Cabozantinib Loaded DSPE-PEG$_{2000}$ Micelles as Delivery System: Formulation, Characterization and Cytotoxicity Evaluation

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Abstract

Cabozantinib, a potent pan-tyrosine kinase inhibitor, has been reported to provide enhanced antitumor efficacy by simultaneously inhibiting both MET and VEGF pathways, which are critical to tumor angiogenesis, survival and migration. Its very poor water solubility prevents its administration by the intra-venous route, which may be important in patients unable to take the drug orally. In this study, we developed an efficient PEG-lipid-based polymeric micelle formulation with enhanced drug solubility and stability for cabozantinib delivery. DSPE-PEG$_{2000}$ micelles encapsulating cabozantinib were prepared by a thin-film rehydration method followed by a lyophilization process to generate the dry dosage form. The average hydrodynamic diameter of freshly pre-pared micelles was 11 nm with a narrow size distribution, and the dry micelle cake could be fully reconstituted by rehydration. Approximately 75% of the drug was encapsulated into the lyophilized cake, and a sustained drug release profile was observed in simula-ted normal physiological release medium. Compared with the free cabozantinib solution, the drug-loaded micelles displayed significantly enhanced intracellular accumulation and cytotoxicity in human glioblastoma cancer cells and non-small lung cancer cells. These results suggest that the micellar formulation of cabozantinib may serve as a promising nanocarrier in anticancer treatments.

Keywords: Cabozantinib; DSPE-PEG; Sustained release; Micellar solubilization; Lyophilization; Cyto-toxicity.

Introduction

Receptor tyrosine kinases (RTKs) are a family of trans-membrane cell surface receptors for diverse cell-signal-ing molecules such as cytokines, growth factors and hor-mones. Functionally, RTKs regulate many critical cellular processes, including cell growth, differentiation and an-giogenesis. Dysregulation of RTK activity is associated with several tumorigenic pathways, including the stimula-tion of malignant transformation, angiogenesis and tumor growth. RTK over expression has been observed in a wide variety of highly invasive human tumors, such as head and neck squamous cell carcinomas [1], medullary thyroid cancer (MTC) [2-5], glioblastoma multiforme (GBM) [6-8] and non small cell lung cancer (NSCLC) [9,10].

Dysregulation of RTKs can be activated via multiple mechanisms such as gene mutations, overexpression of ligands and receptor overexpression. Aberrant activation of mes-enchymal-epithelial transition factor (MET) [11-15] and vascular endothelial growth factor receptor 2 (VEGF-R-2) environment formed by the long fatty acyl chains can accommodate lipophilic drug molecules to efficiently-solubilize these poorly water-soluble drugs and restrict the mobility of the incorporated drugs at the same time, leading to a sustained drug release. Furthermore, the PEG moiety on the hydrophilic shell creates steric hin-drance that stabilizes micelles from aggregation, reduces the clearance rate by the reticuloendothelial system (RES), prolongs the circulation time of the drug-loaded micelles [50,51] and in turn facilitates the tumor accumu-lation of drug-loaded micelles due to the compro-mised leaky vasculature [52] found in many solid tumors.

In this study we have developed a stable caboza-ninib-encapsulated DSPE-PEG$_{2000}$ micelles formulation, with sustained release and enhanced delivery into cancer cells. The micellar formulation showed enhanced cyto-toxicity and comparable cellular uptake of cabozantinib by human glioblastoma cancer cells and non-small lung cancer cells. The micellar formulation of cabozantinib could be stored in the lyophilized form for an extended period of time with 96% drug recovery, making the mi-cell formulation a potential candidate for cancer therapy.

Materials and Methods

Materials

N - (Car-bonyl-meth-oxy-polyethylene-glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine (DSPE-PEG 2000 sodium salt) was purchased from NOF AMERICA CORPORATION (White Plains, NY, USA). Cabozantinib was obtained from Chemi-etek* (Indianapolis, IN, USA). HEPES (BioPerformance Certified, ≥99.5%) and penicillin/streptomycin

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Micelles formation and drug loading

Preparation of cabozantinib encapsulated DSPE-PEG<sub>2000</sub> micelles:

DSPE-PEG<sub>2000</sub> micelles were prepared by the lipid film-rehydration method described elsewhere [53] with modifications. Briefly, 20 mg of DSPE-PEG<sub>2000</sub> and 1.5 mg of cabozantinib were weighed in a 15-mL round bottom flask and dissolved with methanol, followed by bath sonication for 1 min. A thin film of drug-polymer was formed after the organic solvent was removed under the reduced pressure by a rotary evaporator. Residual methanol was removed by placing the flask on a high vacuum pump (< 0.2 mbar) overnight to thoroughly dry the thin film. The resulting dry film was then rehydrated using 2 mL of 10-mM HEPES-buffered saline (HBS) (pH 7.4).

