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Investigation of Hexadecylphosphocholine (miltefosine) usage in Pegylated liposomal doxorubicin as a synergistic ingredient: In vitro and in vivo evaluation in mice bearing C26 colon carcinoma and B16F0 melanoma

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ABSTRACT

In this investigation, Hexadecylphosphocholine (HePC, miltefosine) was being used as a new ingredient in Pegylated liposomal doxorubicin (PLD) and different aspects of this integration such as its effect on doxorubicin (Dox) release and cell uptake, cytotoxicity of liposomes, in vivo distribution and half-life clearance time of Dox as well as median survival time were illustrated. The liposomal formulations were Pegylated liposomal doxorubicin containing 0, 0.5, 1, 2 and 4% mole ratios of HePC (HePC–PLD) and their respective Dox-free liposomes (HePC–PLs). The cells used were colon carcinoma (C26), adriamycin-resistant breast cancer (MCF-7-ADR), and B16F0 melanoma cell lines, of which C26 and B16F0 cells were exploited for tumoring in BALB/c and C57Bl/6 mice, respectively. In most cases, increase in miltefosine percentage resulted in physically liposomal instability, increased Dox delivery and toxicity and reduced blood half-life of Dox. Overall, HePC 4% –PLD and PLD differed significantly in many respects and it was considered too toxic to be injected at the same dose (15 mg Dox/ kg) as PLD. Although HePC 2% –PLD could extend the median survival time marginally in comparison to PLD, the concept of HePC- containing liposomes merits further investigation.

1. Introduction

Elimination of cancerous cells with almost no or less harmful side effects on normal tissues is the main concern with chemotherapy of cancerous diseases. In fact, almost all chemotherapy schedules regardless of either the regimen that are used or the kind of drug that are virtually considered toxic, ultimately failed because of discretionary limitation of drug dose (Boranic and Raci, 1979; Nakazawa et al., 2001; Schumacher, 1999; Webber et al., 2007). As a result, chance of evolving resistant cancerous cell population increases, which in turn, results in malignancy (Gottesman, 2002; Piccart-Gebhart et al., 2005). Regarding this notorious fact, a majority of researchers are trying to find out methodologies for restricting serious side effects of chemotherapeutic agents and targeting cancerous cells (Dark et al., 1997; Huber et al., 1994) and their exclusive environment (Peer et al., 2007).

One promising approach has already been proved that could be achieved by exploiting delivery systems ranging from 1–100 nm in size, in which large amount of drug could be accommodated (Davis et al., 2008; Peer et al., 2007). Among such delivery systems, Pegylated liposomal doxorubicin (PLD, commercially known as Doxil) is the cheap but intelligent system with comparatively enhanced therapeutic effect (Wigler et al., 2002). There are several positive points in PLD that explain why PLD administration results in relatively less adverse side effects and more therapeutic value as opposed to Dox. Firstly, particles of such size exclusively accumulated in solid tumors. Secondly, the long chains of polyethylene glycol polymers create a hydration shell around liposomes that protect them from endocytosis by reticuloendothelial system; therefore, avoiding hepatic engagement and hepatotoxicity, and extending plasma half-lives of liposomes. As a result, the opportunity for their passive accumulation in solid tumors is provided, where the microvessels are leakier

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(Enhanced Permeability and Retention (EPR) effect). And lastly, liposomes could fill up with the enormous amount of Dox through remote loading technique by ammonium sulfate gradient (Barenholz, 2012; Wigler et al., 2002). Although these advantages are important factors that have ultimately led to FDA approval of Doxil in USA for treatment of cancers like ovarian cancer, this system suffers from its inadequate doxorubicin release rate in tumors, where it must be presented in large quantities to produce the desire effect (Barenholz, 2012; Gabizon and Barenholz, 2010; Gordon et al., 2001). Therefore, modification of Doxil with respect to its cargo delivery rate and its intrinsic toxicity is regarded as key to improvement in therapeutic effect (Gabizon and Barenholz, 2010; Needham and Dewhirst, 2001).

Hexadecylphosphocholine (miltefosine, HePC) is the first drug derived from phospholipids with multi-effect properties including induction of cell apoptosis and perturbation of cell membrane due to its detergent-like character (Lindner et al., 2008). So far, it has clinically been approved for the topical treatment of breast cancer skin metastasis (Unger et al., 1992) and for the oral treatment of leishmaniasis (Sundar et al., 2002). It is the representative of a novel class of substances known as alkylphosphocholines, which all chemically resemble a lysophospholipid that lacks the central glycerol molecule consisting of an alkyl chain directly esterified to phosphocholine (Lindner et al., 2008). Unlike lysophospholipids, which have a high turnover in cell membrane, alkylphosphocholines are catabolized more slowly and accumulate in cell membrane of tumors and other tissues (Fleer et al., 1987). These compounds could interfere with membrane lipids like lysophospholipids but because of their lower catabolism rate they have the greater potential for triggering apoptotic cascade (Papagiannaros et al., 2006a). Moreover, their detergent-like characteristic brings about destabilization in cell membrane (Lindner et al., 2008). These are probably the main reasons for appearing hemolysis and thrombophlebitis after parenteral application of HePC (Munoz et al., 2013). However, parenteral application of HePC-containing liposomes or liposomal doxorubicin has already been reported, in which HePC was exploited as anti-proliferative agent and as an ingredient for enhancing cargo release rate from liposome (Lindner et al., 2008).

