European Journal of Pharmaceutics and Biopharmaceutics 90 (2015) 90–96

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Comparison between linear and star-like HPMA conjugated pirarubicin (THP) in pharmacokinetics and antitumor activity in tumor bearing mice

Hideaki Nakamura [a,b], Eva Koziolová [c], Tomáš Etrych [c], Petr Chytil [c], Jun Fang [a,b], Karel Ulbrich [c], Hiroshi Maeda [a,*]

[a] Research Institute for Drug Delivery Science, Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan
[b] Laboratory of Microbiology and Oncology, Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan
[c] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 14 April 2014
Accepted in revised form 15 October 2014
Available online 8 November 2014

Keywords:
HPMA polymer conjugate
Pirarubicin (THP)
Acid-cleavable linkage
EPR effect
Dendrimer-derived polymer conjugate
Chemical carcinogenesis
Controlled drug release

ABSTRACT

Previously we showed that linear poly(*N*-(2-hydroxypropyl)methacrylamide) conjugates of pirarubicin (THP), LP-THP, with MW about 39 kDa, exhibited far better tumor accumulation and therapeutic effect than that of parental free THP. To improve the pharmacokinetics of LP-THP further, high-MW conjugate of poly(amido amine) (PAMAM) dendrimer grafted with semitelechelic HPMA copolymer (PHPMA) was synthesized [star polymer (SP); 400 kDa] and conjugated with THP via hydrazone bond-containing spacer (SP-THP). THP was conjugated to SP to form SP-THP via acid cleavable hydrazone bonding, which responds to acidic milieu of tumor tissue. As a consequence, it would release free THP, by active therapeutic principle. SP-THP exhibits larger hydrodynamic diameter (25.9 nm) in aqueous solution than that of LP-THP (8.2 nm) as observed by light scattering and size exclusion chromatography. Because of the larger size, the tumor AUC_{5h-72h} of SP-THP was 3.3 times higher than that of LP-THP. More importantly, released free THP was retained selectively in the tumor tissue for at least up to 72 h after administration of SP-THP. We found that SP-THP exhibited superior antitumor effect to LP-THP against both S-180 tumor-bearing mice *in vivo*, and with chemically AOM/DSS-induced colon tumor-bearing mice, most probably due to their different molecular size. In our comparison study of *in vitro* and *in vivo* behavior of SP-THP and LP-THP we concluded that SP-THP exhibited enhanced therapeutic efficacy not only in implanted tumor but also in orthotopic/spontaneous tumor despite its higher toxicity compared to LP-THP. Upon these findings further investigation using various tumors including transgenic, and metastatic tumors is going to be conducted soon.

1. Introduction

4′-O-tetrahydropyranyl doxorubicin (pirarubicin, or THP) is an anthracycline antibiotic used for treatment of various cancers in such organs as breast, head and neck, cervix, and lymphoma, etc. [1]. An intrinsic problem of low-MW anticancer drugs is also applicable to THP (MW 628); its body distribution is indiscriminate in all normal tissues and organs before tumor delivery. Thus adverse effects such as bone-marrow suppression, cardiac toxicity as well as others limit the usage of higher dose of THP in clinical setting. Thus improvement of pharmacokinetics, especially tumor selective delivery is the prime requirement.

Poly(*N*-(2-hydroxypropyl)methacrylamide (PHPMA) is highly water-soluble biocompatible macromolecule, namely nontoxic and non-immunogenic [2,3]. After intravenous (i.v.) injection, high-MW PHPMA (more than 40 kDa) is retained in the systemic circulation for longer time (>24 h) at significant concentration, thus it preferentially accumulates in the tumor tissue by enhanced permeability and retention (EPR) effect [4]. By conjugating low-MW antitumor drugs to PHPMA, the accumulation of the antitumor drug in tumor would be enhanced, and the therapeutic response would be improved.