The flask with the cabozantinib loaded DSPE-PEG<sub>2000</sub> micelles was sonicated for 5 min and then placed in a 50°C incubator for 1 h and then cooled to room temperature, followed by passing through a 0.22-μm nylon syringe filter. The un-encapsulated cabozantinib was re-moved by ultracentrifugation (MWCO: 3 kDa) at 3000 rpm for 10 min. Freshly prepared cabozantinib loaded DSPE-PEG<sub>2000</sub> micelles were stored at -20°C overnight and then transferred to -80°C for 2 h, followed by lyophilization below 0.01 mbar to prepare the dry dosage form.

Gel permeation chromatography:

The DSPE-PEG<sub>2000</sub> micelles encapsulating cabozantinib were characterized by gel-permeation chromatography (GPC) on a Shimadzu 2010CHT HPLC with an evaporative light scattering detector (ELSD) (Shimadzu Scientific Instruments, Inc., Columbia, MA, USA). GPC was performed with a Shodex OHpak SB-803 HQ column (Showa Denko America, Inc., New York, NY, USA). Human lung adenocarcinoma epithelial cell line A549 was kindly provided by Dr. Cory Berkland, University of Kansas (Lawrence, KS, USA). Double distilled water (ddH<sub>2</sub>O) was used in syntheses, characterization and cell-culture (sterilized by autoclaving).

The amount of cabozantinib incorporated into the DSPE-PEG<sub>2000</sub> micelles was quantified by the gradient reverse phase high-pressure liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu LC-2010CHT and an SPD-M20A Prominance HPLC Photo Diode Array Detector (Shimadzu Scientific In-struments, Inc., Columbia, MA, USA). An ODS-100 C18 analytical column (250 × 4.6 mm; 5 μm) was used for the analysis. The mobile phase consisted of A (10 mM ammonium acetate, pH 5.2) and B (acetonitrile), and the linear gradient was 30-90% B over 12 min at a flow rate of 1.0 mL/min. UV absorption was measured at 240 nm, and the drug loading efficiency (DL %) and encapsulation efficiency (EE %) of cabozantinib in the DSPE-PEG<sub>2000</sub> micelles were calculated using the equations:

\[
DL\ % = \frac{(Weight\ of\ the\ cabozantinib\ in\ micelles/ Weight\ of\ the\ cabozantinib\ and\ the\ DSPE-PEG_{2000})\ \times\ 100\%}{EE\ % = \frac{(Weight\ of\ the\ cabozantinib\ in\ micelles/ Weight\ of\ fed\ cabozantinib)}{X\ 100\%}}
\]

Micelle size and zeta potential measurements

The hydrodynamic diameter of the micelles was measured by dynamic light scattering (DLS) at 25°C on a ZetaPALS (Brookhaven Instruments Corp., Holtsville, NY, USA), with measurements repeated three times. The zeta potential of micelles prepared in 10-mM HBS was measured at 25°C on the ZetaPALS with measurements repeated three times.

In vitro drug release profile of the cabozantinib from DSPE-Micelles

The release behavior of cabozantinib from micelles was evaluated using a dialysis method under a sink condition. Approximately 5 mL of the micellar formulation of cabozantinib or the free drug solution was trans-ferred into dialysis tubing (Snake SkinTM, MWCO: 3.5 kDa) (Thermo Scientific Inc., Rockford, IL, USA). To prepare the aqueous solution of cabozantinib, the drug was first dissolved in DMSO and then diluted with 1-mM HCl to reduce the DMSO content to below 0.5% (v/v). The dialysis tubing was closed at both ends with clips and placed in 4.0 L of phosphate buffered saline (PBS) (pH 7.4) at 37°C. A sample of 50 μL was withdrawn from the dialysis tubing at pre-determined time intervals, and the PBS was changed every 4 h to ensure sink condition. The drug amount in each sample was determined as previously described.

