Journal of Controlled Release 208 (2015) 106–120

Contents lists available at ScienceDirect

Journal of Controlled Release

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

A comparative study of folate receptor-targeted doxorubicin delivery systems: Dosing regimens and therapeutic index

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ARTICLE INFO

Article history:
Received 20 January 2015
Received in revised form 3 April 2015
Accepted 9 April 2015
Available online 11 April 2015

Keywords:
Angiogenesis
Polymer therapeutics
Pullulan
Liposomes
Doxorubicin
Folate receptor targeting

ABSTRACT

Ligand–receptor mediated targeting may affect differently the performance of supramolecular drug carriers depending on the nature of the nanocarrier. In this study, we compare the selectivity, safety and activity of doxorubicin (Dox) entrapped in liposomes versus Dox conjugated to polymeric nanocarriers in the presence or absence of a folic acid (FA)-targeting ligand to cancer cells that overexpress the folate receptor (FR). Two pullulan (Pull)-based conjugates of Dox were synthesized, (FA-PEG)-Pull-(Cyst-Dox) and (NH_2-PEG)-Pull-(Cyst-Dox). The other delivery systems are Dox loaded PEGylated liposomes (PLD, Doxil®) and the FR-targeted version (PLD-FA) obtained by ligand post-insertion into the commercial formulation. Both receptor-targeted drug delivery systems (DDS) were shown to interact *in vitro* specifically with cells via the folate ligand.
Treatment of FR-overexpressing human cervical carcinoma KB tumor-bearing mice with three-weekly injections resulted in slightly enhanced anticancer activity of PLD-FA compared to PLD and no activity for both pullulan-based conjugates. When the DDS were administered intravenously every other day, the folated-Pull conjugate and the non-folated-Pull conjugate displayed similar and low antitumor activity as free Dox. At this dosing regimen, the liposome-based formulations displayed enhanced antitumor activity with an advantage to the non-folated liposome. However, both liposomal formulations suffered from toxicity that was reversible following treatment discontinuation. Using a daily dosing schedule, with higher cumulative dose, the folated-Pull conjugate strongly inhibited tumor growth while free Dox was toxic at this regimen. For polymeric constructs, increasing dose intensity and cumulative dose strongly affects the therapeutic index and reveals a major therapeutic advantage for the FR-targeted formulation. All DDS were able to abrogate doxorubicin-induced cardiotoxicity. This study constitutes the first side-by-side comparison of two receptor-targeted ligand-bearing systems, polymer therapeutics versus nanoparticulate systems, evaluated in the same mouse tumor model at several dosing regimens.

1. Introduction

For more than a century, since Paul Ehrlich established the "magic bullet" concept [1], generations of chemists and pharmacologists have attempted to devise powerful and specific anticancer drugs, that go directly to their intended targets yet remain harmless to healthy tissues. Despite groundbreaking achievements, most anticancer drugs suffer from narrow therapeutic window and cancer still threatens numerous lives owing to its multi-dimensional complexity. In recent years, different versatile macromolecular systems, commonly defined as nanomedicines, have been designed and developed for cancer therapy [2,3]. Nano-sized carriers can improve the physico-chemical properties of low molecular weight drugs enhancing their therapeutic index by altering their pharmacokinetics and increasing their accumulation in the target tissue exploiting the enhanced permeability and retention (EPR) effect [4,5]. However, this non-selective blood circulation and extravasation-dependent targeted strategy, relying solely on the leaky vasculature of tumors is limited due to the heterogeneity of the angiogenic vasculature characterizing different tumor types [6]. Therefore, conjugation of targeting ligands to nanocarriers might overcome the limitations of non-selective delivery to the tumor site, achieving enhanced tumor selectivity and intracellular uptake.

Drug delivery systems (DDS) include supramolecular assemblies for the (i) physical entrapment of drugs, such as liposomes [7,8] and nanoparticles [9,10] and (ii) chemical covalent binding of

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http://dx.doi.org/10.1016/j.jconrel.2015.04.009
0168-3659/

drugs, such as polymer conjugates [11,12], polymeric micelles [13,14] and polymersomes [15,16], named polymer therapeutics [17].

