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Two step mechanisms of tumor selective delivery of *N*-(2-hydroxypropyl)methacrylamide copolymer conjugated with pirarubicin via an acid-cleavable linkage

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ABSTRACT

N-(2-Hydroxypropyl)methacrylamide copolymer containing hydrazide groups (PHPMA) conjugated with pirarubicin (THP) via a hydrazone bond (PHPMA-hyd-THP) is a drug conjugate that releases THP in the acidic milieu of a tumor. PHPMA-hyd-THP has an apparent Mw of 40,000 and a hydrodynamic diameter of 8.2 ± 1.7 nm but no apparent plasma protein binding. PHPMA-hyd-THP possesses two mechanisms of selectivity toward solid tumors and has potent antitumor action. The first one is drug accumulation in tumors that depends on the enhanced permeability and retention (EPR) effect, which results in a 4–20 times higher concentration of drug in the tumor than in normal tissues such as the heart, lung, and intestine. This accumulation in tumor tissue is in great contrast to that of conventional low-Mw THP. The second one is pH-dependent release of drug from PHPMA-hyd-THP: this conjugate released free THP more efficiently at a lower pH, which exists in tumors, and exerts cytotoxic activity. Free THP is known for its much faster uptake into tumor cells compared with doxorubicin. Thus, in our in vitro study, PHPMA-hyd-THP showed a higher cytotoxicity at the lower pH of tumor tissue than at the neutral pH of normal tissue. Furthermore, much more THP was liberated from the conjugate in acidic tumor tissue than in normal tissue. The EPR effect-dependent accumulation of PHPMA-hyd-THP and tumor-selective THP release in the tumor tissues led to highly tumor-selective drug accumulation, which continued for more than 72 h, whereas the lowest free drug concentration was detected in normal tissues at 24 h and no longer at a later time. In conclusion, we determined in our study here that the acid-cleavable PHPMA-hyd-THP conjugate had an excellent antitumor effect without appreciable adverse effects.

1. Introduction

The tumor-targeting principle of the enhanced permeability and retention (EPR) effect of macromolecules in solid tumor was originally described by Matsumura and Maeda in 1986 [1]. The EPR effect is attributed to (i) the aberrant architecture of tumor blood vessels, (ii) the elevated production of various vascular permeability factors, and (iii) the lack of functional lymphatic drainage in tumor tissue [1–3]. These tumor-specific features lead to the accumulation of biocompatible macromolecules in tumor tissue, which results in a superior antitumor effect with far fewer adverse effects. Recent progress in designing nanomedicines with antitumor effects has utilized the EPR effect as a prime principle [4,5]. For macromolecular drug carriers, e.g. liposome, polyethylene glycol, and poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) conjugates were widely used. PHPMA is nontoxic, non-immunogenic, and ratio of drug to PHPMA can be easily controlled. High Mw PHPMA (more than 40 kDa) remains in the systemic circulation longer, thus it preferentially accumulates in the tumor tissue by enhanced permeability and retention (EPR) effect [6]. Therefore, conjugation of the antitumor drug to the PHPMA enhances the tumor accumulation property of the antitumor drug.

During the past decade, many extensive studies failed to determine the drug characteristics required for selective delivery of the drugs to tumors and for the optimal therapeutic effect. For instance, despite marked accumulation of macromolecular or liposomal antitumor drugs in tumors, many cases showed insufficient antitumor effects [7,8]. This finding may be attributed to inadequate release of drugs at the tumor site and/or poor intracellular uptake of macromolecular drugs even when the drug was delivered to the tumor tissue. In nanomicelles or liposomal drugs, an active pharmaceutical drug is encapsulated in the core of the micelles and must be released from the nanoparticles to exert its therapeutic effect [7,9]. However, these micelles or macromolecular drugs require adequate stability when they exist in the systemic circulation. Thus, to achieve a superior EPR effect,

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the plasma half-life of these macromolecular drugs must be several hours or days. Solving these difficult requirements continues to be a great challenge and has involved various cancer-specific approaches utilizing proteases, lower pH, redox potential, and external stimuli such as heat, ultrasound, and light for tumor-selective drug delivery [8,10].

For this study, we chose a pH-sensitive linker to attach drugs to polymers as a promising strategy for the efficient release of antitumor drugs from macromolecules at tumor sites. Cleavage and release of drugs must proceed in lysosomes, with a pH of 5.0–5.5, and in the tumor tissue (milieu), which has an intrinsic pH of 6.5–6.9. This pH clearly differs from the neutral pH of 7.4–7.6 in blood or normal interstitial tissue [11–13]. We report here the development of pH-sensitive water-soluble polymer-drug conjugates containing a hydrazone bond [14] that linked THP and poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA), that was effectively cleaved at the acidic tumor pH of 6.5–6.9, and that liberated free THP. We showed in an earlier study that an acid-cleavable macromolecular drug with doxorubicin attached via a hydrazone bond had a much higher cytotoxic effect compared with the noncleavable drug [15]. Rihova et al. reported that PHPMA conjugated to doxorubicin via a hydrazone linkage showed pronounced antiproliferative activity in K562 cells with a limited lysosome content [13]. Moreover, an acid-cleavable hydrazone conjugate showed superior cytotoxicity compared with the lysosomal protease-degradable peptide conjugate in an in vitro cytotoxicity assay [15,16]. Yang et al. previously reported that endocytosis, in folate receptor-mediated endocytosis, was not sufficient to cleave the hydrazone linkage in KB cells [17]. These results suggest that acid-cleavable conjugates may be partly cleaved outside the cells and that released free drug is internalized into the cells, which leads to cell death. Related to these results, the lower pH of tumor tissue (6.5–6.9), in contrast to the pH of normal tissue (7.4–7.6), will facilitate cleavage of the hydrazone bond.

