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Halothane, a Novel Solvent for the Preparation of Liposomes Containing 2-4'-Amino-3'-Methylphenyl Benzothiazole (AMPB), an Anticancer Drug: A Technical Note

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INTRODUCTION

Most anticancer drugs have the tendency to target normal as well as tumor cells. Liposomes, especially long-circulating liposomes, are promising carriers for delivering drugs designed specifically to target cancer cells,[1-5] thus increasing anticancer activity and minimizing toxicity.

Anticancer activity has been shown by 2-4'-amino-3'-methylphenyl benzothiazole (AMPB) (**Figure 1**) against a variety of cancers including breast, ovary, lung, and kidney cancers in animal studies[6] (Stevens, unpublished data, 1994, and Stella, unpublished data, 1997) Unfortunately, however, severe dose-limiting hepatotoxicity of AMPB has also been reported in animals[6] (Stevens, unpublished data, 1994, and Stella, unpublished data, 1997).

AMPB has low water solubility (0.54 μg/mL) (Stella, unpublished data, 1997) and therefore must be manipulated through solubilization techniques in order to increase its solubility. Although traditional approaches such as cosolvency, pH control, micellization, and complexation could solubilize this drug, its acute hepatotoxicity cannot be reduced using these techniques. To increase the anticancer activity, as well as minimize toxicity, long-circulating liposomal formulations have been developed in this study.

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The first step in the preparation of liposomes is to dissolve the lipids into organic solvent. The solvent must be volatile and have high solubility for lipids. This requirement has introduced safety issues. Chloroform and ether are the commonly used solvents. Chloroform was used as an anesthetic during surgery for many years before its harmful effects on the liver and kidney were recognized. Based on animal studies, the Department of Health and Human Services (DHHS), the International Agency for Research on Cancer (IARC), and the Environmental Protection Agency (EPA) have determined that chloroform is possibly carcinogenic to humans. The breakdown products of chloroform in air include hydrogen chloride and phosgene, which are even more toxic than chloroform. Ether, while relatively nontoxic, is highly flammable. Both of these agents have been abandoned as anesthetics for clinical use. Even though only a small amount of residue would exist in liposomal formulations, it is still not acceptable. Because chloroform and ether evaporate very quickly, they would be harmful to the lab workers who were exposed to them. Chloroform is also toxic to aquatic and soil organisms after entering the natural environment, which is a probable consequence of using it as a solvent.[7,8,9]

Halothane, a commonly used inhalation anesthetic,[10] was used in this study in place of either chloroform or ether to prepare liposomal formulations. The Occupational Safety and Health Administration does not classify halothane as a carcinogen to humans. Thus halothane is preferred to chloroform and ether because it is safer, more volatile, and an excellent solvent for most lipids.

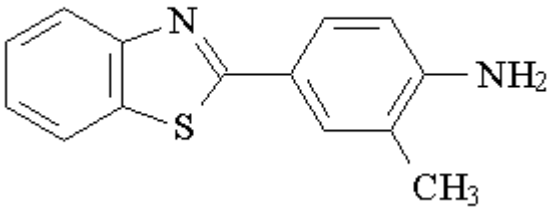

Figure 1. Chemical structure of AMPB.

MATERIALS AND METHODS

Materials

The USP apparatus was modeled using the CFD package Fluent version 5.4.8 (Fluent Incorporated, Canterra Resource Park, NH). Fluent is a state-of-the-art computer program for modeling fluid flow and heat transfer in complex geometries.[13]

High-Performance Liquid Chromatography Assay

A Beckman Gold high-performance liquid chromatography (HPLC) system with a #168 detector and a Pinnacle octyl amine (C8) column (5 µm, 150 × 4.6 mm, cat no: 9183565, Restek Corp (Bellefonte, PA) were used for all assays. The wavelength of 254 nm was chosen for AMPB detection. The HPLC mobile phase was composed of acetonitrile (ACN) and an aqueous solution that contained 0.1% trifluoroacetic acid (TFA) in water. The gradient method was started with 70% aqueous solution and 30% ACN. The percentage of ACN was increased at the rate of 2% per minute until reaching 55% at about 12 minutes. The injection volume was 50 µL, and the flow rate was 1.0 mL/min. The total running time was 13 minutes, and the retention time of AMPB was approximately 9.5 ± 0.3 minutes at ambient temperature. A calibration plot of peak area versus concentration was found to be linear in the range of 1 to 100 µg/mL.

Liposome Preparation: Thin-Film Method

Zhao et al[11] determined that the optimal molar ratio of drug and lipids with AMPB/ EPC/Chol/ DSPE-PEG 2000 is 1:5:5:1. This ratio and a 2 mg/mL concentration of AMPB were used in this study to investigate halothane as a replacement for chloroform to prepare liposomes.

