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Insights into accelerated liposomal release of topotecan in plasma monitored by a non-invasive fluorescence spectroscopic method

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ABSTRACT

A non-invasive fluorescence method was developed to monitor liposomal release kinetics of the anticancer agent topotecan (TPT) in physiological fluids and subsequently used to explore the cause of accelerated release in plasma. Analyses of fluorescence excitation spectra confirmed that unencapsulated TPT exhibits a red shift in its spectrum as pH is increased. This property was used to monitor TPT release from actively loaded liposomal formulations having a low intravesicular pH. Mathematical release models were developed to extract reliable rate constants for TPT release in aqueous solutions monitored by fluorescence and release kinetics obtained by HPLC. Using the fluorescence method, accelerated TPT release was observed in plasma as previously reported in the literature. Simulations to estimate the intravesicular pH were conducted to demonstrate that accelerated release correlated with alterations in the low intravesicular pH. This was attributed to the presence of ammonia in plasma samples rather than proteins and other plasma components generally believed to alter release kinetics in physiological samples. These findings shed light on the critical role that ammonia may play in contributing to the preclinical/clinical variability and performance seen with actively-loaded liposomal formulations of TPT and other weakly-basic anticancer agents.

1. Introduction

Many physiological factors (i.e. age, gender, dose regimen, type or location of cancer, mononuclear phagocyte system [1]) have been proposed to influence the pharmacokinetics (PK) and pharmacodynamics (PD) of nanoparticle formulations of anticancer agents. Unfortunately, the correlation between these factors and nanoparticle efficacy remains largely unknown [2,3]. For liposomal formulations, bilayer integrity may be compromised by the particles' interactions with proteins (e.g. vesicle binding and particle opsonization) [4–7] or osmotic stresses [8,9] either in the circulation or at the tumor site. Actively-loaded liposomal formulations may also exhibit accelerated drug release due to *in vivo* factors that alter the intraliposomal pH [10]. Liposomal formulations that rely on pH gradients for active-loading of anticancer agents are numerous [11–16] and would share in these susceptibilities. Reports describing the effects of such physiological phenomena on *in vivo* release kinetics have been limited due to the lack of available *in-situ* methods to monitor and distinguish entrapped from free drug in physiologically relevant media. Methods to determine the release kinetics of drugs from circulating liposomes and/or at the tumor site are crucial to optimizing the efficacy of liposome-based drug delivery systems.

Ultimately, mathematical models will be needed to interpret release profiles and provide a mechanistic understanding of the *in vivo* factors that lead to variability in the performance of liposomal formulations in order to establish *in vitro–in vivo* correlations. Such models must distinguish between physicochemical release characteristics intrinsic to the drug/particle system and factors contributed by the release environment (i.e. kinetic or thermodynamic effects attributable to the particular medium within which release is determined) [10,17–21].

Topotecan (TPT) is a camptothecin analog known for its topoisomerase-I inhibitory activity and regulation of genes associated with angiogenesis [22]. Several preclinical studies have demonstrated increased anti-tumorigenic efficacy of liposomal formulations of TPT that have reduced systemic clearance, allowing greater uptake and extended tissue exposure in murine solid tumors [14,23,24]. Many of the liposomal formulations of TPT are actively loaded by establishing an acidic intravesicular compartment relative to the extravesicular pH of the loading solution. This process provides high drug loading efficiency while ensuring delivery of the pharmacologically active lactone form of TPT to the tumor. While actively loaded liposomal formulations have often shown prolonged retention in aqueous buffers [14,25,26], the same formulations may exhibit accelerated release in plasma [14,26].

Although the low intravesicular pH has been shown to persist after active drug loading [14,24–27], to the authors' knowledge this is the first report of its use to differentiate between entrapped and free TPT during drug release. Realizing that the fluorescence of TPT is pH-dependent [13,28], changes in TPT fluorescence in aqueous liposomal

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suspensions and in plasma were explored as a potential means of non-invasively monitoring liposomal release in real-time. Analyses of fluorescence spectra confirmed that free TPT exhibits a red shift in its excitation spectrum as pH is increased. Due to this red shift, release of TPT from actively loaded liposomal TPT (ALLT) formulations could be monitored using fluorescence at higher wavelengths (410–430 nm) where entrapped drug at low intravesicular pH does not fluoresce.

The initial aim of this study was to validate a fluorescence method to non-invasively monitor liposomal release of TPT in a physiological environment. During the course of comparing apparent liposomal release profiles in different media including PBS buffer, plasma, and plasma ultrafiltrate using either the fluorescence method or HPLC it became evident that: a) TPT release is dramatically accelerated in human plasma as initially reported by Liu et al. [14]; and b) similar release kinetics were obtained in plasma ultrafiltrates. Recognizing that a non-filterable plasma component must be responsible for the accelerated release and that normal human plasma contains low levels of ammonia [29,30], additional studies were conducted to probe the concentrations of ammonia in the plasma samples and the effect of ammonia on TPT release. To mechanistically rationalize differences in release profiles using different analytical methods and media, mathematical models were developed to account for the effects of liposome concentration, intravesicular pH, TPT ionization, and ammonia concentration on release kinetics.