Liposomal formulations have proven to be among the most successful approaches of drug delivery translated to the clinic. These spheroidal phospholipidic vesicles represent a versatile carrier for both hydrophilic and hydrophobic drugs. Due to the simple production, efficient drug loading and ease of tailoring of their physico-chemical and biopharmaceutical properties, several liposomal formulations for drug delivery have been developed [18]. Nevertheless, the early liposomal formulations were affected by major uptake by the reticuloendothelial system (RES), which dramatically reduced the circulation half-life [19]. Efforts made to avoid clearance by the immune system [20], resulted in the development of long-circulating ("stealth") liposomal formulations with the ability of escaping the RES clearance. The most effective approach is the coating of the vesicle surface with poly(ethylene glycol) (PEG) [21], which provides steric hindrance, avoidance of opsonization and thus, guarantees a prolonged circulation in the bloodstream. These features led to the approval of several liposomal formulations for clinical use [22].

Doxil® is a PEGylated liposomal doxorubicin (PLD) formulation approved for the treatment of breast cancer, ovarian cancer, multiple myeloma, and Kaposi's sarcoma [22,23]. PLD accumulates in cancer tissue via non-selective targeting extravasating through the leaky tumor vasculature (EPR effect). Following accumulation in the target tissue, the liposomes undergo degradation leading to the release of the entrapped doxorubicin (Dox), which is then internalized in the cancer cells as free drug [22]. The advantage of the liposomal formulation compared to free Dox is the reduced cardiac toxicity, while the main adverse effect is the hand-foot syndrome (palmar-plantar erythrodysesthesia), that causes redness, swelling, and pain on the palms of the hands and the soles of the feet. Another toxicity related to PLD is a pseudo-allergic reaction that might appear after the first infusion [22,24].

In parallel, several polymer therapeutics have been developed for the delivery of Dox [25–27]. A few of them have reached clinical trials [28–30], however, none of them have yet received approval by the main authorities, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

On the basis of Ringsdorf's polymeric carrier concept [31,32], we recently developed a conjugate of Dox using pullulan as the polymeric backbone [33]. Pullulan is a natural, non-ionic and linear homopolysaccharide formed by repeating units of maltotriose [34]. It has been used in the formulation of several drug delivery systems [35–38], due to its biodegradability, low immunogenicity, reduced toxicity and its fair solubility in aqueous and a few organic solvents [39]. Furthermore, pullulan bears functional groups along the polymer backbone that allow multivalent derivatization with a variety of pendant functions [40].

Pullulan polymers at the nano scale exploit the leaky tumor vasculature and EPR effect to selectively accumulate in cancer tissue [37,41]. Several pullulan-based delivery systems for anticancer drugs have been developed. These include self-assembling hydrophobized pullulan [42], pH-sensitive pullulan nanoparticles [41] and bioconjugates [37,43–45] for the delivery of Dox, camptothecin, paclitaxel, alendronate, cisplatin, metothrexate and combretastatin A4. Here, we conjugated Dox to the pullulan backbone via an acid sensitive hydrazone bond, which is stable at physiological pH, but hydrolyzes under acidic conditions, such as those found in endosomes or an even lower pH in lysosomes. The bioconjugate was endowed with targeting properties by introducing folate functions in the supramolecular structure.