Three batches of liposomes were made for stability study, and the thin-film method was used in their preparation.[12] Lipids and AMPB were dissolved in chloroform or halothane. The organic solvents were then removed by a rotary evaporator under reduced pressure. A thin film of lipid was observed after the solvents were removed. At least 8 hours in a desiccator under house vacuum was needed to completely remove the residues of organic solvents. Normal saline was then added to hydrate the lipid thin film. The hydration was followed by sonication in a 45°C water bath. A yellowish homogeneous suspension appeared after hydration. The final volume of the liposomal suspension was adjusted to make the desired concentration of AMPB (eg, 2 mg/mL).

Optical Microscope Observation

An optical microscope (Leica Microsystems Switzerland Ltd, Heerbrugg, Switzerland) was used to observe the particle size and shapes before particle size reduction. Particle diameters above 0.3 µm were determined by using a calibrated eyepiece scale. Photographs were taken using a SPOT camera (Spot Image Corp, Chantilly, VA).

Particle Size Reduction

A laboratory homogenizer (EmulsiFlex-C5, Avestin, Ottawa, ON, Canada) was used to reduce the particle size of the liposomes. Multiple passes (5k-10k psi) were often needed to obtain the desired particle size (~200 nm).

Transmission Electron Microscope Observation

A transmission electron microscope (Philips Electron Optics, model 420, Eindhoven, The Netherlands) was used to observe the size and lamellarity of liposomes after size reduction by homogenization. Liposome suspensions were stained by 2% phosphotungstic acid/sodium hydroxide (pH 6.2) and dried on carbon-coated grids for observation.

Particle Size Measurement

The Zetasizer 1000 (Malvern Instrument, Warcestershire, UK) was used to measure the final particle size of liposomes in the range of 1 to 1000 nm. The instrument settings were as follows: temperature, 25°C; scattering angle, 90°; aperture, 400 µm; count rate, 150 ± 50 (kilo counts/sec); analysis mode, auto/continuous; running time, automatic (120-3000 seconds). Samples

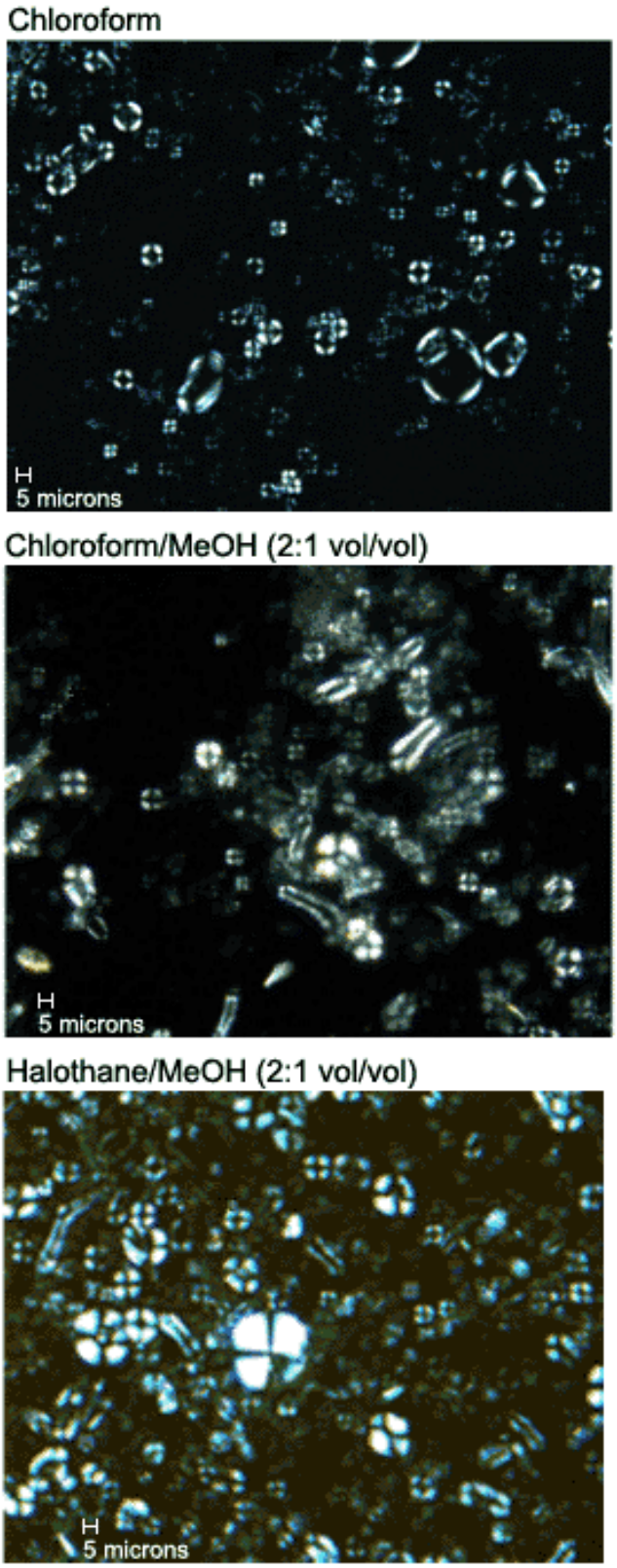

Figure 2. Structure of liposomes containing AMPB (2 mg/mL) under light microscope (×400).

were diluted with deionized water in triplicate. The mean particle size from each of the 3 batches was averaged to get the average mean particle size.

