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In vivo comparative study of distinct polymeric architectures bearing a combination of paclitaxel and doxorubicin at a synergistic ratio

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ABSTRACT

Nowadays, combination therapy became a standard in oncology. In this study, we compare the activity of two polymeric carriers bearing a combination of the anticancer drugs paclitaxel (PTX) and doxorubicin (DOX), which differ mainly in their architecture and supramolecular assembly. Drugs were covalently bound to a linear polymer, polyglutamic acid (PGA) or to a dendritic scaffold, polyglycerol (PG) decorated with poly(ethylene glycol) (PEG), forming PGA-PTX-DOX and PG-PTX-bz-DOX-PEG, respectively. We explored the relationship between the polymeric architectures and their performance with the aim to augment the pharmacological benefits of releasing both drugs simultaneously at the tumor site at a synergistic ratio. We recently designed and characterized a PGA-PTX-DOX conjugate. Here, we describe the synthesis and characterization of PG dendritic scaffold bearing the combination of PTX and DOX. The performance of both conjugates was evaluated in a murine model of mammary adenocarcinoma in immunocompetent mice, to investigate whether the activity of the treatments is affected by the immune system. Drug conjugation to a nano-sized polymer enabled preferred tumor accumulation by extravasation-dependent targeting, making use of the enhanced permeability and retention (EPR) effect. Both PGA-PTX-DOX and PG-PTX-bz-DOX-PEG nano-sized conjugates exhibited superior antitumor efficacy and safety compared to the combination of the free drugs, at equivalent concentrations. However, while PGA-PTX-DOX was more efficient than a mixture of each drug conjugated to a separate PGA chain, as was previously shown, PG-PTX-bz-DOX-PEG had similar activity to the mixture of the PG-PTX-bz-PEG and PG-DOX-PEG conjugates. Our results show that both conjugates are potential candidates as precision combination nanomedicines for the treatment of breast cancer.

1. Introduction

Nanotechnology and combination therapy are two major fields that show great promise in the treatment of cancer. The use of polymers as targetable drug carriers helps to improve drug's therapeutic effectiveness while reducing adverse side effects associated with high dosage by improving their pharmacokinetics [1].

The rationale for using polymers as carriers for the delivery of antitumor agents, even if they are not conjugated to cell-specific molecules, is based on the work of Matsumura and Maeda defined as the enhanced permeability and retention (EPR) effect [2]. We and others utilize the EPR effect in order to design appropriate nano-sized agents, which enable a blood circulation and extravasation-dependent targeting to different types of cancer and believe that it is sufficiently prevalent in human tumors [3,4]. However, distinct tumors differ in the patho-physiological status of their vasculature enabling (or not) the EPR phenomenon of macromolecules. Therefore, using distinct supramolecular polymeric architectures enabled us to explore their suitability to extravasate from diverse hyperpermeable angiogenic vasculature in heterogeneous tumor types. The optimal size of a nano-sized agent, which can deliver an adequate dose of drugs distributed homogeneously and thus induce the most efficient therapeutic effect, is reportedly between 10 nm and 200 nm in diameter [4]. However, the supramolecular structure of the conjugates, whether they are dendritic, hyperbranched or linear, and their biodegradability, can have a major impact on the nanomedicine's performance [5].

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An important advantage of polymeric nanomedicines is their versatility, enabling to tailor different compounds with controlled loading percentage on a single polymeric backbone [6,7]. Paclitaxel (PTX) and doxorubicin (DOX) are potent anticancer drugs used in the clinic as mono-therapies [8,9] or in combination with other modalities to treat various neoplasms [10–13]. However, both drugs suffer from side effects, like neurotoxicity for PTX and dose-dependent cardiotoxicity for DOX, as well as poor pharmacokinetics. These two drugs have dissimilar physico-chemical properties, pharmacokinetics and distinct mechanisms of action, toxicity and drug resistance [14]. As was already shown, binding two synergistic drugs to the same polymeric backbone will most likely result in improved anti-cancer activity and reduced toxicity [15–18].

A chemical conjugation of PTX and DOX to nanocarriers could offer pharmacodynamic and pharmacokinetic advantages by enhanced blood circulation and extravasation-dependent targeting, delivering both drugs to the tumor site at the required ratio for synergism. Undesired toxicity could be overcome, the dose reduced and multiple targets reached, by combining two therapeutic agents resulting in an increased therapeutic effect and an overall increased anticancer activity [11,19]. Such combination of polymer-drug conjugates can be achieved by (a) two polymeric systems, each carrying a different drug, or (b) one single polymeric carrier that is administered bearing two or more different drugs. It has been shown that both approaches yield superior properties as compared to the administration of the two pristine drugs [18,20,21].

Since the polymeric structure can have a major impact on the therapeutic efficacy, we have chosen to compare polymer-drug conjugates based on a linear or a dendritic nanocarrier. Polyglutamic acid (PGA) was chosen as the linear carrier, whereas dendritic polyglycerol (PG) decorated with poly(ethylene glycol) (PEG) was chosen as the dendritic counterpart. It is known that PGA-based conjugates when conjugated to hydrophobic drugs tend to form intramolecular aggregation when dissolved in water [22] yielding surface properties mainly driven by the negatively charged PGA backbone. In the case of PEGylated dendritic polymer-drug conjugates with a core-shell architecture, the surface properties in aqueous solution are mainly driven by the PEG shell [23]. We therefore hypothesize that the two types of conjugates would have different properties in solution due to the differences in their surface properties.

