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Polymer conjugates of doxorubicin bound through an amide and hydrazone bond: Impact of the carrier structure onto synergistic action in the treatment of solid tumours

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ABSTRACT

In this study, we describe the synthesis, physico-chemical characterisation and results of the *in vitro* and *in vivo* evaluation of the biological behaviour of *N*-(2-hydroxypropyl)methacrylamide-based (HPMA) copolymer conjugates bearing doxorubicin (DOX) partly bound via a pH-sensitive hydrazone and partly via enzymatically degradable amide bonds, each contributing to a different anti-tumour mechanism of action of the polymer–doxorubicin conjugate. The following two types of HPMA copolymer drug carriers designed for passive tumour targeting were synthesised and compared: the linear non-degradable copolymer and the biodegradable high-molecular-weight (HMW) diblock copolymer. The HMW diblock copolymer carrier containing a degradable disulphide bond between the polymer blocks showed a rapid degradation in a buffer containing glutathione within the first few hours of incubation. In contrast to the conjugate with the amide bond-bound DOX requiring the presence of lysosomal enzymes to release DOX, the polymer–drug conjugate with the DOX bound via a hydrazone bond released DOX by pH-sensitive hydrolysis, which was significantly faster in a buffer of pH 5.0 (intracellular pH) than pH 7.4, mimicking the conditions in the bloodstream. The significant and comparable *in vivo* anti-tumour activity of the diblock HMW conjugate and an equimolar mixture of the conjugates differing in the DOX attachment method along with the development of cancer resistance during treatment with these conjugates demonstrated the high potential of these compounds in the development of new nanomedicines suitable for the treatment of solid tumours.

1. Introduction

The water-soluble drug conjugates based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers rank among the most intensively studied polymer drug delivery systems (Duncan, 2005; Kopeček, 2013; Ulbrich and Šubr, 2010). HPMA copolymer–DOX conjugates containing doxorubicin (DOX) bound through an amidic bond and an oligopeptide GFLG spacer ($DOX_{(am)}$) have undergone clinical evaluation or experimental therapy (Říhová et al., 2003; Říhová and Kubáčková, 2003; Seymour et al., 2009; Vasey et al., 1995; Vasey et al., 1999; Vicent et al., 2008; Vicent et al., 2009). Subsequently, the anti-cancer drug doxorubicin was attached to the HPMA carriers by a spacer containing a pH-sensitive hydrazone bond ($DOX_{(hyd)}$). These polymer drug conjugates were fairly stable in model buffers at pH 7.4 (simulating the pH of the bloodstream) and released the drug by chemical hydrolysis in a pH range representative of the endosomal and lysosomal environment inside the target cells (pH 5–6) (Etrych et al., 2001; Etrych et al., 2002; Říhová et al., 2001; Ulbrich et al., 2003; Ulbrich et al., 2004b). These HPMA copolymer–DOX conjugates exhibit promising anti-cancer activity *in vivo* (Etrych et al., 2008b; Šírová et al., 2010). The potential of HPMA copolymer–DOX conjugates for use in the treatment of solid tumours can be further improved by increasing the molecular weight of the polymer carrier above the renal threshold, which prevents the polymer–drug conjugate from rapid blood clearance and elimination from the body while enhancing its tumour accumulation due to the enhanced permeation and retention (EPR) effect (Etrych et al., 2012; Maeda, 2012; Maeda et al., 2013; Matsumura and Maeda, 1986). Efficient accumulation of polymer conjugates in solid tumours can only be achieved with a

