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Conjugates of doxorubicin with graft HPMA copolymers for passive tumor targeting

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ABSTRACT

Synthesis, physicochemical behavior, tumor accumulation and preliminary anticancer activity of a new biodegradable graft copolymer–doxorubicin (DOX) conjugates designed for passive tumor targeting were investigated. In the graft high-molecular-weight conjugates the multivalent *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer was grafted with a similar but semitelechelic HPMA copolymer; both types of polymer chains were bearing doxorubicin attached by hydrazone bonds enabling intracellular pH-controlled drug release. The polymer grafts were attached to the main chain through spacers, degradable enzymatically or reductively, facilitating, after the drug release, intracellular degradation of the graft polymer carrier to short fragments excretable from the organism by glomerular filtration. The graft polymer–DOX conjugate exhibited prolonged blood circulation and enhanced tumor accumulation in tumor-bearing mice indicating the important role of the EPR effect in the anticancer activity. The graft polymer–DOX conjugates showed a significantly higher antitumor activity *in vivo* than DOX·HCl or the linear polymer conjugate when tested in mice bearing 38C13 B-cell or EL4 T-cell lymphoma, with a significant number of long-term-surviving (LTS) mice with EL4 T-cell lymphoma treated with a single dose 15 mg DOX equiv./kg on day 10.

1. Introduction

During the last two decades many water-soluble synthetic polymers have turned into intensively studied drug carrier systems [1–3] for delivery of a variety of therapeutics, including anticancer agents. Conjugation of drugs with water-soluble polymers may reduce their toxicity, improve their solubility, bioavailability and stability (enzymatic, thermal, etc.), eliminate undesirable interactions, and prolong circulation time [4,5]. Likewise, polymer–drug conjugates may enable controlled drug release and specific delivery to the target tissue. To reach a tumor tissue-specific or a tumor cell-specific drug release and, consequently, an antitumor activity of a drug, precise selection of the polymer carrier structure and type of linkage between the drug and the carrier are important [5,6]. The drug can be liberated from the conjugate by a specific enzymatic cleavage [7,8] or by pH-controlled hydrolysis [9]. Among the most intensively studied polymer–drug carriers belong the copolymers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) [10,11], some of their drug conjugates being already subjected to clinical trials [3].

The antitumor potential of the polymeric prodrugs can be further improved by coupling the polymer carrier with antibody [12,13] or by increasing its molecular weight [14,15]. Molecular weight of the polymer carrier higher than renal threshold prevents the drug from fast blood clearance and elimination from organism. Furthermore, a higher molecular weight of the polymer carrier is the basis for enhanced accumulation of the prodrug in a solid tumor tissue due to a tumor-related phenomenon described as the enhanced permeability and retention (EPR) effect [16,17], which significantly increases with increasing molecular weight of the delivery system. The effect of molecular weight on accumulation of poly(HPMA) in sarcoma 180 [18] and HPMA copolymers in other tumors [19] was also found in mice. HPMA copolymers are synthetic polymers with the non-degradable main chain. This means that only polymers with molecular weight below the renal filtration limit (ca. 50 kDa) could be easily eliminated from the body via glomerular filtration, the higher-molecular-weight polymers accumulate in the body. For that reason, the high-molecular-weight polymer–drug carriers should be biodegradable or should contain biodegradable linkages enabling degradation of the polymer to smaller fragments excretable from the organism. Various biodegradable high-molecular-weight drug conjugates based on HPMA or PEG were prepared and tested for anticancer activity against selected tumors [20–22]. In recent years we have demonstrated that synthetic water-soluble HPMA copolymers containing anticancer drug doxorubicin (DOX) bound via the hydrolytically degradable hydrazone bond provide a potential drug delivery system [23] enabling achievement of up to 100% long-term survival (LTS) of tumor-inoculated mice treated with a single dose of the conjugate in the therapeutic regime [24–26]. Such polymer–DOX conjugates were fairly stable in aqueous solution at pH 7.4 (pH of bloodstream) and DOX was released at pH 5–5.5 (mimicking pH in endosomes). The conjugates were linear or

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branched water-soluble polymers with enhanced molecular weight. An increase in molecular weight of branched polymers led to improvement of the therapeutic effect of the conjugate [21], which could be ascribed to a higher accumulation of the branched polymer–drug conjugate in the tumor due to the more pronounced EPR effect. A drawback of the branched polymer system is its high polydispersity and lower reproducibility of its synthesis.

