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A novel liposomal formulation of flavopiridol

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ABSTRACT

Flavopiridol has shown promising activities in hematologic and solid tumor models, as well as in clinical trials in chronic lymphocytic leukemia patients. Flavopiridol has relatively low solubility and high plasma protein-binding. To address these issues and to provide an alternative strategy to achieve clinical efficacy, we encapsulated flavopiridol into a liposomal carrier and characterized its physicochemical and pharmacokinetic properties. The liposomes, comprising hydrogenated soy phosphatidylcholine (HSPC), cholesterol and poly (ethylene glycol) 2000-distearoyl phosphatidylethanolamine (PEG-DSPE), were prepared by polycarbonate membrane extrusion and then loaded with flavopiridol by a pH-gradient driven remote loading procedure. The liposomes had a mean diameter of 120.7 nm and a flavopiridol entrapment efficiency of 70.4%. Pharmacokinetic study in mice after i.v. bolus injection showed that the liposomal flavopiridol had an increased elimination phase half-life ($T_{1/2\beta}$, 339.7 min vs. 57.0 min), decreased clearance (CL, 0.012 L/min vs. 0.036 L/min), and increased area under the plasma concentration–time curve (AUC, 10.8 min μmol/L vs. 3.4 min μmol/L) compared to the free drug. This indicates a significant and potentially beneficial change in flavopiridol pharmacokinetics for the liposomal formulation. Further preclinical studies are warranted to define the toxicity and therapeutic efficacy of this novel formulation.

1. Introduction

Flavopiridol is an important experimental drug that is currently under clinical investigation for multiple diseases, including non-Hodgkin's lymphoma, chronic and acute leukemias, and renal, prostate, colon and gastric carcinomas (Zhai et al., 2002; Burdette-Radoux et al., 2004; Tan et al., 2002; Senderowicz et al., 1998; Byrd et al., 2005; Lin et al., 2002; Flinn et al., 2005; Liu et al., 2004; Aklilu et al., 2003; Shapiro et al., 2001). Mechanistic studies have shown that flavopiridol is a potent inhibitor of cyclin-dependent kinases (CDKs), which are key regulators in cell cycling. Flavopiridol acts by inducing programmed cell death, promoting differentiation, inhibiting angiogenic processes and modulating transcriptional events (Sedlacek, 2001). In clinical trials, its anticancer effect has been demonstrated at generally lower concentrations (usually at the nanomolar to low micromolar range) relative to other chemotherapeutics (Senderowicz, 2002).

Flavopiridol is a weak base (Fig. 1) and has poor solubility in water (Li et al., 1999). At pH 7.4, the compound has a solubility of 0.04 mg/mL. For *in vitro* cell dosing, most studies have applied DMSO as a solvent, which is not suitable for *in vivo* applications. Clinically, flavopiridol is solubilized as a citrate/2-hydroxypropyl-β-cyclodextrin (HPβCD) complex (Dannenfelser et al., 1996), although this also requires the use of acidic pH. Another challenge is that flavopiridol has relatively high plasma protein binding affinity, which limits free drug concentrations and compromises activity towards cancer cells. Continuous infusions over 72 h have been applied in an attempt to achieve sufficient steady state plasma concentrations (Senderowicz, 2003). Recent studies suggest that increased exposure to flavopiridol achieved via changes in dose rate can improve response in patients with chronic lymphocytic leukemia (Byrd et al., 2007). Flavopiridol, formulated as a HPβCD and citric acid complex, has been shown to induce diarrhea,

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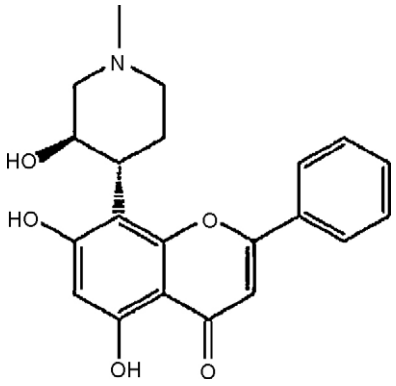

Fig. 1. Structure of flavopiridol.

fatigue, and neutropenia (Byrd et al., 2007; Senderowicz, 2001). A slow-release, long-circulating formulation, based on liposomes, may provide a novel approach to improve the therapeutic index of flavopiridol.