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molecular weight exceeding the renal threshold, which is approximately 50,000 g.mol^{-1} for non-linear structures, e.g., star-like HPMA copolymers, and could be up to 70,000 g.mol^{-1} for linear structures due to "warm-like" movement (Etrych et al., 2012). To avoid the undesirable accumulation of the high-molecular-weight (HMW) polymer carrier in the body after the drug is released, the carrier must be biodegradable, forming polymer degradation products excretable from the organism. Diblock, grafted, micellar and star-like HPMA copolymer–DOX conjugates have been designed for this purpose (Chytil et al., 2008; Chytil et al., 2012; Etrych et al., 2008a; Etrych et al., 2010a; Etrych et al., 2011a; Etrych et al., 2011b). The mechanism of action of the HPMA copolymer conjugate bearing either $DOX_{(am)}$ or $DOX_{(hyd)}$ differs and reflects the diverse method and intensity of their intracellular accumulation. HPMA conjugates containing $DOX_{(am)}$ (bound via an amidic bond) directly penetrate the plasma membrane and are detectable in all of the associated cellular membranes, i.e., membranes of the endocytic compartment, a nuclear membrane and membranes of the Golgi and endoplasmic reticulum. The cytotoxicity of these conjugates appears to be primarily caused by the collapse of the cell metabolism. Conjugates containing $DOX_{(hyd)}$ (bound via a pH-sensitive hydrazone bond) are internalised by endocytosis and fluid phase pinocytosis, and DOX is released from the polymeric carrier at a low pH in endosomes and lysosomes (Hovorka et al., 2006). The primary target is the nucleus, and the cancer cells die by immunogenic cancer cell death, ICD (Kroemer et al., 2013; Šírová et al., 2013). The use of the above-mentioned polymer–DOX conjugates, which differ in their mode of effect on tumour cells, in combination, mixed in ratios resulting in a synergistic effect of both types of the polymer–DOX conjugates, resulting in a particularly significant anti-tumour effect with very low non-specific drug toxicity was described in a patent (Etrych et al., 2010b), and some results have previously been published (Říhová et al., 2010).

Here, we describe the synthesis and properties of linear nondegradable and HMW biodegradable diblock HPMA copolymers as drug carriers enabling combination chemotherapy. We studied in detail the conjugates simultaneously bearing $DOX_{(am)}$ and $DOX_{(hyd)}$ and compared their behaviour with a mixture of respective conjugates bearing single $DOX_{(am)}$ or $DOX_{(hyd)}$. The system was designed with the aim to achieve a combination or even a synergistic effect of the diversely bound DOX on the tumour growth due to different mechanisms of action of the polymer-conjugated $DOX_{(am)}$ or $DOX_{(hyd)}$. The HMW diblock carrier was designed to achieve efficient passive tumour accumulation and subsequent biodegradation, enabling elimination of the polymer from the body. The physico-chemical properties of these conjugates, namely the hydrodynamic radius (R_h) of a polymer coil in aqueous solution, the molecular weight of the polymer conjugates, the stability of the conjugates incubated in aqueous solutions of a specific pH, the biodegradability of the diblock polymer carrier and the *in vitro* cytotoxicity for various cancer cell lines, were studied to evaluate the potential of the conjugates for *in vivo* tumour treatment. These data are completed with selected results of the *in vivo* verification of anti-tumour activity of the conjugates in mice bearing EL4 T-cell lymphoma.

2. Materials and methods

2.1. Chemicals

N,*N*′-dicyclohexylcarbodiimide (DCC), *N*,*N*-dimethylformamide (DMF), 4-(dimethylamino)-pyridine (DMAP), dichloromethane, methanol, ethyl acetate, tetrahydrofuran, 2,2′-azobis (isobutyronitrile) (AIBN), 4,4′-azobis(4-cyanopentanoic acid) (ABIC), cysteamine hydrochloride, di(2-pyridyl) disulphide, 1-aminopropan-2-ol, methacryloyl chloride, methyl 6-aminohexanoate hydrochloride (ah), hydrazine hydrate, *tert*-butyl carbazate, dithiothreitol (DTT), trifluoroacetic acid (TFA), triisopropylsilane (TIS), reduced L-glutathione (GSH) were purchased from Sigma–Aldrich (Prague, Czech Republic), doxorubicin hydrochloride (DOX·HCl) was purchased from Meiji Seiko (Japan) and 2,4,6-trinitrobenzene-1-sulphonic acid (TNBSA) was purchased from Serva, Heidelberg. Solvents were dried and purified by usual procedures.