Here, we describe the synthesis, physicochemical and preliminary biological properties of new graft high-molecular-weight polymer–drug conjugates based on HPMA copolymers designed for passive tumor targeting. The anticancer drug, doxorubicin, is attached to the polymer carrier via pH-sensitive hydrazone bond susceptible to hydrolysis enabling DOX release in mild acid environment (pH 5–5.5). In the conjugates the main polymer chain is grafted with three types of semitelechelic HPMA copolymers thus forming a high-molecular-weight structure with quite a narrow molecular weight distribution. The polymer grafts are attached to the main polymer backbone via spacers susceptible to enzymatic or reductive degradation enabling disintegration of the conjugate to the polymer fragments of molecular weight of the original grafts clearable from a body by glomerular filtration.

2. Experimental part

2.1. Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2,2′-azobis(isobutyronitrile) (AIBN), 6-aminohexanoic acid (ah), methyl 6-aminohexanoate hydrochloride (ah-MeO), *N*,*N*′-dimethylformamide (DMF), dithiothreitol (DTT), *N*-hydroxysuccinimide (HOSu), glutathione, *N*,*N*′-dicyclohexylcarbodiimide (DCC), leucylglycine, glycylphenylalanine, phthalaldehyde (OPA), 2-iminothiolane, *N*-ethyldiisopropylamine (EDPA), dimethyl sulfoxide (DMSO), *tert*-butyl carbazate, 4,5-dihydrothiazole-2-thiol, 3-sulfanylpropanoic acid (SPA) hydrazine hydrate, ethylenediaminetetraacetic acid (EDTA), reduced glutathione (GSH), ethylenediamine (EDA), cathepsin B and trifluoroacetic acid (TFA) were purchased from Fluka. Doxorubicin hydrochloride (DOX.HCl) was purchased from Meiji Seiko, Japan and 2,4,6-trinitrobenzene-1-sulfonic acid (TNBSA) from Serva, Heidelberg, Germany. 2-(2-pyridyldisulfanyl)ethylamine hydrochlorid (PDEA) was synthesized according to [27].

2.2. Synthesis of monomers

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described in [28] 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 a two-step synthesis as described in [25]. M.p. 110–114 °C; purity (HPLC) >99.5%; elemental analysis: calcd. C 57.70 C, H 8.33, N 13.46; found C 57.96, H 8.64, N 13.25.

6-Methacrylamidohexanohydrazide (Ma-ah-$NHNH_2$) was prepared by a two-step procedure (as described in [26]). Briefly: In the first step, methyl 6-aminohexanoate hydrochloride was acylated with methacryloyl chloride in dichloromethane in the presence of anhydrous sodium carbonate. After 2 h reaction, insoluble inorganic compounds were filtered off and dichloromethane was evaporated. The crude oily methyl 6-methacrylamidohexanoate was dissolved in 153 mL methanol and hydrazinolyzed with hydrazine hydrate (32 mL≈33 g, 0.66 mol) for 6 h. Excess of hydrazine hydrate was removed by multiple co-distillation at reduced pressure with propan-2-ol. Then the product was recrystallized from a mixture dichloromethane/ethyl acetate (1:1). M.p. 79–81 °C; purity (HPLC) >99.6%; elemental analysis: calcd., C 56.32, H 8.98, N 19.70; found, C 56.49, H 8.63, N 19.83. ^{1}H NMR 300 MHz ($CDCl_3$, 297 K): 1.35 m (2H, $CH_2(CH_2)_2$–N); 1.50–1.69 m (4H, $CH_2CH_2CH_2CH_2$–N); 1.95 dd (3H, CH_3); 2.17 t (2H, ((C=O)–CH_2); 3.26 dt (2H, N–CH_2); 3.91 s (2H, NH_2); 5.30 t (1H, C=CH_2 E); 5.67 t (1H, C=CH_2 Z); 6.10 s (1H, N*H*NH_2); 7.45 s (1H, N*H*–CH_2).

(*N*-Methacryloylglycyl)-DL-phenylalanylleucylglycinohydrazide (Ma-GFLG-$NHNH_2$) was prepared using the same procedure as described above for Ma-ah-$NHNH_2$ [26]. M.p. 139–140 °C; purity (HPLC) >99.5%; elemental analysis: calcd., C 58.10, H 7.36, N 17.68; found, C 58.21, H 7.39, N 17.54. Amino acid analysis: Gly/D-Phe/L-Phe/D-Leu/L-Leu 2.03:0.0.47:1.00:0.01. HPLC showed two peaks of equal areas at 9.39 min (L-Phe peptide) and 9.91 min (D-Phe peptide).