Drug Encapsulation Efficiency Determination

The liposomes (approximately 200-300 nm) were diluted 10 times by normal saline. The diluted solutions were gently vortexed for about 20 seconds and were free from any visible aggregation and precipitation.

Duplicate 1-mL samples from each batch were placed in ultracentrifugation tubes (Nalge Nunc International, Rochester, NY). The instrument (L8-55 ultracentrifuge, Beckman Countler, Inc, Allendale, NJ) was set at 50 000 rpm and 10°C for 3 hours with a temperature pre-equilibrated head. Separated phases (supernatant and pellets) in the centrifuge tubes were observed after ultracentrifugation. The supernatant was pipetted out and the drug concentration (C_s) in it was determined by HPLC. The pellet was washed with normal saline 3 times before it was well hydrated and dispersed by adding 1 mL normal saline and sonicated in a water-bath sonicator for several minutes. Dilution was conducted by adding pure ACN, and the drug concentration (C_p) in the pellet was determined by HPLC. The encapsulation efficiency (*EE*) equals the concentration of drug in the pellet divided by the total concentration of drug in the formulations, as described by the following equation:

$$EE = \left(\frac{C_p}{C_p + C_s} \right) \times 100\% \quad (1)$$

RESULTS AND DISCUSSION

Liposomal formulations were prepared using chloroform, chloroform/methanol (2/1, vol/vol) and halothane/methanol (2/1, vol/vol). No obvious differences of liposomal formulations mediated by halothane and chloroform were observed under the microscope. The maltisian structures, which are due to the multi-bilayers of liposomes produced by the thin-film method before particle size reduction, were observed in all formulations (**Figure 2**). This was supported by transmission electron microscope (TEM) observation (**Figure 3**). Multilamellar structures were observed for all formulations under TEM and the particle size for liposomes after homogenization ranged from 100 to 200 nm. Encapsulation of AMPB between the lipid bilayers of liposomes (**Figure 3A, B, C**) had little effect on the shape of the liposomes except to produce a thicker

membrane compared with liposomes without the drug (**Figure 3D**).

The data indicate that all 3 liposomal formulations have high encapsulation efficiency, are stable for at least 3 months at 4°C, and show a similar trend of encapsulation efficiency decreasing over time. Also, the 3 formulations show a similar increase in particle size with time (**Figure 4**).

The slight difference in the particle size of the liposomes may be because of the multiple steps of hydration and homogenization. After the solvents (halothane or chloroform) were removed, lipids formed a thin film at the bottom of the round-bottom flask. At the same experimental conditions (temperature and pressure), halothane is easier to remove. The lipid thin films mediated by halothane are more homogeneous after drying than are the chloroform-mediated films, and the halothane-mediated thin films are also easier to hydrate using sonication.

Since AMPB is a lipophilic compound, most of the drug was encapsulated within the bilayers of the liposomes. After reaching equilibrium partitioning, liposomes encapsulating AMPB are quite stable. More than 90% of the drug remains inside the liposomes after 3 months of storage at 4°C. The tendency of slight leakage of the drug is probably due to lipid oxidative degradation, which may also influence the packing of the lipids in the liposomes. Loss of residual solvent by evaporation may also play a role in reducing encapsulation.

Additional AMPB liposomal formulations containing different ratios of DSPC, EPC, cholesterol, and DSPE-PEG 2000 were prepared by using halothane and chloroform, respectively. Furthermore, liposomal formulations of fenretinide (a lipophilic compound), a hydrophilic prodrug of AMPB, and some proprietary compounds provided by the National Cancer Institute were prepared. In all cases, no significant differences in the physicochemical properties (particle size, size distribution, drug encapsulation efficiency) were observed between halothane-mediated and chloroform-mediated liposomes. These studies indicate that halothane, a safer solvent, is equivalent to chloroform as a vehicle for liposome preparation.

