanied by the formation of dense taxane-rich crystals. A reduction in the drug/lipid ratio, accompanied by a reduction in the phospholipid concentration, may result from either loss of the taxane from the liposome
or the formation of dense liposome aggregates. Optical microscopy (above) can be used to discriminate these possibilities.

**Filtration.** Various types of filters permit the passage of small liposomes and retain larger aggregates or precipitates. The polycarbonate filters used for extrusion (above) are one example; a solvent-resistant polycarbonate filter having pores larger than the liposomes but smaller than drug precipitates can be incorporated into a syringe-compatible holder (Avestin, Inc., Ottawa, Canada), which permits the analysis of small-volume samples. Filtration of the SUV formulation is performed at intervals, and both drug and phospholipid concentrations in the filtrate are determined (above). A reduction in the drug/lipid ratio without a reduction in the phospholipid concentration in the filtrate is interpreted as destabilization accompanied by drug precipitation. By optical microscopy, the observation of taxane-rich crystals would be expected. If both the drug concentration and the phospholipid concentration of the filtrate decrease, dense liposome aggregates may be forming. In parallel, drug may be precipitating. Optical microscopy (above) can be used to discriminate between these possibilities.

**Combined Methods.** In practice, filtration or centrifugation alone may be unsatisfactory. We have observed that filtration alone can be difficult to perform if excessive drug precipitation or liposome aggregation occurs. Also, immediately after sonication, crystals may be reduced to a size range that is able to pass through filter pores and thus lead to an underestimate of the amount of precipitated drug. Centrifugation may be inadequate for removing very small drug precipitates, particularly when destabilization is proceeding rapidly. As a result, we developed a combined procedure, in which the preparation is centrifuged and filtered repeatedly (Sharma and Straubinger, 1994). This process continues until no visible precipitate can be seen after centrifugation. Each successive filtrate or supernatant is collected after the separation step; this fraction should contain stable taxane-containing liposomes. The drug and phospholipid concentrations are assayed for each filtrate and supernatant; if the process has completely removed any drug precipitates and liposome aggregates, then the drug/lipid ratio will reach a constant value and indicate that a stable drug/lipid ratio has been achieved.

**Determination of Physical Stability by Spectroscopic Techniques**

**Circular Dichroism Spectropolarimetry (CD).** The taxanes possess several chromophores linked through chiral centers and therefore are CD active (Balasubramanian and Straubinger, 1994; Balasubramanian et al., 1994). The conformation of the taxanes, and therefore the taxane
CD spectrum, is sensitive to the polarity of the molecular environment (Balasubramanian et al., 1994; Williams et al., 1993; Vander Velde et al., 1993) (Fig. 3). This phenomenon can be used to monitor both the conformation and stability of taxanes incorporated into liposomes (Balasubramanian and Straubinger, 1994; Campbell et al., 2001) or other carriers (Alcaro et al., 2002; Sharma et al., 1995b). Because CD measurements are rapid and nondestructive, this technique provides a useful adjunct to the centrifugation/filtration method of investigating taxane formulation stability (Balasubramanian and Straubinger, 1994; Campbell et al., 2001).

Fig. 3. Circular dichroism spectra of paclitaxel. Paclitaxel was dissolved at 0.1 mM in ethanol (filled circles) or suspended in water (open circles). CD spectra were acquired at 25°C over the range of 360–215 nm, using a Jasco J500 spectropolarimeter calibrated with d_{10} camphor sulfonic acid and a 1-mm pathlength cuvette. (Reprinted with permission from Campbell et al., 2001.)
Interpretation of Paclitaxel CD Spectra. Figure 3 shows the CD spectrum of 100 \( \mu M \) paclitaxel dissolved in methanol or suspended in water. In methanol, the soluble drug shows a deep, symmetrical negative band centered at 295 nm, which arises from a \( \pi-\pi^* \) transition of the aromatic rings in the C13- and C2–O-benzoyl side chains (Fig. 1); this band shifts to shorter wavelengths in solvents of lower polarity (Balasubramanian et al., 1994). A shoulder at \( \sim 265 \) nm may be more or less prominent, depending on solvent polarity, and this band corresponds to the conformation of the small \( O \)-benzoyl side chain at C2 (Fig. 1). Both the C2–O-benzoyl ring and the C13 side chain are markedly sensitive to solvent polarity (Balasubramanian and Straubinger, 1994; Balasubramanian et al., 1994; Vander Velde et al., 1993). A positive band at approx. 230 nm exists in methanol and arises from n–\( \pi^* \) transitions involving C\( \subseteq\)O groups at C3\( ^0 \) and C9\( ^0 \) and several chiral monoolefins (Balasubramanian and Straubinger, 1994; Balasubramanian et al., 1994).

The CD spectrum of paclitaxel in water (Fig. 3) represents aggregated, precipitating drug. As the drug aggregates, the negative 295-nm band decreases drastically in intensity and shifts to higher wavelengths, indicating a change in the conformation and environment of the C13 side chain. The formation of exciton split bands at \( > 305 \) nm marks the formation of dimeric drug aggregates. Changes in the intensity of the 265-nm band implicate changes in the C2–O-benzoyl side chain as well.

The CD spectrum of paclitaxel incorporated into liposomes at low drug/lipid ratios resembles the CD spectrum of fully soluble drug in 70–100% methanol (Balasubramanian and Straubinger, 1994). As the drug/lipid ratio increases to proportions that destabilize the liposome formulations, the 265-nm band may become more prominent, and the 295-nm band both shifts to longer wavelengths and decreases in intensity. Both changes signify drug aggregation in the liposome membrane. As destabilization and precipitation of drug occur, the CD spectrum resembles that of paclitaxel in water (Fig. 3).