In vitro cytotoxicity of cabozantinib from DSPE-Micelles

Two human malignant glioblastoma cell lines (U87 and U251) and a human lung adenocarcinoma epithelial cell line (A549) were used to investigate the in vitro cytotoxicity of the cabozantinib micelles. The cell lines were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere. Cancer cells in growth medium were seeded at a concentration of 3,000 cells per well in 96-well flat-bottomed plates and allowed to attach overnight. The cabozantinib aqueous solu-tion (≤ 0.5% v/v DMSO) or micellar drug solution was incubated with cells at different final concentrations from 5 to 20 μM. Trichloroacetic acid (TCA) and 10-mM HBS were added as positive and negative controls, respectively. Cell media were refreshed 24 or 48 h after the treatment, and a resazurin-based

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colorimetric assay was used to assess viability of cell cultures at 72 h post treatment using GraphPad Prism 6 (Graph-Pad Software Inc., La Jolla, CA, USA).

**Determination of cellular uptake of the cabozantinib DSPE-PEG<sub>2000</sub> micelles**

In order to evaluate the cellular uptake and accumulation of the cabozantinib micelles, cell lines were seeded at a density of 2 × 10<sup>4</sup> cells/mL in a 12-well plate. The cells were incubated with free drug solution or cabozantinib micelles at final drug concentrations of 10 or 20 μM for 2 h at conditions described in previous section. Non in-ternalized drug in the medium was removed, and the at-tached cells were then washed three times with ice-cold PBS. The cell pellets were collected at 5000 rpm for 10 min at 4°C after the trypsinization, and they then were resuspended in 0.5 mL of methanol for the quantification following the procedures as previously described.

**Statistical analysis**

All values are expressed as the mean ± standard deviation. One phase exponential association was applied for the curve fitting of the in vitro drug release kinetics to calculate the release half-life (t<sub>1/2</sub>). Statistical analyses were performed using two-way ANOVA with GraphPad Prism 6 and the significance was set at P < 0.05.

**Results and Discussion**

**Preparation of cabozantinib in DSPE-PEG<sub>2000</sub> micelles and determination of drug loading**

The poorly-water soluble drug cabozantinib was successfully encapsulated into the DSPE-PEG<sub>2000</sub> micelles by a thin-film rehydration method, and the resulting purified micelle solution was clear without drug precipitates or aggregates (Figure 1A left). A lyophilization process was performed in this study to prepare the dry dosage form and to enhance physical stability for long-term shelf storage. The lyophilized cake (Figure 1A middle) could be redispersed in water (Figure 1A right) within 30 s. The cell pellets were collected at 5000 rpm for 10 min at 4°C after the trypsinization, and they then were resuspended in 0.5 mL of methanol for the quantification following the procedures as previously described.

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The CMC of DSPE-PEG<sub>2000</sub> micelles is as low as approximately 0.5 – 1.0 μM in HEPES buffer [54] resulting from the strong hydrophobic interaction among the saturated C<sub>18</sub> acyl chains [54,55]. The final concentration of the DSPE-PEG<sub>2000</sub> in our micellar formulation was 7 mM, which is about 7000 fold above the CMC, leading to a successful micelle formation and efficient drug loading.

Drug leakage from colloidal drug delivery systems and subsequent drug precipitation and degradation during extended periods of storage have been observed in sev-eral studies due to the nanoparticle aggregation and the hydrolysis of the building blocks of the polymers that formed the nanoparticles [56,57]. Lyophilization has been used to stabilize a variety of drug nanocar-riers [58-62]. Sugars such as trehalose, sucrose, glu-cose and mannitol are often added at 5–20% (w/v) into the particulate systems as cryo/lyoprotectants to spare the products from the freezing/drying stress that may induce aggregation or fusion of nanoparticles.

However, collapse of the lyophilized cake was observed for some formulations containing glucose (5% and 10%, w/v) and mannitol (5%) [63]. In this study, a ly-ophilized micellar formulation of cabozantinib was pre-pared without adding cryo/lyoprotectants, and the re-constitution of the resultant lyophilized polymer-drug with water successfully regenerated the micelles. The GPC of the reconstituted drug-loaded micelles (Figure 1C) was found to be same as that of the freshly pre-pared micelle formulation. The ability of PEG-DSPE micelles to reconstitute without cryoprotectant is probably due to the large hydrophilic-lipophilic bal-ance of the micelles. Successful reconstitution of the indisulam-encapsulated DSPE micelle formulations without a cryoprotectant has also been reported [64].

