European Journal of Pharmaceutics and Biopharmaceutics 105 (2016) 40–49

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

# Anti-cancer activity of doxorubicin-loaded liposomes co-modified with transferrin and folic acid

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ARTICLE INFO

*Article history:*
Received 3 February 2016
Revised 21 May 2016
Accepted in revised form 30 May 2016
Available online 2 June 2016

*Keywords:*
Liposomes
Dual-targeting
Nanoparticles
Doxorubicin
Cancer
HeLa
Xenograft
Folic acid
Transferrin
A2780-ADR
Receptor targeting
Nanomedicine

ABSTRACT

Cancer-specific drug delivery represents an attractive approach to prevent undesirable side-effects and increase the accumulation of the drug in the tumor. Surface modification of nanoparticles such as liposomes with targeting moieties specific to the up-regulated receptors on the surface of tumor cells thus represents an effective strategy. Furthermore, since this receptor expression can be heterogeneous, using a dual-combination of targeting moieties may prove advantageous. With this in mind, the anti-cancer activity of PEGylated doxorubicin-loaded liposomes targeted with folic acid (F), transferrin (Tf) or both (F + Tf) was evaluated. The dual-targeted liposomes showed a 7-fold increase in cell association compared to either of the single-ligand targeted ones in human cervical carcinoma (HeLa) cell monolayers. The increased penetration and cell association of the dual-targeted liposomes were also demonstrated using HeLa cell spheroids. The *in vitro* cytotoxicity of the doxorubicin liposomes (LD) was then evaluated using HeLa and A2780-ADR ovarian carcinoma cell monolayers. In both these cell lines, the (F + Tf) LD showed significantly higher cytotoxic effects than the untargeted, or single-ligand targeted liposomes. In a HeLa xenograft model in nude mice, compared to the untreated group, though the untargeted LD showed 42% tumor growth inhibition, both the (F) LD and (F + Tf) LD showed 75% and 79% tumor growth inhibition respectively. These results thus highlight that though the dual-targeted liposomes represent an effective cytotoxic formulation in the *in vitro* setting, they were equally effective as the folic acid-targeted liposomes in reducing tumor burden in the more complex *in vivo* setting in this particular model.



## 1. Introduction

Cancer is still one of the leading causes of death worldwide and arises when a normal cell undergoes a series of genetic mutations resulting in its uncontrolled cell growth and proliferation, causing malignancy. Though the fight against cancer has been long-standing, mortality rates for a variety of cancer types have only decreased by less than 2% [1]. The current standard of care for solid tumors comprises of debulking surgery followed by treatment with chemotherapeutic drugs. Since the conceptualization of the "magic bullet" concept, the development of target-specific drugs has always been widely pursued [2]. Although a number of novel anti-cancer drugs have been developed, the use of existing drugs such as doxorubicin (Dox), paclitaxel and cisplatin is still predominant in the clinic [3]. More specifically, anthracycline drugs such as Dox are considered one of the most effective anticancer drugs ever developed [4]. It has been shown that Dox induces caspase-dependent apoptosis in cancer cells due to oxidative DNA damage in addition to topoisomerase II inhibition [5,6]. However, its use has been considered a double edged sword as though it is a potent anticancer molecule, it is also known to target other healthy tissues in the body resulting in toxicity to vital organs such as the heart.

The use of nanoparticles such as liposomes represents an efficient strategy to mitigate these effects by allowing for the

*Abbreviations:* PEG, polyethylene glycol(methoxy-PEG$_{2000}$-DSPE); Tf, transferrin; TfR, transferrin receptor; FR, folate receptor; Dox, doxorubicin; CPP, cell penetrating peptides; MPS, mononuclear phagocytic system; EPR, enhanced permeability and retention; ePC, egg phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; CHEMS, cholesteryl hemisuccinate; DCC, dicyclohexylcarbodiimide; MWCO, molecular weight cut-off; FBS, fetal bovine serum; TLC, thin-layer chromatography; PBS, phosphate buffered saline; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; PL, untargeted PEGylated rhodamine-labeled liposomes; (F)L, folic acid-targeted rhodamine-labeled liposomes; (Tf)L, transferrin-targeted rhodamine-labeled liposomes; (F + Tf)L, folic acid- and transferrin-targeted rhodamine-labeled liposomes; LD, doxorubicin liposomes; (F) LD, folic acid-targeted doxorubicin liposomes; (Tf) LD, transferrin-targeted doxorubicin liposomes; (F + Tf) LD, folic acid- and transferrin-targeted doxorubicin liposomes; UT, untreated.