PGA is a potent polymeric carrier which is synthesized by ring-opening co-polymerization of the corresponding *N*-carboxyanhydrides (NCA), initiated by amines or nucleophilic agents. It is water-soluble, non-toxic, biodegradable and non-immunogenic at the required concentrations to exert its anticancer activity when bound to anticancer drugs. Cysteine proteases, particularly cathepsin B, an enzyme that is highly expressed in most tumor tissues, play a key role in the lysosomal degradation of PGA [24]. In addition, PGA has a γ-carboxyl group in each repeating unit of L-glutamic acid that offers multiple attachment of drugs [18,25]. Those features make PGA an attractive drug carrier, and indeed PGA-PTX (OPAXIO™, former names XYOTAX and CT-2103) is the most progressed polymer–drug conjugate in the pipeline for market approval [26,27].

Our second selected polymeric carrier, PG, has outstanding properties as a drug delivery system regarding its structure, biocompatibility and water solubility. PG can be prepared in a controlled synthesis *via* anionic ring-opening multibranching polymerization (ROMBP) [28]. It is characterized by the combination of highly branched, stable, with very low polydispersity, consisting of a biocompatible polyether scaffold, and a compact, well-defined dendrimer-like architecture that has great impact on their physical and chemical properties [29]. A significant amount of research has been realized towards the design of many different architectures, where the PG hydroxyl groups have been modified into different functionalities, demonstrating a promising therapeutic approach. Drug conjugation to PG through pH-sensitive [30] or enzymatically-cleavable linkers [31] have demonstrated the great potential of PG conjugates against cancer cells *in vitro* as well as *in vivo*. Recently, we reported the therapeutic potential of a multifunctional drug immunoconjugate for targeting cancer cell lines which express the epidermal growth factor receptor (EGFR) *in vitro* [32]. All these polymer drug conjugates showed optimal properties for *in vitro* and *in vivo* applications because of their appropriate size for passive tumor targeting, their high water solubility, pronounced environment responsive properties, a high stability at physiological conditions, cellular internalization, and a favorable tolerability profile.

The aim of this study was to synthesize two different nanocarriers bearing a combination of synergistic drugs at an appropriate ratio, and to determine their advantage *in vivo* over drugs conjugated to separate polymer chains and to free drugs, as well as to evaluate the effect of the polymeric architecture (linear PGA *versus* dendritic PG) on the antitumor activity and safety profile of the conjugates.

2. Materials and methods

2.1. Chemical data

All chemicals were of analytical grade and purchased from Fluka (Germany), Aldrich (Germany), or Merck (Germany), and used as received unless otherwise stated. Chemical reagents included: *N*,*N*-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazol (HOBt), diisopropylethylamine (DIPEA), *N*-hydroxysuccinimide (OHSuc), *N*,*N*-dimethylaminopyridine (DMAP), anhydrous *N*,*N*-dimethylformamide (DMF), and anhydrous tetrahydrofuran (THF). Maleimido-poly(ethylene glycol) (PEG-mal) with MW = 2 kDa was purchased from Rapp Polymer, Germany. The hydrazone derivative of DOX (DOX-EMCH, *i.e.*, DOX bound to 3,3′-N-[ε-maleimidocaproic acid]) as well as the hydrazone derivative of paclitaxel (PTX-bz-EMCH) were prepared as described previously [33–35]. The Indodicarbocyanine maleimide dye (IDCC) was obtained from Epiios Therapeutics GmbH and used to fluorescently label the PG-PTX-bz-DOX-PEG.

Dendritic PG (average MW 10 kDa, PDI = 1.6, approximately 135 OH groups) was prepared according to published procedure [36]. PG amine with 30% of the total hydroxyl groups converted to amino groups (*ca.* 95 OH and 40 NH_2 groups per PG scaffold) was synthesized according to previously reported methodologies [37]. Briefly, PG amine was prepared by a three-step reaction starting from PG and a conversion of OH groups into mesyl (Ms) groups, followed by transformation of Ms. groups into azide (N_3) functionalities, and finally reduction of the N_3 groups to amine (NH_2) groups by using triphenylphosphine as reducing agent. Water of Millipore quality (resistivity ~18 MΩ cm^{-1}, pH 5.6 ± 0.2) was used in all experiments and for preparation of all samples. If not otherwise specified, sodium phosphate buffer (50 mM) was used for the pH of 7.4, and acidic pH values were reached by a sodium acetate buffer (pH 4, 50 mM) and a Britton-Robinson buffer (pH 2.0). All measurements were carried out with freshly prepared solutions at 25 °C. pH values were measured with a Scott instruments HandyLab pH meter at 25 °C.

Size exclusion chromatography (SEC) of PG conjugates was performed with Sephadex G-25 superfine or Sephadex LH-20 (GE Healthcare) respectively under ambient pressure and temperature. All reactions that involved air or water sensitive compounds were carried out in dried flasks under an argon atmosphere and dried solvents from the solvent purification system MB SPS 800, M. Braun Inertgas-Systeme GmbH, Garching, Germany. Size exclusion chromatography analysis was performed using UltiMate 3000 LC System (Thermo Scientific) with photodiode array (PDA)-UV detector and Shodex RI-101 detector (Showa Denko America, Inc.), with Zenix SEC-100 (Sepax) column in phosphate buffer pH = 7.0, flow 1 mL min^{-1}. Reversed phase (RP) high pressure liquid chromatography (HPLC) analysis was performed using UltiMate 3000 LC system (Thermo Scientific) with PDA-UV detector and C18 LiChroCART® Purospher® STAR 250 × 4.6 mm column (5 μm) (Merck Millipore). The mobile phase was a gradient of water (A) and acetonitrile (ACN) (B) both containing