To improve the tumor accumulation of PHPMA drug conjugates, molecular size of PHPMA may be increased; either by branching or

Abbreviations: THP, 4′-O-tetrahydropyranyl doxorubicin; DOX, doxorubicin; SP, star polymer; LP, linear polymer; AUC, area under the curve; AOM, azoxymethane; DSS, dextran sodium sulfate; HPMA, *N*-(2-hydroxyproyl)methacrylamide; EPR, enhanced permeability and retention; PEG, polyethylene glycol; PAMAM, polyamido amine; ABIC, 4,4′-azobis(4-cyanovaleric acid); DIPC, *N*,*N*′-diisopropylcarbodiimide; EDPA, *N*-ethyldiisopropylamine; TNBSA, 2,4,6-trinitrobenzene-1-sulfonic acid; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

* Corresponding author. Institute for DDS, Sojo University, Ikeda 4-22-1, Nishi-ku, Kumamoto 860-0082, Japan. Tel.: +81 96 326 4114; fax: +81 96 326 3185.
E-mail address: hirmaeda@ph.sojo-u.ac.jp (H. Maeda).

http://dx.doi.org/10.1016/j.ejpb.2014.10.007
0939-6411/

grafting HPMA copolymers or by their self-assembly to form high-MW micellar structures [5,6]. However, the synthesis of explicitly branched side chains or graft polymers in comb structure is relatively difficult to control; high polydispersity index and lower reproducibility become a concern. On the other hand, dendrimer is nearly monodisperse, and its surface can be freely and explicitly modified, e.g. by attaching semitelechelic PHPMA. Grafting of the dendrimer by PHPMA decreases the toxicity of the dendrimer as well. Along this line, we previously reported that star-like HPMA copolymer, PHPMA-modified PAMAM dendrimer (SP) conjugated with doxorubicin (DOX), showed superior tumor accumulation, and also antitumor activity to that of linear HPMA copolymer conjugates of DOX [6,7]. Although previous star polymer-DOX conjugate showed high effectivity *in vivo*, a problem was that free DOX liberated from the conjugate exhibited slow intracellular uptake into tumor cells compared to free THP. Namely, DOX required about 100 times longer uptake time, probably it only depended on free diffusion mechanism. Consequently, slow uptake by tumor cells would result in wash out from the tumor site back into systemic circulation, followed by excretion from the body. Therefore, THP polymer conjugate would offer more advantage in *in vivo* setting, but not as much in *in vitro* culture cell system, and thus we have undertaken the present study.

In these polymer conjugates we utilized biodegradable linkers to attach the drug to the polymer [8,9]. Advantage of using biodegradable linker is that the drug can be released from the polymer at or near the pathological region and then exhibits better therapeutic effect. One problem is connected with the use of some highly biocompatible polymer conjugates such as PEG or HPMA copolymer-conjugated drugs. They have lower intracellular uptake velocity than free drug, and consequently lower interaction with the target molecules resulting in lower therapeutic effect than one could expect [10–12]. Therefore, good release of low-MW drugs from the conjugates at tumor site is a critical issue for achieving the superior therapeutic effect, in order to exert drug action to the tumor [10]. For conjugating low-MW drugs, peptide, ester, disulfide and hydrazone bonds were used for conjugating the polymer and drugs [13,14]. These chemical bonds can be cleaved by proteases and esterases which exist more dominantly in the tumor tissue. In addition, more acidic pH favors the spontaneous cleavage of hydrazone bond. Therefore, we designed conjugates with pH-controlled drug release taking place preferentially at the tumor tissue and at pathological lesions.

Here we describe the synthesis of SP-THP designed for efficient tumor accumulation due to the EPR effect of its macromolecular nature and we present results of the biological evaluation of novel dendrimer attached star-like PHPMA copolymer conjugate of THP (SP-THP), in which surface of 2nd generation PAMAM (polyamido amine) dendrimer was grafted with chains of HPMA copolymers, which are conjugated with THP. For the detailed chemical structure of HPMA copolymer THP conjugate see [17]. THP was conjugated to the PHPMA via hydrazone bond, thus efficient drug release occur in the acidic environment of lysosomal pH and/or of tumor tissue. When we compared antitumor activity of SP-THP conjugate with linear HPMA copolymer-THP conjugate (LP-THP) using two types of mouse tumor models, implanted and chemically induced tumor, we found superior tumor accumulation of SP-THP, and thus better therapeutic effect.