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http://dx.doi.org/10.1016/j.ejpb.2016.05.023
0939-6411/

increased accumulation of drugs at the target site while at the same time minimizing drug interaction with healthy cells. Since the conceptualization of their use as drug carriers in the 1970s, liposomes have made a lot of progress being constantly streamlined and have become the carrier of choice for the delivery of a number of promiscuous drug candidates [7–9]. PEGylation of these nanoparticles is known to impart "stealth" properties to liposomes allowing longevity by screening the adsorption of proteins onto their surface thereby minimizing uptake by the mononuclear phagocyte system (MPS) [10–12]. In addition, being in the nanometer size range allows them to accumulate preferentially into tumor tissues by the enhanced permeability and retention (EPR) effect [13].

In order to enhance their cell-specific as well as intracellular delivery, liposomes can be further modified with targeting ligands in order to exploit tumor characteristics since cancer cells are known to over-express a number of cell surface proteins such as integrins, growth factor receptors, glucose transporters, folate receptors (FRs) and transferrin receptors (TfRs) among others [14].

FR is a very well-studied protein in cancer therapy as folic acid plays a vital role in DNA synthesis to support the proliferation of cancer cells. While FR alpha (FR-α) is overexpressed in a variety of cancer types, normal tissues contain very low levels of the receptor and in some cases they are located on the apical side away from circulation such as in the kidney tubules and air sacs of the lungs, thereby minimizing the potential for off-target toxicities [15,16]. As a result, a number of macromolecular drugs as well as nanoparticles have been targeted with folic acid to enhance their antitumor efficacies both *in vitro* and *in vivo* [17–19]. Similarly, the TfR has been widely exploited for drug delivery into cancer cells as it is overexpressed in many cancer types due to its role in iron homeostasis and cell proliferation as well as its receptor mediated endocytosis [20]. For example, Tf-targeted liposomes have been developed for the delivery of anticancer drugs such as oxaliplatin, doxorubicin, 5-fluorouracil, ceramide as well as DNA and siRNA [21–26].

In order to further explore the concept of targeting, the concept of 'dual-targeting' was pursued, which employs the use of two different target-specific ligands on the surface of the same nanocarrier to allow for synergistic effects. Inspired by the strategy employed by natural viruses, it may be advantageous to develop a combination of ligands that can interact with the various cell-surface antigens in order to overcome the heterogeneous pattern of expression in various tumor types and increase the number of potential binding sites for nanomedicines on the surface of tumor cells and within the tumor [27,28]. The different dual-targeted strategies reported recently, comprise of targeting with cell penetrating peptides (CPPs) and hyaluronic acid [29], anti-myosin antibody and TAT peptides [30], αCD19 and αCD20 antibodies [31], as well as targeting the FR and the epidermal growth factor receptor [32].

Though in general the use of targeting has proven advantageous to the delivery of drugs, there have been many instances where there has not been a significant improvement in antitumor efficacy *in vivo* due to the heterogeneous nature of cancer [22,33]. The *in vivo* setting is fairly complex and there are a number of barriers to the delivery of nanoparticles [34]. Therefore we decided to investigate whether targeting would allow for better cell association and antitumor efficacy *in vitro* and *in vivo*, as well as to ascertain whether there was a good correlation among the three experimental models for the various targeted groups. PEGylated Dox-loaded liposomes targeted with folic acid, Tf as well as both were developed and their anticancer potential was then evaluated *in vitro* in cancer cell monolayers and spheroids as well as *in vivo*.