3-(6-Methacrylamidohexanoyl)thiazolidine-2-thione (Ma-ah-TT) was synthesized as described in [29]. Briefly: 6-Methacrylamidohexanoic acid (1.0 g, 5 mmol) and 4,5-dihydrothiazole-2-thiol (0.6 g, 5 mmol) dissolved in 12 mL of tetrahydrofuran (THF) and DCC (1.24 g, 6 mmol) dissolved in ethyl acetate (3 mL) were cooled to −18 °C, mixed with DMAP (~30 mg) and the solution was stirred for 1 h at −15 °C and then kept overnight at 5 °C. The reaction was finished by addition of 0.1 mL of acetic acid at room temperature. The precipitated *N*,*N*'-dicyclohexylurea (DCU) was filtered off, solvents evaporated in vacuum and the product Ma-ah-TT was crystallized from an ethyl acetate/diethyl ether mixture at −18 °C. The product was filtered off, washed with diethyl ether and dried in vacuum. Yield: 1.18 g (78%). Purity (HPLC) >99.5%.

^{1}H NMR [$(CD_3)_2SO$]: δ 1.28 m, 2H (CH_2-γ); 1.41 m, 2H (CH_2-β); 1.58 m, 2H (CH_2-δ); 1.83 s, 3H (CH_3); 3.08 m, (CH_2-α, CH_2-ε); 3.33 t, 2H (CH_2S); 4.48 t, 2H (CH_2N); 5.28 s, 1H (CH_2=); 5.61 s, 1H (CH_2=); 7.87 t, 1H (NH).

3-(*N*-methacryloylglycyl)-DL-phenylalanylleucylglycylthiazolidine-2-thione (Ma-GFLG-TT) was prepared according to [29]. Briefly: Ma-Gly-DL-PheLeuGly-OH (2.0 g, 4.34 mmol) and 4,5-dihydrothiazole-2-thiol (0.544 g, 4.56 mmol) were coupled in 12 mL of DMF using the DCC (1.06 g, 5.14 mmol) method. The final product Ma-Gly-DL-PheLeuGly-TT was purified by column chromatography using Kieselgel 60 (Merck) as a sorbent and ethyl acetate–acetone 3:1 as eluent. Yield: 0.954 g (39%). Purity (HPLC) >99.2%.

^{1}H NMR [$(CD_3)_2SO$]: δ 0.84 d, 3H (CH_3-Leu); 0.89 d, 3H (CH_3-Leu); 1.30–1.70 m, 3H ($CHCH_2$-Leu); 1.84 s, 3H (CH_3); 2.70–3.10 m, 2H (β-Phe); 3.45 t, 2H (CH_2S); 3.50–3.75 m, 2H (Gly); 4.15–4.35 m, 1H (α-Leu); 4.45–4.75 m, 5H (CH_2N, Gly, α-Phe); 5.35 s, 1H (CH_2=); 5.69 s, 1H (CH_2=); 7.20 m, 5H (arom.); 8.01 d, 1H (NH-Phe); 8.10 m, 2H (NH-Gly); 8.25 t, 1H (NH-Leu).

Purity of all the mentioned monomers was examined by HPLC (Shimadzu, Japan) using a reverse-phase column Chromolith Performance RP-18e 100-4.6 with UV detection at 230 nm, eluent water–acetonitrile with acetonitrile gradient 0–100 vol.%, flow rate 0.5 mL/min.

2.3. Synthesis of polymer precursors

Semitelechelic polymer precursor (polymer **1**, Table 1) containing Boc-protected hydrazide groups was prepared by radical precipitation copolymerization of HPMA with MA-ah-NHN-Boc in acetone performed in the presence of a chain transfer agent, SPA. HPMA (1.0 g, 7 mmol), Ma-AH-NHNH-Boc (170 mg, 0.56 mmol) and AIBN (11 mg, 0.067 mmol) were dissolved in acetone (9 mL) containing 11.6 μL SPA (14 mg, 0.13 mmol). The solution was placed in a polymerization ampoule, bubbled with nitrogen, and the ampoule was sealed. Polymerization was carried out for 22 h at 50 °C. The polymer precursor was purified by double precipitation from methanol solution into a twenty-fold excess of an acetone–diethyl ether mixture (3/1). The polymer was filtered off, washed with diethyl ether, and dried in vacuum. The yield was 530 mg (45.2%). The number-average molecular weight of polymer **1** was determined by the end COOH group titration with 0.05 M NaOH. The COOH group of the polymer was converted into the hydroxysuccinimide active ester (OSu) by the reaction with HOSu using the DCC method [30].