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Encapsulation in a rapid-release liposomal formulation enhances the anti-tumor efficacy of pemetrexed in a murine solid mesothelioma-xenograft model

Noha Essam Eldin [a,b], Amr S. Abu Lila [a,b], Kazuyoshi Kawazoe [c], Hanan M. Elnahas [a], Mahmoud A. Mahdy [a], Tatsuhiro Ishida [b,*]

[a] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt
[b] Department of Pharmacokinetics and Biopharmaceutics, Institute of Biomedical Sciences, Tokushima University, Tokushima 770-8505, Japan
[c] Department of Clinical Pharmacy, Institute of Biomedical Sciences, Tokushima University, 2-50-1 Kuramoto-cho, Tokushima 770-8503, Japan

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ABSTRACT

We recently developed a PEG-coated liposome encapsulating the anti-folate drug pemetrexed (PMX). Such liposomal formulations have shown potent cytotoxic effects against malignant pleural mesothelioma (MPM) cells in vitro. In the present study, we investigated the pharmacokinetics, bio-distribution and in vivo anti-tumor efficacy of two liposomal PMX formulations with different drug release rates in a murine mesothelioma-xenograft model. Liposomes with different PMX release rates were prepared via manipulating liposomal membrane fluidity through incorporating either a solid-phase (HSPC) or a fluid-phase (POPC) phospholipid. Both liposomal PMX formulations showed prolonged plasma pharmacokinetics and were accumulated to a similar extent in tumors and other tissues, presumably, due to surface modification with polyethylene glycol (PEG). In a murine mesothelioma-xenograft model, interestingly, PMX encapsulated in a fast-release POPC liposome produced superior tumor growth suppression compared with either free PMX or PMX encapsulated in a slow-release HSPC liposome. Such in vivo anti-tumor efficacy was accomplished mainly by a potent induction of apoptosis within tumor tissue by the released PMX from POPC liposomes. Our results clearly emphasize the therapeutic efficacy of liposomal PMX over free PMX in conquering aggressive solid tumors such as malignant mesothelioma. A guarantee of the targeted delivery of PMX to tumor cells helps overcome some of the major shortcomings encountered with the use of free PMX.

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1. Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive malignancy of the pleura and peritoneum. MPM represents a great challenge to both clinicians and researchers as its incidence is increasing worldwide due to the unrestricted deployment of asbestos in several developing countries (Chen and Pace, 2012; Stahel and Weder, 2009). Many novel chemotherapeutic agents and/or therapeutic modalities have been adopted in the effort to conquer this aggressive disease.

Pemetrexed (PMX), a new multi-target anti-folate, is clinically approved as a single agent or in combination with other chemotherapeutic agents for the treatment of a wide variety of solid tumors, including MPM (Nowak et al., 2012; Richards et al., 2011). PMX is converted by folylpolyglutamate synthetase into a series of active polyglutamate metabolites (Rothbart et al., 2010). These metabolites inhibit 3 folate-dependent enzymes that are involved in de novo purine/pyridine synthesis: dihydrofolate reductase (DHFR), thymidylate synthase (TS), and glycinamide ribonucleotide formyltransferase (GARFT) (Chattopadhyay et al., 2007; Misset et al., 2004). Despite the fact that the combination of PMX and cisplatin has prolonged the survival of malignant mesothelioma patients for as long as 12 months, their response rates are still considered dismal (Uemura et al., 2010).

One of the problems in cancer treatment with chemotherapeutic agents is the non-selective distribution of the active drug to tumor cells as well as to normal cells (Pluen et al., 2001). This problem poses a huge impediment to the continuation of treatment due to either the failure in delivering efficient concentrations of the active drug to the tumor site or to dose-limiting side effects. Many strategies have been adopted to alleviate such problems. Among them, encapsulating/entrapping the drug within nanocarrier drug delivery systems elevated the therapeutic efficacy of encapsulated drugs while minimizing the side effects (Abu-Lila et al., 2009; Mura et al., 2013; Su et al., 2014).

Liposomes are one of the most clinically established nano-platforms for drug delivery. Their efficacy relies mainly on elevating the accumulation of encapsulated drugs in tumor tissue via the enhanced permeation and retention "EPR" effect while reducing systemic effects and toxicity (Fang et al., 2011; Maeda, 2001). Furthermore, modification of

* Corresponding author.
E-mail address: ishida@tokushima-u.ac.jp (T. Ishida).

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the outer liposome surface with hydrophilic polymers such as polyethylene glycol (Klibanov et al., 1990) attenuates liposomal clearance giving the modified liposomes excellent pharmacokinetic profiles in vivo (Bamrungsap et al., 2012).

Recently, we developed a polyethylene glycol (PEG)-coated liposomal PMX formulation (Essam Eldin et al., 2015). Such formulations show an efficient cell growth inhibitory effect against MPM cells in vitro. In the present study, we extended our work to investigate the in vivo fate and anti-tumor efficacy of two liposomal PMX formulations with different drug release rates in a murine mesothelioma-xenograft model. Liposomes with different PMX release rates were prepared via manipulating liposomal membrane fluidity by changing the hydrocarbon chain length and/or degree of saturation of the phospholipid component of the liposome. Phospholipids, namely HSPC (with a T_m 55 °C) and POPC (with a T_m −2 °C), were utilized in the preparation of liposomes and were found to affect drug release, and, thus, the in vivo therapeutic efficacy of the prepared PMX liposomal formulations.