4′-*O*-Tetrahydropyranyldoxorubicin (pirarubicin, or THP), used in this study, is an anthracycline antibiotic originally discovered by Umezawa et al. [18]. THP is approved in Japan for treatment of various cancers such as breast cancer, head and neck cancer, uterine cancer, leukemia, and lymphoma. Despite its superior antitumor effect, THP, as with other low-Mw anticancer agents, widely distributed throughout the body, thus causes adverse effects such as bone marrow suppression and myocardial infarction. Thus, improved control of the body distribution of THP is essential.

An important reason for choosing THP, rather than other anthracycline antibiotics such as doxorubicin, as the conjugation drug is that free THP has an intracellular uptake that is about 100 times faster than that of other drugs [19]. After free THP is delivered to the area around tumor cells, the cells internalize it very quickly, and it exerts rapid potent cytotoxic effects—a clear advantage. The slower cell uptake of doxorubicin, in contrast, may cause diffusion into the systemic circulation and result in toxicity in the normal tissue.

We expected this pH-sensitive polymer-conjugated drug to accumulate in tumor tissue via the EPR effect and to release free THP selectively in the tumor tissue outside and/or inside the cells, but not much would be released in normal tissues with pH values at or above 7.0. This means that the THP-polymer conjugate with the hydrazone bond to PHPMA (PHPMA-hyd-THP) would confer two types of tumor selectivity, one via the EPR effect and the other via the lower pH environment of tumor tissue. The result would be excellent antitumor activity with fewer adverse effects.

2. Materials and methods

2.1. Materials

Male ddY mice were purchased from Kyudo Co., Ltd., Saga, Japan. Dulbecco-MEM (DMEM) was purchased from Nissui Seiyaku, Tokyo, Japan. Reagent-grade salts and solvents were purchased from Wako Pure Chemical Industry, Osaka, Japan. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo Chemical Laboratories, Kumamoto, Japan. Fetal bovine serum was purchased from Nichirei Bioscience, Tokyo, Japan.

2.2. Synthesis of PHPMA-hyd-THP

PHPMA-hyd-THP was synthesized as described in a previous paper but with pirarubicin instead of doxorubicin [13].

2.3. High-performance liquid chromatography (HPLC)

Gel permeation chromatography was performed with a Shodex multimode size exclusion column, Asahipak GF-310 HQ (Showa Denko, Tokyo, Japan) (300 mm × 7.5 mm), with photodiode array detection at 488 nm; elution was with dimethylformamide at 0.5 ml/min. Size exclusion chromatography was performed by using a OHpak SB-804 HQ column (Showa Denko, Tokyo, Japan) (300 mm × 8.0 mm), with photodiode array detection at 488 nm and 280 nm; elution was with 0.01 M phosphate-buffered 0.15 M saline (PBS) at 0.5 ml/min. Molecular weight and polydispersity of the PHPMA-hyd-THP conjugate was determined with a Shimadzu HPLC system equipped with RI, UV and multiangle light scattering DAWN EOS (Wyatt Co., USA) detectors using a TSKgel G3000SWXL column (300 × 7.8 mm, 5 μm) with a mobile phase consisting of 20% 0.3 M acetate buffer (pH 6.5; 0.5 g/L NaN_3) and 80% methanol at a 0.5 ml/min flow rate.

2.4. Dynamic light scattering and zeta potential

The polymer conjugate PHPMA-hyd-THP was dissolved in PBS at 1 mg/ml and was filtered through a 0.2-μm filter attached to a syringe before analysis at room temperature. The particle size and surface charge (zeta potential) were measured in PBS by using the ELS-Z2 dynamic light scattering instrument (Otsuka Photal Electronics Co. Ltd., Osaka).

2.5. Assay to measure release of free THP

PHPMA-hyd-THP was dissolved in 0.1 M sodium acetate buffer (pH 5.5) or in 0.2 M phosphate buffer (pH 6.8 or 7.4) and was then incubated at 37 °C for the indicated time periods. To quantify the released free THP from the conjugates, an aliquot of the buffered solutions mentioned above was added with an equal volume of 0.2 M sodium bicarbonate buffer (pH 9.8) and two times the volume of chloroform followed by vortexing to extract the free THP in the chloroform phase. The chloroform phase was then evaporated to dryness in vacuo, and the pellet was dissolved in acetonitrile. The amount of THP was quantified by means of HPLC.

2.6. Cytotoxicity assay

HeLa cells (human cervical carcinoma) or B16-F10 cells (mouse melanoma) were maintained in DMEM supplemented with 10% fetal calf serum with 5% CO_2/air at 37 °C. To prepare the types of DMEM with different pH values (pH 7.4, 6.9, and 6.5), $NaHCO_3$ was added to DMEM (pH 6.5, 0.5 g/l; pH 6.9, 1.0 g/l; pH 7.4, 3.7 g/l). HeLa cells were then incubated with PHPMA-hyd-THP or free THP for 72 h at different pHs. The MTT assay was performed to quantify the cytotoxicity, with absorbance at 570 nm as usual.

2.7. Analysis of intracellular uptake of THP

HeLa cells were treated with 100 μg/ml THP-equivalent dose of PHPMA-hyd-THP for the indicated time periods. After treatment, cells were washed with PBS and were resuspended in PBS followed by sonication (Hielscher, Teltow, Germany) (30 W, 30 s). To measure the total