In this study we encapsulated flavopiridol in a liposomal carrier, and characterized its physicochemical and pharmacokinetic properties. Our results provide the first evidence that liposomal formulation presents a viable approach to increase the systemic exposure and potential anticancer activity of flavopiridol.

2. Materials and methods

2.1. Flavopiridol

(NSC 649890) was supplied by the NCI's Developmental Therapeutics Program. Hydrogenated soy phosphatidylcholine (HSPC) and poly (ethylene glycol) 2000-distearoyl phosphatidylethanolamine (PEG-DSPE) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol) was purchased from Avanti Polar Lipids (Alabaster, AL). Sepharose CL-4B, Tween-80, HPβCD, β-cyclodextrin sulfate (βCDS) and other chemicals were purchased from Sigma Chemical (St. Louis, MO).

2.2. Liposome preparation

Liposomes were prepared by thin-film hydration, polycarbonate membrane extrusion, followed by remote loading of flavopiridol. Lipids were dissolved in $CHCl_3$ and dried in a round-bottom flask on a rotary evaporator and then under vacuum. The resulting lipid film was hydrated with 300 mM $(NH_4)_2SO_4$ or citric acid. The lipid suspension was then extruded 3 times each through a 200 nm and then a 100 nm pore size polycarbonate membrane on a nitrogen-driven Lipex^{TM} lipid extruder (Northern Lipids Inc., Burnaby, BC, Canada). The liposomes were then subjected to diafiltration using a Spectrum Microkros hollowfiber cartridge to replace $(NH_4)_2SO_4$ outside of the liposomes with phosphate buffered saline (PBS, pH 7.4). For drug loading, flavopiridol was dissolved in a small volume of citric acid (20 mM, pH 4) and added to the liposomes. After 30 min incubation at 60 °C, the liposomes were purified by size exclusion chromatography on a Sepharose CL-4B column equilibrated in PBS (pH 7.4). To verify that flavopiridol loading was indeed driven by a pH gradient, liposomes were also synthesized in the presence of HEPES-buffered saline (HBS, 20 mM HEPES, 145 mM NaCl, pH 7.4) and incubated with flavopiridol.

2.3. Characterization of the liposomes

Liposome size distribution was determined by dynamic light scattering on a NICOMP Submicron Particle Sizer Model 370 (NICOMP, Santa Barbara, CA).

To determine the drug content, the liposomes were lysed by isopropanol. The flavopiridol concentration in the lysate was determined by absorption at 269 nm on a Shimadzu UV–vis spectrophotometer based on the molar extinction coefficient of flavopiridol. Loading efficiency of flavopiridol in liposomes was calculated based on the amount ratio of free drug and liposomal drug, which were separated by the Sepharose CL-4B column equilibrated by PBS (pH 7.4).

The liposomes were observed under transmission electron microscopy (TEM). Briefly, the liposomes were diluted ten-fold with distilled water and applied to 300 mesh, formvarcarbon-coated Cu grids. Liposomes were then negatively stained with 2% uranyl acetate (pH 4.8) for 30 s. Stained samples were characterized using Philips CM 12 TEM (Philips Ltd., Eindhoven, The Netherlands) at a final magnification of 120,000×. The magnification of the microscope was calibrated with standard latex spheres. Three grids were prepared for each sample and the grid openings were randomly selected and viewed.

2.4. In vitro release of flavopiridol from the liposomes

Rates of drug release from liposomes were determined by measuring retention of the drugs in the liposome fraction. Liposomes (100 μL) were added to 900 μL of PBS (pH 7.4) or 55% fetal bovine serum (FBS) and incubated at 37 °C. At various time points (2, 4, 12, 24, 36 and 48 h), samples were removed from the 37 °C water bath. Free and liposome encapsulated flavopiridol were separated by a Sepharose CL-4B column, as described above. As a reference control, 50% FBS was eluted from the same column and shown to elute in fractions between the liposomal and free drug fractions. For drug concentration analysis, liposomes were lysed by isopropanol as described above. The percentages of drug remaining in the liposome fractions were calculated.

