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Anticancer polymeric nanomedicine bearing synergistic drug combination is superior to a mixture of individually-conjugated drugs

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ABSTRACT

Paclitaxel and doxorubicin are potent anticancer drugs used in the clinic as mono-therapies or in combination with other modalities to treat various neoplasms. However, both drugs suffer from side effects and poor pharmacokinetics. These two drugs have dissimilar physico-chemical properties, pharmacokinetics and distinct mechanisms of action, toxicity and drug resistance. In order to target both drugs selectively to the tumor site, we conjugated them at a synergistic ratio to a biocompatible and biodegradable polyglutamic acid (PGA) backbone. Drugs conjugation to a nano-sized polymer enabled preferred tumor accumulation by passive targeting, making use of the enhanced permeability and retention (EPR) effect. The rational design presented here resulted in co-delivery of combination of the drugs and their simultaneous release at the tumor site. PGA–paclitaxel–doxorubicin nano-sized conjugate exhibited superior anti-tumor efficacy and safety compared to the combination of the free drugs or a mixture of the drugs conjugated to separate polymer chains, at equivalent concentrations. This novel polymer-based multi-drug nano-sized conjugate allowed for true combination therapy since it delivered both drugs to the same target site at the ratio required for synergism. Using mice bearing orthotopic mammary adenocarcinoma, we demonstrate here the advantage of a combined polymer therapeutic bearing two synergistic drugs on the same polymer backbone, compared to each drug bound separately to the backbone.

1. Introduction

The conjugation of anticancer drugs with biocompatible polymers offers many advantages over small molecular therapeutics. These include: (i) improved solubility and stability, (ii) preferred passive accumulation of the drug at the tumor site by the enhanced permeability and retention (EPR) effect [1,2], (iii) reduced systemic toxicity and decreased or even abrogated immunogenicity, (iv) ability to overcome cancer cells resistance, and (v) increased therapeutic efficacy [3]. Many of the above advantages arise from the nano-scaled size of the polymer therapeutic. The high molecular-weight nanocarrier can only extravasate through the leaky blood vessels at the tumor area and internalize into target cells via endocytosis, resulting in a longer circulation time of the conjugate in the bloodstream compared with the free drugs [3]. This phenomenon of passive diffusion through the hyperpermeable neovasculature and localization in the tumor interstitium is observed in many solid tumors for macromolecular agents and lipids [4]. Currently, a number of polymer–drug conjugates are available for cancer treatment, and more are in the pipeline for clinical studies [5,6].

Another advantage of polymeric nanomedicines is their versatility, enabling to tailor different compounds with controlled loading percentage on a single polymeric backbone [7,8]. This property can be utilized for the coupling of two chemotherapeutic drugs, with different mechanisms of action, and different resistance and toxicity profiles, on the same nanocarrier. Importantly, coupling two agents that act synergistically will allow the administration of lower concentrations of each agent, increasing their combined anti-tumor efficacy and decreasing their toxicity. These multivalent polymeric nanocarriers serve as ideal platforms for true combination therapy, where the therapeutics are given simultaneously in one injection and share the same pharmacokinetic profile [9–11].

The anthracycline antibiotic, doxorubicin (DOX) and the microtubule-interfering agent, paclitaxel (PTX), are clinically well-established and highly effective anti-neoplastic medications as mono-therapies [12,13] and as sequential combination therapy [14–17]. As small Mw agents, they both suffer from different side effects, like neurotoxicity for PTX and cumulative dose-related cardiotoxicity for DOX [18]. Furthermore, since PTX is not water-soluble, it is administered with a solubilizing agent cremophor EL, which causes hypersensitivity reactions by itself [19].

A chemical conjugation of PTX and DOX to a nanocarrier could offer pharmacodynamic and pharmacokinetic advantages by passively targeting both drugs to the tumor site at the required ratio for synergism. Polyglutamic acid (PGA) is a water-soluble multivalent

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polymer which allows the conjugation of several compounds or targeting moieties within the polymer backbone [20–22]. It is non-immunogenic, is non-toxic, and is biodegradable by cathepsin B, an enzyme that is highly expressed in most tumor tissues [23–26]. PGA enables multivalent binding of synergistic drugs and selective delivery to tumors when used at an appropriate nano-scaled size. Those features make PGA an attractive drug carrier. Indeed, PGA–PTX (OPAXIO™) is currently being evaluated in Phase III clinical trials for ovarian cancer as a single agent [27,28], and for non-small-cell lung cancer in combination with carboplatin [29]. A Phase III clinical trial was recently concluded for OPAXIO™ as an orphan drug in combination with temozolomide and radiotherapy for patients suffering from glioblastoma multiforme (GBM) [30]. OPAXIO™ could not be safely combined with temozolomide due to Grade 4 hematologic toxicity. However, the favorable progression-free and overall survival suggested that OPAXIO™ may enhance radiation for GBM [30]. It is increasingly clear that combination therapy is likely to provide a long-term solution for the treatment of metastatic and/or resistant disease. Recently, several studies have explored the advantage of using a combination therapy in polymer–drug conjugates [31–35].

The aim of this study was to synthesize a polymer therapeutic combining two synergistic drugs on the same polymer chain at an appropriate ratio, and to determine its *in vivo* advantage over drugs conjugated to separate polymer chains and to free drugs.