<sup>1</sup>H NMR measurements were used to further charac-terize the encapsulation of the cabozantinib inside the micelle core formed by the DSPE segments [46,65]. <sup>1</sup>H NMR spectra of cabozantinib

![Figure 1](image-url)
dissolved in CDCl₃ (Figure 2A) and the DSPE-PEG₂₀₀₀ in D₂O (Figure 2B) clearly showed the distinct resonance peaks of the drug and the polymer, respectively, which include protons of methoxy groups (4.1240 ppm and 4.1834 ppm), aromatic rings (6.7053-8.4693 ppm), amino groups (10.2444 ppm) and cyclopropane (1.2754 ppm) for cabozantinib, and the di-saturated C₁₈ acyl chains at 1.2183 ppm and PEG block at 3.6241 ppm for DSPE-PEG₂₀₀₀. By comparison, only prominent peaks corresponding to DSPE-PEG₂₀₀₀ could be observed in the ¹H NMR spectrum of the cabozantinib loaded micelles (Figure 2C) that were prepared with D₂O, due to the restricted motion of the drug molecules in the micelles' hydrophobic cores, indicating efficient drug incorporation. When the lyophilized cabozantinib loaded micelles were reconstituted in the organic solvent, CDCl₃, resonance peaks of both the drug and polymer were present in the ¹H NMR spectrum (Figure 2D), as a consequence of the disrupted micelles structure in the organic solvent, which in turn released the drug from the core of micelles.

The loading efficiency (DL %) and encapsulation efficiency (EE %) of the cabozantinib into the DSPE-PEG₂₀₀₀ micelles were quantified by RP-HPLC and found to be 5.45 ± 0.09 % and 78.37 ± 1.34 %, respectively. Cabozantinib in the neutral form was practically insoluble in water. Remarkably, the aqueous solubility of cabozantinib was increased to 2.23 ± 0.01 mg/mL after being encapsulated into the DSPE-PEG₂₀₀₀ micelles. Cabozantinib loaded micelles in the lyophilized cake could be easily rehydrated with water, and the detected drug amount was 96.00 ± 0.87 % of the freshly prepared micelles solution. This high drug recovery indicates a promising prolonged shelf life of the lyophilized cabozantinib micelles for clinical applications.

Micelle Characterization

The average hydrodynamic size of the oblate spheroidal cabozantinib loaded micelles was 11.7 nm with a narrow size distribution (Figure 3), which is favorable for sustained circulation for in vivo delivery of the drug-in-corporated micelles. It has been reported that micelles with size in the nano-scale range (10 - 100 nm) can resist the systemic clearance by renal filtration and the reticuloendothelial system after administration [51,56], thus providing a prolonged pharmacological effect. In addition, cabozantinib molecules incorporated into the hydrophobic core of micelles are protected from the liver enzyme metabolism. Consequently, drug transportation to targeted extravascular tumor tissues through diffusion from the leaky blood vessels [52] is expected to be significantly enhanced due to the prolonged systemic circulation of the drug.
loaded micelles.

The zeta potential value indicates the potential colloidal stability of the micellar formulation. Colloidal systems with zeta potential above ± 30 mV have been generally accepted as moderately stable to prevent aggregation. The zeta potential of the cabozantinib-loaded micelles was -15.70 ± 1.24 mV, indicating an incipient instability. However, the hydrophilic PEG segments on the surface could prevent aggregation of micelles in solution due to its steric hindrance.

**In vitro drug release profile of cabozantinib from DSPE-PEG<sub>2000</sub> micelles**

The in vitro release behaviors of the micellar formula-tion of cabozantinib and the free drug were evaluated in PBS (pH 7.4) solution at 37°C under the sink conditions to simulate the significant dilution of the adminis-tered micelle solution by physiological fluids. As shown in (Figure 4), approximately 81 % of cabozantinib in the free drug solution was released within 4 h. DSPE-PEG<sub>2000</sub> micelles containing cabozantinib, on the other hand, exhibited sustained drug release over a period of greater than 10 days, with only 22 % of the cabozan-tinib released in the first 8 h. Release half-times of the drug in both forms were calculated from a curve fitting using one phase exponential association analysis.

The t<sub>1/2</sub> was significantly enhanced from less than 10 min to 80 h after the drug was incorporated into the micelles formulation, and similar in vitro controlled drug release profiles of DSPE-PEG micelles have been reported when hydrophobic drugs such as doxorubicin [67] and ridaforolimus [68] were encapsulated.