## 2. Experimental section

### 2.1. Materials, cell culture and animals

FITC-labeled mouse monoclonal anti-transferrin receptor antibody (ab47095) was purchased from Abcam (San Francisco, CA); goat anti-folate receptor alpha primary antibody (sc-16386), FITC-labeled secondary donkey anti-goat antibody (sc-2024) and FITC-labeled normal mouse IgG (sc-2855) (as negative control) were purchased from Santa Cruz Biotechnology (Dallas, TX); nitrophenylcarbonyl-PEG$_{3400}$-nitrophenylcarbonyl (NPC-PEG-NPC) and amino-PEG$_{3400}$-DSPE were from Laysan Bio (Arab, AL); egg phosphatidylcholine (ePC), cholesterol, cholesteryl hemisuccinate (CHEMS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG), (lissamine rhodamine) DPPE (rhodamine) were from Avanti (Alabaster, AL); Dragendorff's reagent, Molybdenum blue, triethylamine (TEA), Sepharose CL-4B (40–165 μm), transferrin, folic acid, dicyclohexylcarbodiimide (DCC) and Ninhydrin were from Sigma-Aldrich (St. Louis, MO); microBCA™ protein assay kit, Whatman nuclepore polycarbonate membranes 19 mm at 0.2 μm, 0.1 μm and 0.05 μm pore sizes, bovine serum albumin (BSA) and agarose were from Fisher/Thermo Scientific (Waltham, MA); Spectra/Por pre-wetted 300,000 MWCO dialysis membranes and Spectra/Por 12–14 kDa MWCO dry membranes were from Spectrum Labs (Rancho Dominguez, CA); CellTiter-Blue® cell viability assay was from Promega Corporation (Madison, WI); and doxorubicin hydrochloride was from LC Labs (Woburn, MA) and Lancrix Chemicals (Shanghai, China).

Streptomycin (25 μg/mL)/Penicillin (10,000 U/mL) solution, RPMI media, Cellstripper™ as well as Trypsin/EDTA were purchased from Corning/Mediatech (Manassas, VA); RPMI without folic acid was purchased from Life Technologies (Carlsbad, CA); F-12K media, normal human skin fibroblasts (CCD-27Sk), human cervical cancer cells (HeLa) and human umbilical vein endothelial cells (HUVEC) were from ATCC (Manassas, VA); fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA); adriamycin-resistant human ovarian carcinoma cells (A2780-ADR) were purchased from Sigma-Aldrich. The HeLa and A2780-ADR cells were grown in RPMI media. Additionally, HeLa were also grown in folic acid deficient RPMI for 2 weeks for the cell association experiments. HUVEC cells were grown in F-12K media supplemented with 0.1 mg/mL Heparin and 0.03 mg/mL endothelial cell growth supplement. All media were supplemented with 10% FBS and 1% penicillin-streptomycin solution. Cells were all grown at 37 °C with 5% $CO_2$.

Matrigel® matrix used for tumor inoculation was purchased from Corning. 6–8 week old female NCR nude mice ($Nu^-/Nu^-$) were purchased from Taconic Biosciences (Rensselaer, NY).

### 2.2. Synthesis of pNP-PEG$_{3400}$-DOPE

The pNP-PEG-DOPE was synthesized and purified by slight modifications to a previously established method [35]. 64.6 μmol NPC-PEG$_{3400}$-NPC (or pNP-PEG$_{3400}$-pNP) was first dissolved in 1 mL chloroform and then reacted with 12.9 μmol DOPE in the presence of 38.8 μmol TEA. The reaction was incubated overnight at room temperature (RT) in an argon atmosphere under constant stirring. The reaction was monitored using thin-layer chromatography (TLC) in a mixture of chloroform-methanol as an eluent at an 80:20 volumetric ratio. Dragendorff's reagent and Molybdenum blue were used to visualize the PEG and DOPE respectively. After completion of the reaction, the solvent was removed from the mixture using rotary evaporation followed by freeze-drying on a FreeZone 4.5L Freeze Dry system (Labconco, Kansas City, MO).