Acquisition of CD Spectra for Taxane Liposomes. To acquire CD spectra of taxanes incorporated in liposomes, the liposomes must be prepared in solutions that do not contribute to the CD spectrum in the range of 350–200 nm; water or physiological saline (0.9\% w/v) is acceptable. Typically, liposomes are prepared by the MLV method described above. Large liposomes cause an intense scattering signal, and two steps may be taken to reduce interference in the CD spectra. First, the concentration of liposomes can be reduced, and a short pathlength (0.1 mm) cuvette may be used. Second, the liposome diameter may be reduced, usually by brief sonication, to limit the delay between liposome preparation and the initiation of CD observations. Spectra are acquired
repetitively over the range of 350–200 nm to capture time-dependent changes that may signify liposome destabilization. In performing experiments to investigate the role of membrane composition on formulation stability, liposome preparations containing a wide range of drug/lipid ratios are prepared, and spectra are acquired for each to determine the time until the spectral signature of drug aggregation/precipitation occurs. Because formulation destabilization may be time delayed, measurements include not only the immediate (<2 h) temporal changes following hydration of the dried drug–lipid mixture but also include more detailed measurements at times that reflect the desired duration of stability in the hydrated state. Typically, 24-h stability in the hydrated state is required for formulations to have a clinical potential.

Quantitative Interpretation of Data. The complex CD spectral changes that occur during drug aggregation and precipitation may be simplified by analysis to produce a simple means to represent stability data (Balasubramanian and Straubinger, 1994; Campbell et al., 2001). Conversion from ellipticity to molar ellipticity normalizes for differences in drug concentration in the formulation. Therefore, comparison of molar ellipticity at wavelengths that are particularly sensitive to destabilization (e.g., the negative band at ~295 nm) provides a relative measure of formulation stability. Typically, molar ellipticity of the ~295 nm band as a function of time enables comparison of the temporal stability of various formulations.

A second manipulation of the CD spectral data permits estimation of the fraction of taxane incorporated in the liposome membrane (Balasubramanian and Straubinger, 1994; Campbell et al., 2001). For this method, CD spectra are acquired for 100 μM taxane in water and in 70% (v/v) methanol. Molar ellipticity is calculated, and the value of the negative-going peak at ~295 nm in methanol is taken as the molar ellipticity of taxane that is 100% incorporated in the membrane. The molar ellipticity at the same wavelength for drug suspended in water is taken to represent 0% incorporation. Time-dependent changes in the percentage incorporated in membranes is taken as an indication of destabilization. The benefit of this second method is that if separation techniques are used to remove precipitated material, calculation of the molar ellipticity reflects the fraction of original drug retained in the membrane and thus provides a means to compare formulations.

Determination of Physical Stability by Other Techniques

Differential Scanning Calorimetry (DSC). The formation of packing defects in the membrane bilayer lipids, mediated by taxane aggregation,
is one mechanism hypothesized for formulation destabilization. DSC is a relatively simple technique that can reveal drug partitioning into the bilayer and the presence of packing defects in the bilayer. Liposomes composed of phospholipids that possess defined acyl chains exhibit a sharp, cooperative transition at characteristic temperatures as the bilayer changes from the gel to the liquid crystalline phase. Drug interaction with the membrane lipids thus can be observed easily with liposomes composed of phospholipids that contain defined hydrocarbon moieties such as dimyristoyl-, dipalmitoyl-, or distearoyl-acyl chains. The incorporation of the drug into the liposome membrane can be observed readily, as the drug may alter the phase transition temperature or broaden the thermotropic peak (Pedroso de Lima et al., 1990). At lower drug/lipid ratios, taxane incorporation induces transition peak broadening (Balasubramanian and Straubinger, 1994), indicating that the drug partitions in the upper portion of the bilayer (i.e., the domain bordering the aqueous interface). Such partitioning is expected, based on the hydrophobic nature of the taxanes. At higher taxane/lipid ratios, a shift in the peak position can be observed, indicating the appearance of packing defects in the bilayer that can induce instability. At high drug/lipid ratios that exceed the capacity of the membrane to incorporate the drug, the DSC thermogram reverts to a sharp peak similar to that of membranes in the absence of drug. This observation indicates that destabilization of the formulation results in the loss of most drug from the membrane bilayer upon drug precipitation.

Typically, thermograms are recorded at heating rates of 2.5–5°K/min. For each thermogram, 15 μl of a 40 mM liposomal solution is loaded into the sample pan, and the samples are held at the initial temperature for 15 min. The DSC instrument is calibrated with standard samples covering a wide range of temperatures. The peak position and peak width at half height are generally indicative of liposome–drug interaction. A broadening of the main transition without any change in the peak melting is classified as an A type change. A shift in transition temperature in addition to peak broadening suggests the formation of packing defects. The appearance of new peaks on either side of the main transition indicates the formation of discrete domains—drug-rich or drug-poor—and may indicate phase separation and immiscibility.

Concluding Remarks

The unusual molecular structure of the active taxanes appears to contribute to both poor solubility and to the desired pharmacological activity. Although a wide variety of new, more water-soluble analogues are under investigation, drug carrier approaches that address the solubility problems
of the currently used agents, which have been studied extensively in large numbers of clinical trials, provide a rational and attractive alternative. Novel drug delivery approaches may provide a useful formulation tool that can eliminate vehicle toxicity and potentially improve the antitumor efficacy of these clinically important agents.

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