The overall goal of the present investigation is to see how HePC as a component of Doxil formulation could affect Dox release, cell uptake, cell toxicity, half-life clearance time and distribution in mice bearing C26 colon carcinoma and B16F0 melanoma. Furthermore, the impact of such alteration on treatment of cancerous tumors was assessed as compared to Doxil®. Therefore, we prepared liposomal formulations that resembled Doxil® in all terms except that 0.5, 1, 2, and 4% mole ratio of HePC were included as a new ingredient in liposomal preparation (HePC–PLD stand for Hexadecylphosphocholine–Pegylated liposomal doxorubicin containing 0.5 to 4% of HePC). For comparison, their respective HePC containing but Dox-free liposomes (HePC–PLs) were prepared.

2. Materials and Methods

2.1. Materials

Hydrogenated soya phosphatidylcholine (HSPC) and Methoxypolyethylene glycol (Mw 2000)-distearylphosphatidylethanolamine (mPEG2000-DSPE) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol, doxorubicin hydrochloride (Dox), Dowex® and RPMI 1640 culture medium were purchased from Sigma-Aldrich (St. Louis, MO); and MTT [3-(4,5-dimethylthiazol-2-yl)-2], diphenyltetrazolium bromide was obtained from Promega (Madison, WI). Acidified isopropyl alcohol (90% isopropanol/0.075 M HCl) was prepared by addition of 7.5 ml HCl, 1 M and 2.5 ml water to 90 ml isopropanol (Merck, Darmstadt, Germany). All other solvents and reagents were used as chemical grade. Commercially available Caelyx® was purchased from Behestan Darou Company (Tehran, Iran).

2.2. Preparation of Liposomes

Liposomes were prepared by thin lipid film hydration and extrusion method and Dox was encapsulated in liposomes via remote loading using ammonium sulfate gradient technique (Bolotin et al., 1994). The composition and nomenclature of the liposomes are given in Table 1. Briefly, appropriate amounts of lipids, which were previously dissolved in chloroform and were used as stock solutions, were added to a round-bottomed flask. The solvent was evaporated under vacuum in rotary evaporator until a thin and homogenous film was formed and then, freeze dried overnight. Subsequently, the lipid films were hydrated with an ammonium sulfate solution (250 mM) at 70 °C under argon atmosphere, sonicated for 15 min, and extruded through polycarbonate nanopore filters of 200 nm and 100 nm pore size (Avestin, Canada). Dox-free liposomes were hydrated with sucrose solution 10% (w/v). Liposomes were dialyzed against HEPES buffered sucrose (HEPES 10 mM, pH 7.0) in dialysis cassettes (Pierce, Rockford, IL) with 12 to 14 kDa molecular weight cut off (MWCO) and then, incubated with doxorubicin solution (1 mg doxorubicin per 7 μmol of total lipid) at 60 °C for 90 min and left at room temperature to cool. To remove free Dox, the resulting liposomal preparation was mixed with Dowex® resin, gently shaken for 60 min and pumped through Poly-Prep columns (Bio-Rad Laboratories Inc.).

2.3. Characterization of Liposomes

Liposome size distribution and polydispersity index were monitored by Dynamic Light Scattering instrument (Nano-ZS; Malvern, UK) after preparation and during the next four months. The Dox concentration of each liposomal preparation was determined and then, adjusted to 2 mg/ml by addition of an appropriate amount of sucrose solution. Hence, aliquots of each preparation were dissolved in acidified isopropyl alcohol below Dox self-quenching concentration, incubated at 60 °C for about half an hour in bath water, and Dox concentration was measured spectrofluorimetrically (ex: 470 nm/em: 590 nm) using Dox calibration curve. For HePC–PLs, the total lipid of each preparation was determined according to Bartlett phosphate assay and all adjusted to 28 mM total phosphate (Bartlett, 1959). Furthermore, the efficacy of Dox encapsulation was confirmed through determination of Dox in pre- and post- purified aliquots.

2.4. Release of Dox from Liposomes In Vitro

Leakage stability of the liposomes was monitored in RPMI 1640 media containing 30% fetal calf serum (FCS). Liposomes were added to the media (1:9 v/v) and incubated at 37 °C, from which samples (0.5 ml) were taken at several intervals until 48 h. The same process was repeated at 42 °C. For control, three samples were taken before incubation ($t = 0$). Then, 120 mg Dowex Ion-exchange resin was added to samples and mixed gently for about half an hour. The Dox concentration was measured as previously described.

2.5. Cell Culture

C26 colon carcinoma (Eppelheim, Germany) and MCF7-ADR breast cancer cell lines with Adriamycin resistance (National Cell Bank of Pasteur Institute, Tehran, Iran) were grown in RPMI 1640 medium. B16F0 melanoma cell line (Sigma-Aldrich, St. Louis, MO) was cultured in Dulbecco's Modified Eagle's medium (DMEM). Both media were supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, as well as 100 IU/ml penicillin and 100 mg/ml streptomycin and the cells incubated at 37 °C in a humidified incubator with a 5% CO2-atmosphere. Cell number and viability were assessed by trypan blue dye exclusion test (Strober, 2001) at the beginning of each experiment.