2.5. Pharmacokinetic studies

Plasma clearance rates of free flavopiridol and drug formulated in the liposomes were compared in ICR mice (Charles River Lab, Wilmington, MA, USA). Mice, in groups of three, received intravenous injections of flavopiridol at 2.5 mg/kg body weight via tail vein. Blood samples were collected in heparin-containing tubes at various time points. Plasma was isolated by centrifugation at 1500 × *g* for 10 min and stored at −20 °C. Flavopiridol in plasma was subsequently extracted by acetonitrile (ACN) and analyzed by modification of a validated LC-MS method (Phelps et al., 2008). During the analysis, each 50 μL plasma sample was mixed with 200 μL cold ACN containing 1 μM internal standard, genistein. After centrifugation at 16,000 × *g* and 4 °C for 10 min, 215 μL of supernatant was dried and reconstituted in 150 μL 60/40 MeOH/H_2O for LC-MS analysis. WinNonlin Version 3.2 (Pharsight Co., CA) was used to determine pharmacokinetic parameters, including area under the curve (AUC), total body clearance (CL) and plasma half-life.

2.6. Statistical analysis

Data were represented as mean ± standard deviations (S.D.) and were analyzed by 2-tailed Student's *t*-tests using the MiniTAB software (Minitab Inc., State College, PA). $p < 0.05$ was used as the cutoff for statistically significant differences.

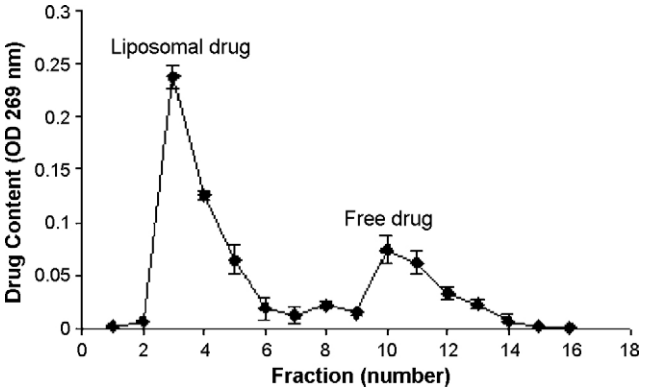

Fig. 2. Separation of liposomal flavopiridol from free flavopiridol by a Sepharose CL-4B column. Drug-containing HSPC/Chol/PEG-DSPE liposome was loaded on the top of a Sepharose CL-4B column equilibrated in PBS (pH 7.4) and the eluate was collected for drug concentration analysis by UV spectrophotometry. *X*-axis indicates the elution volume (mL) and the *Y*-axis is the absorbance of flavopiridol in each fraction after subtraction of the absorbance values of the empty liposomes. Results are the mean of 3 separate experiments. Error bars stand for standard deviations.

3. Results

3.1. Preparation of liposomes and optimization of formulation

To obtain flavopiridol liposomes with small particle size, homogenous distribution and high drug entrapment efficiency, different compositions were explored, including HSPC/Chol (molar ratio 55/45), HSPC/Chol/PEG-DSPE (molar ratio 55/40/5) and HSPC/Chol/Tween-80 (molar ratio 55/40/5). Entrapment efficiency of flavopiridol was obtained by separating liposomal and free drug using a 10 mL Sepharose CL-4B column (Fig. 2). Mean diameters of the liposome preparations were between 120 and 142 nm with relatively narrow distribution. In contrast, entrapment efficiency significantly varied among these formulations, with a range of 37.7 ± 0.7 to 81.2 ± 0.5%. Liposomes composed of HSPC/Chol had the highest drug loading. The addition of PEG-DSPE decreased entrapment efficiency to 69.8 ± 0.3%. Tween-80 reduced the drug content to the lowest value among tested compositions in this study (Table 1). Therefore, in the following experiments, we evaluated flavopiridol liposomes composed of HSPC/Chol/PEG-DSPE.

