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The structure of polymer carriers controls the efficacy of the experimental combination treatment of tumors with HPMA copolymer conjugates carrying doxorubicin and docetaxel

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ABSTRACT

The tumor-specific targeting of cancerostatics using polymer drug carriers represents a potential strategy to achieve an effective treatment with reduced side toxicity. Synthetic water-soluble copolymers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) are carriers with tunable architecture and drug loading, tumor-specific accumulation of the drug, and its controlled release. We describe a combination treatment of murine EL4 T cell lymphoma with HPMA-based star conjugates (M_w 250,000 g mol^{-1}) of doxorubicin (Dox) or docetaxel (Dtx) designed for enhanced tumor accumulation and combination therapy. Although the combination of linear conjugates (M_w = 28,000 g mol^{-1}) containing Dox or Dtx resulted in an additive effect in the treatment of the lymphoma, the opposite was observed in the combination of two star conjugates with Dox or Dtx, as the star Dtx conjugate decreased the treatment efficacy of the star Dox conjugate. The Dtx conjugate alone was virtually ineffective in the reduction of tumor growth or survival time extension; thus, a curative effect could be solely attributed to the Dox-containing conjugate. When Dtx was delivered to the tumor on the same polymer carrier as Dox, the efficacy of the Dox-induced treatment was reduced to a lesser extent. No reduction was found when Dtx was delivered by a linear polymer or applied as a free drug. The phenomenon was strictly related to the enhanced permeability and retention (EPR) effect, as it was not observed in BCL1 leukemia, a model without EPR. The diminished treatment outcome in the combination therapy with the two star conjugates was underlined by the significantly decreased accumulation of Dox in the tumor. The use of the drug-free polymer carrier instead of the Dtx-containing star conjugate did not reduce the treatment efficacy of the Dox conjugate. Thus, the physicochemical characteristics of the polymer carrier designed for tumor-specific drug delivery systems control the activity of the respective drug, leading to changes within the tumor microenvironment that can determine ultimate efficacy of the combination therapy.

1. Introduction

The combination of two or more *anti*-cancer drugs with non-overlapping systemic toxicities is the cornerstone of current cancer therapy. The anthracycline doxorubicin (Dox) has a broad range of actions in solid and hematologic malignancies and is still an essential component of many chemotherapy regimens. Its mechanisms of action include DNA intercalation, the inhibition of topoisomerase II, the formation of reactive oxygen species, and impact on membrane fluidity. The clinical application of Dox is challenged by cumulative and irreversible cardiomyopathy, which often develops within months to years [1]. Docetaxel (Dtx) is a potent *anti*-cancer agent frequently used in the treatment of patients with breast, ovarian, prostate, lung, and other cancers. Dtx interferes with microtubule disassembly, thereby inhibiting cell division and inducing cell death. As with many other chemotherapeutic agents, the metronomic dosing of Dtx has been shown to exert *anti*-angiogenic activity in solid tumors [2]. However, one of the major disadvantages of Dtx is its prominent hydrophobicity. In general, these low-molecular weight (LMW) cytotoxic/cytostatic drugs are characterized by inconvenient pharmacokinetics, systemic toxicity, and the frequent development of drug resistance. The development of efficient tumor-specific drug delivery systems should provide safe and effective treatment not burdened by these disadvantages.

High-molecular weight (HMW) polymer conjugates represent an advanced tumor-selective drug delivery system characterized by pharmacokinetics driven by the enhanced permeability and retention (EPR) effect. Nanosized therapeutic devices, including liposomes,

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micelles, nanoparticles, and polymer conjugates, are restricted from extravasation into normal tissues, as normal vasculature has a continuous endothelial lining with small pores (usually 1–2 nm). Nanotherapeutics preferentially accumulate in the solid tumor tissue because of its leaky vasculature with large fenestrations (100–200 nm) and its poorly developed, or even absent, lymphatic drainage [3]. The size of the polymer therapeutics designed for enhanced passive accumulation in solid tumor tissue by EPR effect was identified as a critical parameter governing their ability to penetrate the tissue upon extravasation. Structures of a size less than 20 nm are considered to diffuse efficiently through tumor tissue [4].

Copolymers based on *N*-2-(hydroxypropyl)methacrylamide (HPMA) are water-soluble polymer carriers of various LMW drugs. The main advantages of HPMA-based conjugates are increasing drug solubility and bioavailability, prolonged circulation time, and improved pharmacokinetic/biodistribution profiles of LMW drugs [5,6]. It is a tunable system that allows the tailor-made synthesis of the desired carrier structure, size, and content of the drug bound by stimuli-sensitive linkages to the carrier, such as a pH-sensitive hydrazone bond [7,8]. By definition, the active drug is liberated by acid-sensitive hydrolysis that occurs mainly in the endosomal and lysosomal compartments of cells and also within the tumor microenvironment, which is characterized by low pH. HPMA-based conjugates of cytotoxic drugs have shown the *in vivo* ability to deliver the cytostatic/cytotoxic drugs directly to the tumor site while maintaining high *anti*-tumor efficacy and low systemic exposure and toxicity. Ample evidence shows that HPMA copolymer conjugates carrying Dox or Dtx can induce the complete regression of several types of syngeneic murine tumors [9–12]. Treatment-dependent, immunologically mediated tumor resistance was regularly observed in the cured animals, suggesting the involvement of immunogenic cancer cell death induced by HPMA-based copolymer conjugates carrying drugs bound *via* a hydrazone bond [13]. The HPMA copolymer conjugates of Dtx have been shown to be a feasible system for solubilizing the drug, ensuring its favorable pharmacokinetics, and tumor delivery [11,14].