2. Materials and methods

2.1. Materials

Powders of 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC, >99% purity) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG2K, MW = 2806, >99% purity) were purchased from Avanti Polar Lipids (Alabaster, AL). Topotecan hydrochloride was purchased from AK Scientific (Union City, CA). Heparinized human plasma samples from seven healthy donors of different ethnicity were purchased from Innovative Research (Novi, MI), aliquoted and stored at −20 °C. An additional complement-protected plasma sample was also obtained from the same source by special request. Benzene sulfonic acid sodium salt (sodium besylate) was purchased from Spectrum Chemicals (New Brunswick, NJ). Millipore ultrafiltration cartridges (Amicon® Ultra 0.5 mL centrifugal filter device with 3000 MWCO Ultracel® membrane), Nuclepore polycarbonate membranes (0.1 μm), Dowex 50WX8-200 resin in the H^+ form, solvents, and buffer salts were purchased from Fisher Scientific (Florence, KY). All solvents were HPLC grade.

2.2. Liposome preparation

Large unilamellar liposomes were prepared based on previously reported methods [10,17,19,20,31,32]. Briefly, powders of DSPC and DSPE-PEG2K were dissolved in chloroform at a molar ratio of 95:5, then dried under nitrogen, and finally under vacuum (−30 in Hg) at 35 °C for 6 h. After drying, the films were hydrated in either 0.3 M ammonium besylate, 1 mM TPT in 50 mM pH 3.75 formate buffer, or pH 7.4 phosphate buffered saline (PBS) solutions to produce 30 mg/mL lipid suspensions. These suspensions were vortexed at 60 °C, then extruded through two 100 nm polycarbonate membranes 10 times at 40 psig and 60 °C to yield suspensions of ammonium besylate-loaded liposomes (ABLs), passively-loaded TPT-containing liposomes at pH 3.75, or blank liposomes, respectively.

The ammonium besylate solutions (0.3 M) used for liposome hydration were prepared in a manner similar to that previously used to make other amino-based salts [15,16]. Solutions of sodium besylate (0.6 M) were passed through an ion exchange column loaded with Dowex 50WX8-200 resin in the H^+ form. The eluted solutions were subsequently titrated with ammonium hydroxide (3.0 M) to the equivalence point and diluted to the desired concentration.

2.3. Active loading of TPT into ammonium besylate liposomes

Previous studies have shown that active-loading of weakly basic drugs results in high encapsulation efficiency and possibly longer drug retention *in vitro* and *in vivo* [14,24]. Actively-loaded liposomal suspensions of TPT were prepared with the aim of evaluating a fluorescence method to analyze drug release *in vivo* or *ex vivo*. Active loading was performed by generating a low intravesicular pH via an ammonia gradient [12,14]. This gradient was established when extravesicular ammonium besylate was removed by passing the suspension through a Sephadex G-25 column similar to previous reports [14]. In this case, 0.4 mL of the ABL suspension was passed through the column equilibrated with 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.5 buffer and the first 5 mL of eluted suspension was collected for loading studies. Next, 1.5 mL of the eluted suspension was added to an equal volume of TPT dissolved in the same pH 5.5 buffer to achieve a total TPT suspension concentration of 60 or 180 μM and a lipid concentration of 0.92 mg lipid/mL. Loading occurred over a 72 hour period within a 37 °C incubator.

ALLT suspensions were prepared for release studies by removing extravesicular buffer and any remaining unloaded drug by applying 0.5 mL of ALLT to a Sephadex G-25 column equilibrated with PBS similar to previous reports [10,18]. The first 2.5 mL fraction eluted from the column was discarded. ALLT eluted in the next 2.5 mL fraction was collected for use in release studies monitored by fluorescence or HPLC.

2.4. Liposome characterization

Particle size was determined for ALLT and PLLT using dynamic light scattering (DLS) using a Beckman Delsa™ Nano C Particle Sizer as previously reported [20,21]. Lipid content was monitored by HPLC using an evaporative light scattering detector (ELSD). A Waters Alliance 2695 separations module equipped with an Allsphere (Alltech Associates, Inc., Deerfield, IL) silica column (4 × 150 mm, 5 μm) and guard column (20 × 4.0 mm, 5 μm) and a mobile phase consisting of 80% of solvent A (80% chloroform:19.5% methanol:0.5%(v/v) NH_4OH) and 20% of solvent B (80% methanol:19.5% water:0.5% (v/v) NH_4OH) flowing at 1 mL/min was used to quantify DSPC in conjunction with an ELSD (Sedere, Inc., Lawrenceville, NJ) operated at 40 psig and 40 °C. Standards of DSPC were dissolved in mobile phase A (0.05–0.3 mg DSPC/mL). Log–log plots of peak area versus concentration were linear over this concentration range. Samples (100–250 μL) were dried at room temperature under N_2, then dissolved in chilled solvent A before analysis.

2.5. Fluorescence method development and validation

2.5.1. TPT excitation spectra

Samples and standards from validation and release studies were placed in 1 mL quartz cuvettes (NSG Precision Cells, Inc. Farmingdale, NY) for spectrometric analysis. Fluorescence excitation spectra (290–500 nm) were collected with a FluoroMax-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ) operating at a constant emission wavelength of 550 nm, slit width of 1.5 nm, and a 0.5 second integration time. The temperature of the sample chamber was maintained at 37 °C.

Excitation spectra of free TPT (2.5 μM) and PLLT (2.5 μM total suspension concentration of TPT after Sephadex removal of unentrapped drug) in pH 3.75 formate buffer were analyzed to compare the excitation spectra of free and entrapped TPT under acidic conditions. These spectra were compared to excitation spectra of free TPT (2.5 μM) at pH 7.4 and ALLT suspensions in pH 7.4 PBS (2.5 μM suspension TPT, 37 μg lipid/mL) to determine if ALLT spectra were indicative of an acidic intravesicular environment and whether spectra of entrapped and unentrapped drug were different.