Regarding the internal buffer, 300 mM $(NH_4)_2SO_4$ retained ~20% more drug inside of liposome than 300 mM citric acid ($p < 0.05$). In addition, 70 mM HPβCD combined with citrate could promote the drug entrapment to the same degree as $(NH_4)_2SO_4$ (Table 2).

Table 3
Effect of pH gradient on entrapment efficiency

pH of external buffer	pH of internal buffer	Entrapment efficiency (EE) (%)
7.4	4.0	70.1 ± 1.3
7.4	7.4	0.8 ± 0.7

Data represent the mean ± S.D. of three separate experiments ($n = 3$).

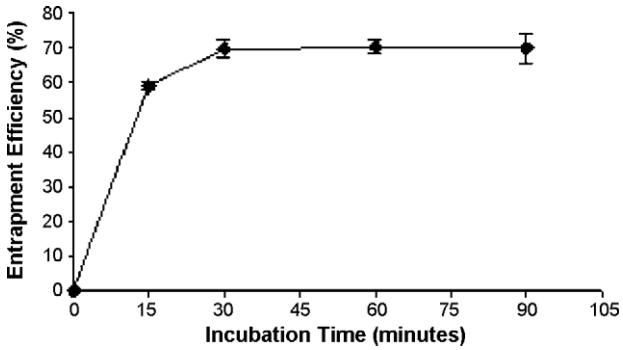

Fig. 3. Flavopiridol entrapment efficiency over drug loading time. Flavopiridol was incubated with empty HSPC/Chol/PEG-DSPE liposome in PBS at 60 °C for different loading times (minutes) and the drug entrapment efficiencies (EE) were measured respectively. Results are the mean of 3 separate experiments. Error bars stand for standard deviations.

We also tried to incorporate βCDS into the formulation, but did not find any improvements in drug entrapment efficiency. No drug loading was found for liposomes entrapping HBS (pH 7.4) as the internal buffer (Table 3), suggesting an important role of a pH gradient.

Drug loading into liposomes composed of HSPC/Chol/PEG-DSPE (molar ratio 55/40/5) increased with the incubation time up to 30 min. Further incubation beyond 30 min did not increase the drug entrapment efficiency (Fig. 3). Under TEM, the liposomes appeared as round particles with a homogenous distribution (Fig. 4).

3.2. In vitro release studies

The *in vitro* release kinetics of flavopiridol from HSPC/Chol/PEG-DSPE liposomes was determined. As shown in Fig. 5, the formulation was stable in PBS and in serum at 37 °C. The release over a time period of 24 h was <25%, while at 48 h the liposomes still retained over 60% flavopiridol.

Table 1
Effect of lipid composition on encapsulation efficiency and particle size

Liposome composition (molar ratio)	Size (nm)	Polydispersity index (PI)	Entrapment efficiency (EE) (%)
HSPC/Chol (55/45)	142.3 ± 1.5	0.178 ± 0.061	81.2 ± 0.5
HSPC/Chol/PEG-DSPE (55/40/5)	120.7 ± 0.9	0.112 ± 0.072	69.8 ± 0.3
HSPC/Chol/Tween-80 (55/40/5)	138.1 ± 1.2	0.318 ± 0.028	37.7 ± 0.7

Data represent the mean ± S.D. of three separate experiments ($n = 3$).

Table 2
Effect of internal buffer on encapsulation efficiency and particle size

Internal Buffer	Size (nm)	Polydispersity index (PI)	Entrapment efficiency (EE) (%)
300 mM $(NH_4)_2SO_4$	120.7 ± 0.9	0.112 ± 0.072	69.8 ± 0.3
300 mM $(NH_4)_2SO_4$, with 70 mM HPβCD	132.4 ± 0.3	0.278 ± 0.031	70.1 ± 0.6
300 mM citric acid	118.3 ± 0.7	0.182 ± 0.044	48.2 ± 0.1
300 mM citric acid, with 70 mM HPβCD	120.3 ± 0.7	0.361 ± 0.019	64.3 ± 0.4
100 mM βCDS	127.7 ± 1.1	0.312 ± 0.055	37.8 ± 0.9

Data represent the mean ± S.D. of three separate experiments ($n = 3$).