In order to confer cellular targeting properties to DDS, many targeting agents have been evaluated [46–50]. Folic acid (FA) is an attractive targeting agent to a large number of cancer cell types that overexpress the folate receptor (FR). This small molecule lacks immunogenicity and can be easily conjugated to supramolecular and macromolecular structures. FR-targeted precision nanomedicines can be exploited for the treatment of cancers and other difficult-to-treat diseases overexpressing folate receptor, such as polycystic kidney disease [51], and inflammatory diseases (e.g. adjuvant arthritis) targeting activated macrophages [52]. Thus, folic acid has been widely used for conjugation to DDS [22]. Aimed at evaluating the targeting properties of FA, we conjugated it both to a doxorubicin-loaded liposome (via ligand post insertion) [53] and to a Dox-pullulan conjugate. Previous studies showed that Dox loaded folated liposomes and Dox conjugated folated pullulan have suitable biopharmaceutical behaviors that make them promising for improved therapeutic performance as compared to their non-folated counterparts. However, in view of exploiting these novel drug delivery systems for an efficient and targeted cancer treatment, *in vitro* and *in vivo* comparative studies were undertaken to compare the biopharmaceutical and therapeutic performance of the liposomal vs polymeric systems. The comparative studies were performed according to advanced and validated *in vitro* and *in vivo* protocols, which could provide some elucidation of the influence of the architecture on the DDS behavior in terms of efficacy and safety. The effect of the supramolecular structures on the antitumor and antiangiogenic activity and on the targeting properties conferred by the bound FA was investigated testing Dox-equivalent doses in human cancer cell line and on endothelial cells as well as *in vivo* on tumor-bearing mice.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride was purchased from Iffect Chemphar Co., LTD. (Shenzhen, P.R. China). Doxil® was supplied by Johnson & Johnson (New Jersey, USA). [^{3}H]-folic acid (FA) sodium salt was bought from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). 3,3′-N-(ε-maleimidocaproic acid)-hydrazidetrifluoroacetic acid salt (EMCH) was obtained from Molecular Biosciences, Inc. (Boulder, CO, USA). Diaminopolyethylene glycol ($PEG_{1900}(NH_2)_2$), ~100 kDa pullulan (Mw/Mn 2.03), folic acid (FA), cholesterol, sodium borohydride ($NaBH_4$), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), and all the solvents were purchased from Fluka (Buchs, Switzerland). Cysteamine (Cyst), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), mannitol, sodium cyanoborohydride ($NaCNBH_3$), 2,4,6-trinitrobenzenesulfonic acid (TNBS), triscarboxyethylphosphine (TCEP), dithiothreitol (DTT), Masson's Trichrome, and pullulan standard set for gel permeation analyses were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogenated soybean phosphatidyl-choline (HSPC) was purchased from Lipoid (Duisburg, Germany). Distearoyl-phosphatidylethanolamine conjugated to 2 kDa monomethoxy-poly(ethylene glycol) ($mPEG_{2000}$-DSPE) was obtained from Bio-lab (Jerusalem, Israel). Folate derivatized $mPEG_{3350}$-DSPE (FA-PEG_{3350}-DSPE) was supplied by Shaare Zedek Experimental Oncology Lab (Jerusalem, Israel). ProLong Gold antifade with DAPI mounting medium was purchased from Invitrogen (Eugene, OR, USA). Nu/nu mice and folate depleted diet were purchased from Harlan (Rechovot, Israel). All tissue culture reagents were purchased from Biological Industries Ltd. (Beit Haemek, Israel), unless otherwise indicated.

2.2. Methods

2.2.1. Synthesis of polymeric conjugates of doxorubicin

Pullulan derivatized with PEG and cysteamine (NH_2-PEG)-Pull-(Cyst), pullulan derivatized with PEG, cysteamine and FA, (FA-PEG)-Pull-(Cyst), pullulan derivatized with PEG, cysteamine and Dox, (FA-PEG)-Pull-(Cyst), and pullulan derivatized with PEG, cysteamine, FA and Dox, (FA-PEG)-Pull-(Cyst-Dox), were synthesized according to previously published procedures [33] described in the Supporting Information.

2.2.2. Liposomal formulation

The PEGylated liposomal doxorubicin formulation used in these studies was Doxil®, a product of Johnson & Johnson, marketed in Israel by Janssen Pharmaceuticals (Shefayim, Israel) in 10 mL vials at a doxorubicin concentration of 2 mg/mL. Control drug-free PEGylated