Repeat-dose toxicity and toxicokinetic studies with cabozantinib in rats that received daily oral gavage have shown debilitating side effects, including hematopoietic, hepatic, gastrointestinal or renal toxicity [40]. Polymeric mi-celles that incorporate PEG segment have been em-ployed as long-circulating drug vehicles [65,69,70], as the presence PEG in the outer hydrophilic shell spares micelles from aggregation and also decreases the ad-sorption of protein such as immunoglobulins to the hydrophobic surfaces, thus reducing the fast clearance of the micelles by RES [71,72]. Moreover, following the intravenous injection, the nanoscale drug-loaded mi-celles have been found to preferentially accumulate in the tumor tissues from their leaky endothelial vasculature, due to the enhanced permeability and retention effect [73-75]. Our study demonstrated a significantly prolonged in vitro release profile of the cabozantinib from the micellar formulation due to the strong drug interaction and association with the hydro-phobic micelle core, which is responsible for maintain-ing a steady-state drug stability and increased systemic circulation. After intravenously administered into the patients, cabozan-tinib-loaded DSPE-PEG2000 micelles are expected to target the tumor tissue and then controllably release the active chemotherapeutic agent. Consequently, the systemic toxicity of the drug could be minimized as the chemotherapy exposure to normal tissues, especially hematopoietic cells and epithelial cells in the gastrointesti-nal tract, is reduced.

**Cellular uptake of cabozantinib from DSPE-PEG2000 Mi-celles**

Human lung adenocarcinoma epithelial cell line A549 and human malignant glioblastoma cell lines U87 and U251 were treated with free cabozantinib solution or drug loaded DSPE-PEG<sub>2000</sub> micelles for 2 h, and the drug amounts internalized in cells were then quantified using the RP-HPLC. As shown in (Figure 5), micellar formulation of cabozantinib significantly enhanced the cellular accumulation of the drug in all cell lines under study at the concentration of 10 μM. Similarly, enhanced cellular uptake of hydrophobic drug-loaded micelles has been reported in the case of doxorubicin [76]. The enhanced intracellular uptake was possibly due to the different internalization mechanisms employed by the micellar formulation and the free drug. Several groups have investigated the mechanisms of micelle internal-ization by cancer cells using biocompatible fluorescent micelles [77]. Drug-loaded micelles have been shown to enter the cytoplasmic compartment by the endocy-tosis process, followed by the diffusion of the incor-porated molecules into organelles such as lysosomes, mitochondria and the Golgi apparatus. In comparison, free drug molecules slowly traversed through the cell membrane and then entered the cytoplasmic space. To our surprise, no statistical significance was observed at the fed cabozantinib concentration of 20 μM. We
In vitro cytotoxicity of cabozantinib from DSPE-PEG\textsubscript{2000} Micelles

Cabozantinib is currently being tested in cancer patients with non-small cell lung cancer, glioblastoma, melanoma, ovarian cancer and hepatocellular carcinoma, etc. We chose one human non-small cell lung cancer cell line A549 and two human malignant glioblastoma cell lines U87 and U251 in this pilot study to investigate the cytotoxicity of the cabozantinib-loaded DSPE-PEG\textsubscript{2000} micelles. The results of cytotoxicity study on free cabozantinib and cabozantinib-loaded micelles solution against the three cell lines are shown in (Figure 6). Overall, we observed strong dose and time-dependent inhibitory activity in all cell lines under study from 5 to 20 μM. Compared to the free drug solution, the cabozantinib micellar formulation exhibited significantly enhanced cytotoxicity after 24 or 48 h of incubation. Higher cytotoxic activity has also been demonstrated in other anticancer drug-loaded nanoparticles. For example, a doxorubicin-loaded polymeric nanoparticle was found to be over 30 times more active in drug-resistant MCF-7
tumor cells than free drug, which was attributed to the increased intracellular drug concentration. Diao et al later reported reversed multidrug resistance using doxorubicin-loaded PEG-PCL copolymer micelles in multidrug-resistant K562 cells [76]. Among the three human tumor cell lines, the U87 and A549 cell lines were more sensitive to the treatment than the U251 cell line, probably due to varying levels of VEGFR-2/MET expression and intracellular drug concentration.

Conclusions

In the present work, we successfully solubilized cabozantinib, a dual MET/VEGFR2 inhibitor, in an 11-nm DSPE-PEG2000 micellar formulation with an encapsulation efficiency of approximately 80%. The drug-loaded micelles were stabilized in the lyophilized form. Therefore, it could provide extended shelf life without using cryo/lyoprotectants. The sustained in vitro release profile indicated a potentially prolonged in vivo circulation after administration for the long-acting chemotherapy. Compared with the free cabozantinib solution, drug-loaded micelles exhibited increased intracellular drug uptake and higher cytotoxicity in one human lung adenocarcinoma epithelial cell line and two human malignant glioblastoma cell lines. In conclusion, cabozantinib can be formulated as a highly concentrated drug in an aqueous injectable solution above the solubility limit of the free drug, thus lowering the required dosage volume. Therefore, administration of cabozantinib-in-corporated micelles may serve as a promising approach to parenteral formulation of a chemotherapeutic agent against human malignant solid tumors.

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