Table 4
Pharmacokinetic parameters of flavopiridol after intravenous administration (2.5 mg/kg)

	$T_{1/2\beta}$ (min)	V_d (L/kg)	AUC (min μmol/L)	CL (L/min)
Liposomal flavopiridol	339.7(8.6%)	2.4(11.9%)	10.8(10.3%)	0.012(10.3%)
Free flavopiridol	57.0(15.3%)	1.2(12.1%)	3.4(7.5%)	0.036(7.4%)

Data represents WinNonlin parameter estimate (standard error of estimate represented as CV%).

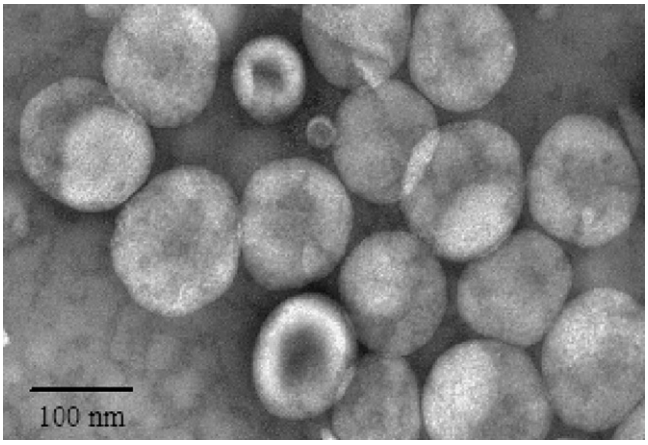

Fig. 4. TEM micrograph of flavopiridol-entrapped HSPC/Chol/PEG-DSPE liposomes.

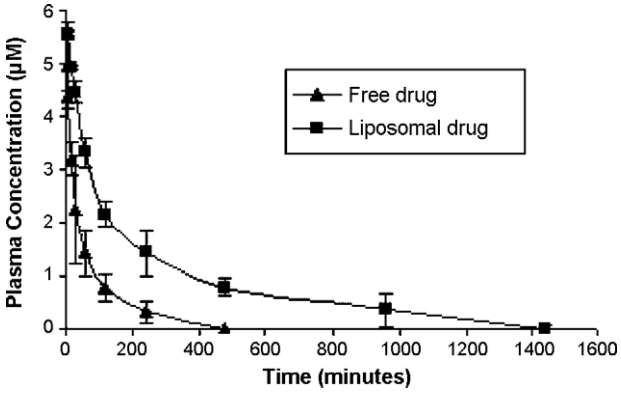

Fig. 6. Plasma concentration-time curve of flavopiridol after i.v. bolus injection of drug-entrapped liposome in mice. Liposomal flavopiridol was injected through the tail vein (2.5 mg/kg) in groups of 3 mice each. At indicated time points mouse plasma was collected, and flavopiridol was extracted for concentration analysis by LC-MS. Error bars stand for standard deviations ($n = 3$).