2. Materials and methods

2.1. Materials

Pemetrexed disodium (PMX; Alimta®), a freely water soluble crystalline powder with an octanol/water partition coefficient of 0.16, was purchased from Eli Lilly (Indianapolis, IN, USA). Dioleoyl-phosphatidylethanolamine (DOPE), hydrogenated soy phosphatidylcholine (HSPC), palmitoyloleoyl phosphatidylcholine (POPC), and 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were kindly provided by NOF (Tokyo, Japan). A cationic lipid, O,O′-ditetradecanoyl-N-(alpha-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was obtained from Sogo Pharmaceutical (Tokyo, Japan). Cholesterol (Chol) was purchased from Wako Pure Chemical (Osaka, Japan). All other reagents were of analytical grade.

2.2. Animals and tumor cell line

BALB/c *nu/nu* mice (male, 5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were acclimatized under controlled environmental conditions that included constant temperature, lighting and humidity. All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University.

A human malignant pleural mesothelioma cell line (MSTO-211H) was generously supplied by Dr. Cheng-Long Huang (Department of Thoracic Surgery, Faculty of Medicine, Kyoto University, Kyoto, Japan). The cells were cultured in RPMI-1640 medium (Wako Pure Chemical) supplemented with 10% heat-inactivated FBS (Corning, Corning, NY, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (ICN Biomedicals, CA, USA) and incubated at 37 °C in 95% relative humidity under 5% CO_2.

2.3. Preparation of liposomes

Solid-phase cationic liposomes modified with PEG-derivative were composed of HSPC/CHOL/DC-6-14/$mPEG_{2000}$-DSPE (5/3/2/0.25 M ratio). Fluid-phase cationic liposomes modified with PEG-derivative were composed of DOPE/POPC/CHOL/DC-6-14/$mPEG_{2000}$-DSPE (3/2/3/2/0.25 M ratio). DOPE was added to act as a membrane fusion promoter (Litzinger and Huang, 1992). To follow the bio-distribution of the liposomes, they were labeled with the non-metabolized lipid marker ^{3}H-CHE (40 μCi/μmol lipid) as a non-exchangeable lipid phase marker. Liposomes were prepared using the reverse-phase evaporation technique as described previously (Essam Eldin et al., 2015). Briefly, lipids (50 mmol) were dissolved in 6 ml of chloroform/diethyl ether (1 : 2 v/v) and then 2 ml of PMX solution (25 mg/ml) in phosphate buffered saline (pH 7.4) was dropped into the lipid mixture to form a water/oil (W/O) emulsion. Sizing of the prepared liposomes was conducted via extruding the liposomal dispersion ten times through polycarbonate membrane filters (Nuclepore, CA., USA) with pore sizes of 200, 100, and 80 nm using an extruder device (Lipex Biomembranes Inc., Vancouver, Canada). The temperature of extrusion depended on the transition temperature of phospholipid used; POPC-liposomes (with T_m −2 °C) were extruded at room temperature, while, HSPC-liposomes (with T_m 55 °C) were extruded at 65 °C. The phospholipid concentrations were evaluated following extrusion via phosphorus determination by colorimetric assay (Rouser et al., 1970). Un-encapsulated free PMX was removed by column chromatography using Sepharose CL-4B (GE Healthcare Bioscience, Buckinghamshire, UK), and free PMX was determined using HLPC, as described previously (Essam Eldin et al., 2015). The physicochemical properties of the prepared liposomes, as determined by using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), are summarized in Table 1.

2.4. In vitro release of PMX from liposomes

Solid-phase liposomes and fluid-phase liposomes were mixed with mouse serum (1:1 v/v (%)) and incubated at 37 °C for 24 h. Free PMX released from either solid-phase or fluid-phase liposomes was separated by gel filtration chromatography with Sepharose CL-4B. The liposome fractions were collected, and the PMX that remained in the liposomes was quantified via HPLC, as described previously (Essam Eldin et al., 2015).

2.5. In vivo anti-tumor activity of liposomal PMX in a subcutaneous MSTO 211H tumor-bearing mice model

Male BALB/c *nu/nu* mice were inoculated subcutaneously (s.c.) at the back region with MSTO-211H cells (5×10^6) in 100 μl PBS. On days 10, 13, 16, 19, 22, and 25 after inoculation of the tumor cells, each group (6 mice) received an intravenous injection of either 9% sucrose (control), free PMX (25 mg/kg), or liposomal PMX (25 mg PMX/kg corresponding to 125 mg total lipid/kg). The dose of PMX was selected based on our preliminarily experiments and on the doses reported in preclinical studies (Iwahori et al., 2011; Takezawa et al., 2011).

Estimation of tumor volume was conducted every 3 days using a caliper, and tumor volume was calculated using the following formula:

$$\text{Tumor volume } (\text{mm}^3) = (\text{width})^2 \times (\text{length})/2$$

(Abu Lila et al., 2009).

Median survival time (MST (day)) was defined as the time at which the percentage surviving is 50%. The percentage of increased life span (ILS (%)) was calculated using the following equation:ILS (%) = [MST of treated group/MST of control group] − 1] × 100(Kviecinski et al., 2008; Lopes de Menezes et al., 2000).

Body weight was measured simultaneously to investigate any remarkable systemic toxicity.

2.6. Bio-distribution study of PEG-coated liposomes in tumor-bearing mice

To assess the tissue distribution of either PEG-coated cationic HSPC liposomes or PEG-coated cationic POPC liposomes, ^{3}H-CHE-labeled PEG-coated liposomes (25 mg total lipid/kg) were intravenously injected into mice bearing MSTO-211H tumors when the tumors had grown to 200–300 mm^3. At different time points post-liposomal injection (5 and 30 min; 1, 2, 4, 6, and 24 h), blood (100 μl) was withdrawn via cardiac puncture. After withdrawing blood samples, the mice were euthanized and the kidneys, lungs, spleens, livers, and tumors were collected. Radioactivity in blood and tissues was assayed, as described

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