2. Materials and methods

2.1. Materials

1-Aminopropan-2-ol, methacryloyl chloride, 4,4′-azobis (4-cyanovaleric acid) (ABIC), 6-aminohexanoic acid (ah), *N*,*N*′-dimethylformamide (DMF), *N*,*N*′-diisopropylcarbodiimide (DIPC), *N*-ethyldiisopropylamine (EDPA), dimethyl sulfoxide (DMSO), *tert*-butyl carbazate, trifluoroacetic acid (TFA) and 2,4,6-trinitrobenzene-1-sulfonic acid (TNBSA) were purchased from Sigma–Aldrich. Pirarubicin was purchased from Abbliss Chemicals, Houston, USA and poly(amido amine) (PAMAM) dendrimers with 1,4-diaminobutane core were purchased from Dendritic Nanotechnologies, Inc., Atlanta, USA. Male ddY mice and ICR mice were purchased from Kyudo Co., Ltd., Saga, Japan. Dulbecco-MEM (DMEM) was purchased from Nissui Seiyaku, Tokyo, Japan. Azoxymethane, dextran sodium sulfate, and reagent grade salts were purchased from Wako Pure Chemical Industry, Osaka, Japan. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1-Methoxy-5-methylphenazinium methylsulfate (WST-1) was purchased from Dojindo Chemical Laboratories, Kumamoto, Japan. Fetal calf serum was purchased from Nichirei Bioscience, Tokyo, Japan.

2.2. Synthesis of monomers

***N*-(2-Hydroxypropyl)methacrylamide** (HPMA) was synthesized as described in [15] using K_2CO_3 as a base. m.p. 70 °C; purity > 99.8% (HPLC); elemental analysis: calc., 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 [16]. 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. Purity of both monomers 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.%, flow rate, 0.5 mL/min, and ^{1}H NMR Bruker spectrometer (300 MHz).

2.3. Synthesis of star-like polymer precursor

The star polymer precursor was prepared by grafting PHPMA with terminal thiazolidine-2-thione (TT) chain end group onto the 2nd generation PAMAM dendrimers containing terminal amino groups as described recently [17]. Briefly: Poly(HPMA-co-methacryl-amidehexanoyl-NHNH-Boc) terminated with TT group (**copolymer I**) was prepared by radical solution copolymerization in DMSO initiated with azo-initiator ABIC-TT. Consequently, the star-like polymer precursor (MW 256,000, Section 2.1) was prepared by grafting the semitelechelic **copolymer I** onto PAMAM dendrimer (aminolysis of the copolymer TT group with amino groups of PAMAM in methanol). After 2 h, low-MW impurities were removed by gel filtration (Sephadex LH-20, solvent methanol) and the star hydrazide groups-containing polymer precursor (**copolymer II**) was isolated, after deprotection of the Boc protecting groups with concentrated TFA, by precipitation in ethyl acetate.

2.4. Synthesis of star polymer conjugate

The star polymer conjugate with pirarubicin attached via a pH-sensitive hydrazone bond was prepared by the reaction of the hydrazide groups-containing **copolymer II** with pirarubicin in methanol in the dark. After 18 h the conversion reached 98% (determined by HPLC in methanol/aqueous buffer solvent as described below) and the star polymer-drug conjugate was purified from low-MW impurities (pirarubicin or its degradation products) by precipitation into ethylacetate. MW = 400,000 g/mol; I_n = 2.3, content of THP = 10.9 wt.%; R_h = 13 nm. LP-THP was synthesized as describe before [17].