HMW star polymer drug nanocarriers prepared by grafting poly(amido amine) (PAMAM) dendrimers by hetero-telechelic *N*-(2-hydroxypropyl)methacrylamide (HPMA) represent well-defined drug delivery systems with remarkably low polydispersity [14–16]. It was shown that these conjugates carrying either Dox or Dtx as the cytotoxic drug are able to induce more potent *anti*-cancer effect compared with a linear HPMA-based polymer conjugate and the respective free drug [14,17,18]. This is indeed related to the enhanced capacity of the *star*-shaped polymer conjugates to accumulate in solid tumors [14,15]. Herein, the structure of the conjugates was designed to be biodegradable in tumor cells because of the presence of proteolytically degradable tetrapeptide Gly-Phe-Leu-Gly (GFLG) spacers linking the dendrimer core with the copolymer chains. Thus, the biodegradable polymer carrier ensured the enhanced accumulation in the tumor because the HMW of the conjugate, followed by the drug release and degradation of the HMW polymer carrier structure into polymer fragments excretable from the body *via* renal filtration.

Clinically, the combination of Dox and Dtx is burdened by considerable systemic toxicity. Herein, a star polymer conjugate was studied carrying respective drugs and compared with the combination of two respective conjugates carrying either drug. Surprisingly, the combination treatment worsened the curative effect of the carrier-delivered Dox. Therefore, this strong antagonism of two star conjugates, not apparent in combination of two linear conjugates, was studied in more detail. We showed that the antagonism is attributable to diminished Dox accumulation in the tumor. Thus, the proper selection of the polymer carrier size and architecture as well as the active pharmacological ingredients could be fundamental for the resulting outcome of the combination treatment of solid tumors with polymer-drug conjugates.

2. Material and methods

2.1. Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2.2′-azobis(isobutyronitrile) (AIBN), 4.4′-azobis(4-cyanovaleric acid) (ABIC), 6-aminohexanoic acid (ah), *N*,*N*′-dimethylformamide (DMF), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*,*N*′-diisopropylcarbodiimide (DIPC), 1-hydroxy benzotriazole (HOBT), *N*-ethyldiisopropylamine (EDPA), dimethyl sulfoxide (DMSO), Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, *tert*-butyl carbazate, 4.5-dihydrothiazole-2-thiol, ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA) and trinitrobenzene-1-sulfonic acid (TNBSA) were purchased from Fluka. Doxorubicin hydrochloride (Dox·HCl) was purchased from Meiji Seika, Japan. 3,3′-[Azobis(4-cyano-4-methyl-1-oxobutane-4.1-diyl)]bis(thiazolidine-2-thione) (ABIC-TT) was synthesized according to [19]. Poly(amido amine) (PAMAM) dendrimers with 1,4-diaminobutane core in the center were purchased from Dendritic Nanotechnologies, Inc., USA.

2.2. Synthesis of monomers, oligopeptide and Dtx derivative

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described in [19] using K_2CO_3 as a base. M.p. 70 °C; purity > 99.8% (HPLC); elemental analysis: calcd., C 58.72%, H 9.15%, N 9.78%; found, C 58.98%, H 9.18%, N 9.82%.

N-(*Tert*-butoxycarbonyl)-*N*′′-(6-methacrylamidohexanoyl)hydrazine (Ma-ah-NHNH-Boc) was prepared in two-step synthesis as described in [20]. M.p. 110–114 °C; purity (HPLC) > 99.5%; elemental analysis: calcd. C 57.70C, H 8.33, N 13.46; found C 57.96, H 8.64, N 13.25.

6-Methacrylamidohexanohydrazide (Ma-ah-$NHNH_2$) was prepared in two-step synthesis as described in [21]. M.p. 79–81 °C; elemental analysis: calc. C 56.32%, H 8.98%, N 19.70%; found C 56.49%, H 8.63%, N 19.83%.

The derivative of Dtx with levulic acid (Dtx-LEV) was synthesized using the carbodiimide coupling method as described in [11]. The chemical structure of the Dtx-LEV derivative was determined and proved by NMR spectroscopy. HPLC showed 95.3% purity (peak maximum at 12.58 min). MS (APCI): *m*/*z*: 904.25 $[M-H]^-$.

The linear tetrapeptide H-Gly-Phe-Leu-Gly-OH (H-GFLG-OH) was assembled by the automatic solid phase peptide synthesis using automatic peptide synthesizer, starting from the C-terminus using standard Fmoc procedures as described in [18]. The product was characterized by MALDI-TOF MS (392.5 M + H) and reverse-phase high-performance liquid chromatography (HPLC) showing single peak with a retention time of 18.0 min.

Purity of all the monomers and oligopeptide mentioned above was examined by HPLC (Shimadzu, Japan) using a reverse-phase column Chromolith Performance RP-18e 100–4.6 with PDA detection , eluent water–acetonitril with acetonitril gradient 0–100 vol.%, 0.1% TFA, flow rate 5 mL/min.

2.3. Synthesis of polymer precursors and polymer-drug conjugates

Statistical copolymer of HPMA and Ma-ah-$NHNH_2$ (polymer LP, Table 1) containing hydrazide groups was prepared by radical copolymerization in methanol as previously described [22].

Semitelechelic copolymer of HPMA and Ma-ah-NHNH-Boc terminated with chain-end thiazolidine-2-thione (TT) group (polymer LP-TT, Table 1) was prepared by radical solution copolymerization [HPMA (2.0 g, 0.014 mol) with Ma-ah-NHNH-Boc (378 mg, 1.21 mmol)] in DMSO initiated with azo-initiator ABIC-TT as described [19]. The content of end-chain TT groups was determined spectrophotometrically