2. Materials and methods

2.1. Ethics statement

All animal procedures were performed in compliance with Tel Aviv University guidelines approved by the Institutional Animal Care and Use Committee.

2.2. Materials

All chemicals and solvents were A.R. or HPLC grade. Chemical reagents were purchased from Sigma-Aldrich (Israel) and Merck (Israel). HPLC grade solvents were purchased from Biolab (Israel). Paclitaxel and doxorubicin hydrochloride were purchased from Petrus Chemicals and Materials Ltd. (Israel). All tissue culture reagents were purchased from Biological Industries Ltd (Beit Haemek, Israel), unless otherwise indicated. PGA was purchased from Alamanda Polymers (Huntsville, AL, USA).

2.3. Chemical data

All reactions requiring anhydrous conditions were performed under a nitrogen atmosphere. Size exclusion chromatography (SEC) 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. 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® STAR250 × 4.6 mm column (5 μm) (Merck Millipore). The mobile phase was a gradient of water (A) and acetonitrile (ACN) (B) both containing 0.1% (vol/vol) trifluoroacetic acid (TFA), 20% to 100% solvent B in 15 minutes. Chromelion software was employed for data analysis. Polymer conjugates were purified by SEC on Sephacryl S-200 HR (GE Healthcare, Buckinghamshire, UK), using water as eluent. 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).

2.4. Synthesis of PGA–PTX–DOX

In this combined conjugate PTX is bound directly to the PGA by an ester bond and DOX is bound through an acid-sensitive hydrazone bond. PGA–PTX–DOX conjugate was synthesized using the following steps (Scheme 1):

2.4.1. Synthesis of PGA–PTX

PTX (29.7 mg, $3.48 * 10^{-2}$ mmol) was conjugated to PGA (150.0 mg, 1.16 mmol; 100 units, Mw ~13 kDa) by carbodiimide coupling (DIC/HOBt) in anhydrous DMF (10 ml). After 24 hours the solvent was evaporated under high vacuum and the residue washed in chloroform/acetone (4:1). The resulting precipitate was washed in chloroform/acetone (2:2) and dried under vacuum to obtain PGA–PTX conjugate. The supernatant of the chloroform/acetone washing mixture was kept to determine drug loading by measuring the amount of unreacted PTX by analytical HPLC.

2.4.2. Synthesis of SH–PGA–PTX

Cysteamine (12.7 mg, $1.65 * 10^{-1}$ mmol) was bound to the PGA–PTX by carbodiimide coupling in anhydrous DMF (15 ml), in the presence of 50 mM dithiothreitol (DTT) as reducing agent. After 24 hours the solvent was evaporated under high vacuum and the residue dissolved in MilliQ water. This aqueous solution was purified using Sephacryl S-200 HR column to remove unreacted cysteamine. The appropriate fractions were lyophilized to obtain SH–PGA–PTX (elution fractions 85–160 ml). Presence of thiol groups on the polymer was confirmed by Ellman's test [36].

2.4.3. Synthesis of PGA–PTX–DOX

DOX-3,3′-*N*-[ε-maleimidocaproic acid] hydrazide (EMCH) was coupled with SH–PGA–PTX, by selective reaction of the maleimide moiety of the linker with SH groups on PGA, to form the final conjugate PGA–PTX–DOX. Reaction was done in dry DMF (10 ml) with the addition of *tris*(2-carboxyethyl)phosphine (TCEP) as a reducing agent. The water-soluble sodium salt of the conjugate was obtained by dissolving the product in 0.25 M $NaHCO_3$ after evaporation of DMF. This aqueous solution was purified using Sephacryl S-200 HR column, removing unreacted drugs and low molecular weight contaminants, and lyophilized to obtain the final product as a red powder (elution fractions 85–160 ml). DOX loading was determined by measuring the absorbance of the conjugate ($\lambda_{EX} = 495$ nm) and by using the molar absorbance of DOX–EMCH ($\varepsilon = 9250\ M^{-1}\ cm^{-1}$). The conjugate was synthesized in 100 mg scale. Appropriate controls were synthesized (i.e., PGA–PTX (300 mg scale), PGA–DOX (50 mg scale)).

2.4.4. Synthesis of DOX–EMCH

DOX (100.7 mg, $1.74 * 10^{-1}$ mmol) was coupled to an acid-sensitive, EMCH (118.0 mg, $3.48 * 10^{-1}$ mmol) linker in a procedure developed by Willner et al. [37]. MS (ES^+): *m/z*: 752.3 [M], 775.5 $[M + Na]^+$.

2.5. Physico-chemical characterization of the conjugates

2.5.1. Nuclear magnetic resonance (NMR) measurements

^1H NMR was performed on 400 MHz Avance, Bruker (Karlshruhe, Germany) system with tetramethylsilane (TMS) as an internal standard. The spectra were recorded at room temperature (RT) in deuterium oxide (D_2O), except for PTX, which was recorded in deuterated chloroform ($CDCl_3$).

2.5.2. Surface charge measurements of the conjugates

Zeta potential measurements were performed on a Zetasizer Nano ZS analyzer with an integrated 4 mW He–Ne laser ($\lambda = 532$ nm; Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). To elucidate the surface charge of the conjugates, PGA–PTX, PGA–DOX and PGA–PTX–DOX potentials were measured in 20% aqueous phosphate buffered