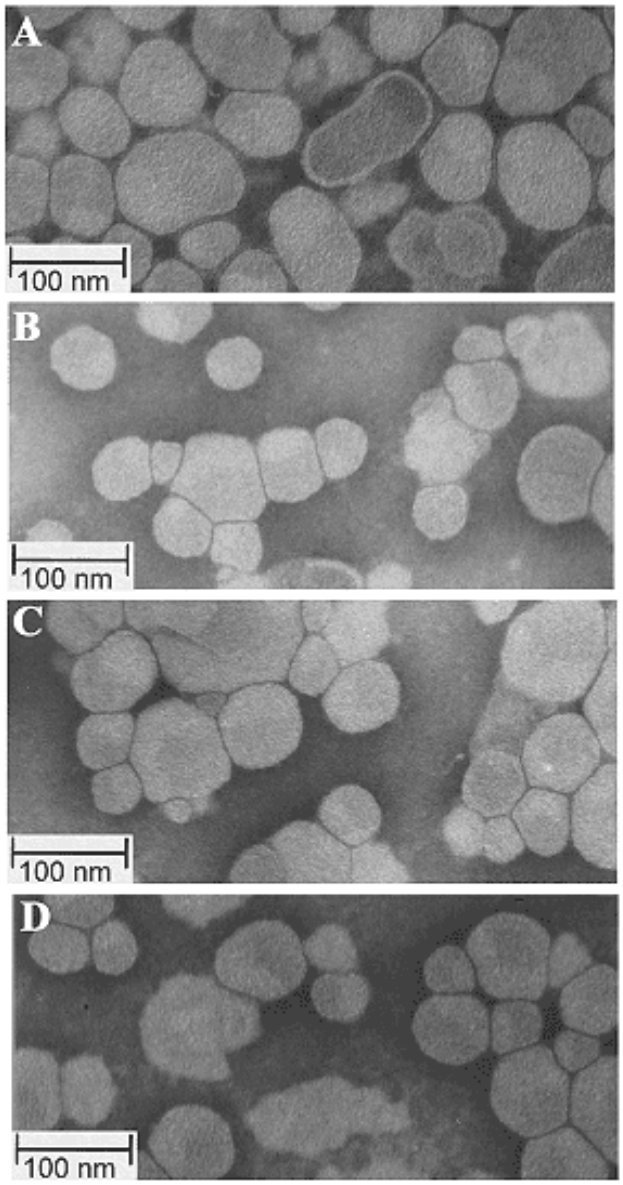

Figure 3. TEM micrograph of liposomes after homogenization (×164k): (A) liposomes with AMPB by using chloroform; (B) liposomes with AMPB by using chloroform/MeOH; (C) liposomes with AMPB by using halothane/MeOH; (D) liposomes without AMPB by using halothane.

CONCLUSION

Chloroform and ether are currently the solvents of choice despite the fact that chloroform is a known carcinogen and ether is highly flammable. Halothane is volatile yet nonflammable and is currently used as an inhalation anesthetic; hence it is safe.

This study uses halothane in place of chloroform or ether to prepare liposomal formulations for the antitumor drug 2-4'amino-3'-methylphenyl benzothiazole (AMPB or NSC 674495). The preliminary data show

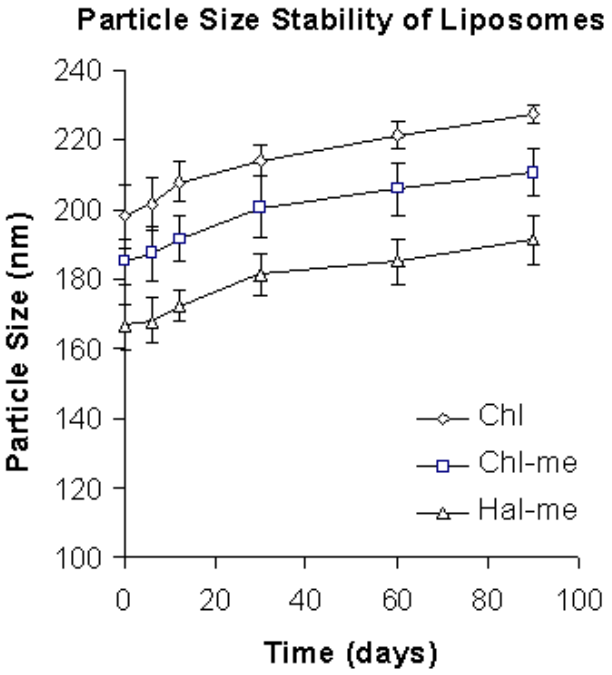

Figure 4. Particle size stability of 3 liposomal formulations of AMPB at 4°C (n = 3; error bars represent standard deviation of the mean).

that there are no significant physicochemical property (particle size, encapsulation efficiency, and vesicle shapes) differences between the halothane- and chloroform-mediated formulations. Similar results have been observed for other anticancer drugs tested.

Based on its success with AMPB, halothane may be a promising replacement for chloroform and ether in preparation of liposomes for other anti-cancer drugs.

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