3.3. Pharmacokinetic study

Pharmacokinetics of flavopiridol-containing liposomes composed of HSPC/Chol/PEG-DSPE was determined in mice. As shown by the plasma flavopiridol concentration–time plot (Fig. 6), the flavopiridol in the liposomes was cleared at a slower rate compared to free flavopiridol. At 24 h after i.v. bolus administration, about 20.4% of the injected liposomal flavopiridol remained in the plasma as compared to undetectable for free flavopiridol. Plasma concentration data were fitted into a two-compartment model and pharmacokinetic parameters were calculated using WinNonlin. Table 4 shows the pharmacokinetic parameters of liposomal flavopiridol in comparison with those after free drug administration, with a half-life ($T_{1/2\beta}$) of 340 min, apparent volume of distribution (V_d) of 2.4 L/kg, area under curve (AUC) of 10.8 min μmol/L and clearance (CL) of 0.012 L/min. Compared to parameters of the free drug ($T_{1/2\beta}$ 57.0 min, CL 0.036 L/min, AUC 3.4 min μmol/L, V_d 1.2 L/kg), V_d of liposomal flavopiridol was 2 times greater. Meanwhile, AUC value of free drug in plasma was 3 times lower than that of the liposomal formulation. These data show that the liposomal flavopiridol had prolonged blood circulation time and decreased clearance.

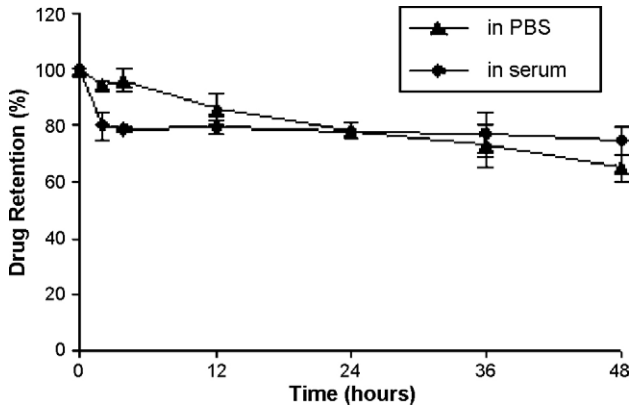

Fig. 5. *In vitro* release of flavopiridol from the HSPC/Chol/PEG-DSPE liposome. Flavopiridol-entrapped HSPC/Chol/PEG-DSPE liposome in PBS was incubated at 37 °C. At different time points, the liposomes were separated from the released free drug by Sepharose CL-4B column followed by the measurement of drug amount retained in liposome. Results are the mean of 3 separate experiments. Error bars stand for standard deviations, $n = 3$.

4. Discussion

As biodegradable and essentially non-toxic vesicles, liposomes have been used as delivery vehicles for several anti-cancer agents, such as doxorubicin in Doxil®, daunorubicin in DaunoXome®, vincristine in Marqibo®, and paclitaxel in LipoTaxen® (Hofheinz et al., 2005). In this study, we loaded flavopiridol into a PEGylated liposomal carrier, and characterized its physicochemical and pharmacokinetic properties.

Flavopiridol is weakly basic (Li et al., 1999). Entrapment of this compound in HSPC/Chol/PEG-DSPE liposomes was achieved by pH-gradient driven remote loading. Through size measurement, evaluation of encapsulation efficiency and *in vitro* drug release studies, the composition of the lipids and the loading method for the compound were optimized. A loading efficiency of 70% was obtained.

Pharmacokinetic study demonstrated that liposomal flavopiridol had prolonged blood circulation time and decreased clearance compared to the free drug. Currently, clinical dosage formulation of flavopiridol, which contains citrate and HPβCD, is administered via either a single infusion (ranging from 1 to 72 h) or a 30 min infusion followed by a 4 h infusion. Minimal efficacy was observed with the single infusions, while nearly 50% of CLL patients benefited from the 30 min/4 h schedule (Liu et al., 2004; Aklilu et al., 2003; Shapiro et al., 2001; Byrd et al., 2007). All infusion schemes, however, resulted in significant and potentially severe toxicities. Switching to a liposomal formulation offers the potential to alter the pharmacokinetics of flavopiridol and provide a more favorable efficacy/toxicity profile for this promising chemotherapeutic agent.

In conclusion, a novel liposomal flavopiridol formulation has been designed and evaluated. This formulation provides characteristics such as high drug encapsulation ratio, low *in vitro* release rate, and slow drug clearance and prolonged circulation time *in vivo*. This provides an alternative solubilization vehicle for administration of flavopiridol. Further preclinical studies are warranted to define the safety and therapeutic efficacy of this novel formulation.

Acknowledgments

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