2.2. Synthesis of monomers

***N*-(2-Hydroxypropyl)methacrylamide** (HPMA) was synthesised by methacryloylation of 1-aminopropan-2-ol as described previously using Na_2CO_3 as a base (Ulbrich et al., 2000). M.p. 64–66 °C; elemental analysis: Calcd. C 58.74, H 9.0, and N 9.79; Found C 58.79, H 9.05, N 9.81.

6-Methacrylamidohexanoylhydrazine (Ma-ah-$NHNH_2$) was prepared as described in the literature (Etrych et al., 2008b). M.p. 79–81 °C; elemental analysis: Calcd. C 56.32, H 8.98, and N 19.70; Found C 56.43, H 8.72, N 19.88.

***N*-(*tert*-butoxycarbonyl)-*N*′-(6-methacrylamidohexanoyl) hydrazine** (Ma-ah-NHNH-Boc) was prepared in a two-step synthesis as described previously (Ulbrich et al., 2004a). M.p. 110–114 °C, elemental analysis: Calcd. C 57.70, H 8.33, N 13.46; Found C 57.89, H 8.59, N 13.59.

6-Methacrylamidohexanohydrazone–doxorubicin (Ma-ah-NHN = DOX) was prepared by the reaction of Ma-ah-$NHNH_2$ with DOX·HCl (Etrych et al., 2008b). M.p. 172–175 °C; TLC on Silicagel 60 F_{254} (methanol:chloroform:accetic acid 2:8:1) one spot at R_f = 0.9. MALDI-TOF MS: 762.2 (M + Na).

Methacrylglycyl-D,L-phenylalanylleucylglycyl doxorubicin (Ma-GFLG-DOX) was prepared as described earlier (Ulbrich et al., 2000). Mass spectra MS ESI: 1008.4 (M + Na) (LCQ Fleet, Thermo Scientific), RP HPLC showed single peak at 18.2 min (UV detection at 484 nm), amino acid analysis: Gly:D-Phe:L-Phe:L-Leu = 2.03:0.46:0.54:1.0.

The purity of the monomers was examined by a Shimadzu HPLC system equipped with a reverse-phase column (Chromolith Performance RP-18e, 100 × 4.6 mm) eluting with water-acetonitrile (gradient 0–100 % acetonitrile) utilising UV–VIS detection (Shimadzu SPD-M10A vp) (220 nm).

2.3. Synthesis of functionalised azo initiators

The synthesis of 2-(2-pyridyldisulfanyl)ethylamine hydrochloride (PDEA) was accomplished according to the reference (Ebright et al., 1992). The azo initiator *N*,*N*′-bis[2-(pyridin-2-yldisulfanyl]-4,4-azobis[(4*R*)-4-cyanopentamide] (ABIC-PDS) was synthesised using 4,4′-azobis(4-cyanopentanoic acid) and PDEA using the carbodiimide method as previously described (Etrych et al., 2010a).

2.4. Synthesis of the polymer precursors and polymer conjugates

The multivalent polymer precursor poly(HPMA-*co*-Ma-ah-$NHNH_2$) **1** containing hydrazide groups distributed along the polymer chain was prepared as previously described (Etrych et al., 2008b). Semitelechelic polymer precursor **2** bearing Boc protected hydrazide groups and a polymer chain terminating in a PDS group was prepared by solution radical copolymerisation of HPMA (0.4 g, 2.8 mmol) with Ma-ah-NHNH-Boc (74 mg, 0.347 mmol) initiated with ABIC-PDS (0.162 g) and reacted in DMSO (2.9 ml) at 60 °C for 6 h. The polymer was isolated by precipitation into a mixture of acetone–diethyl ether (1:3), filtered off and dried in a vacuum. The Boc protecting groups were removed by TFA.