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Intracellular Delivery of Mitomycin C with Targeted Polysaccharide Conjugates Against Multidrug Resistance

YU CAO,[1,2] DIDI CHEN,[1] PEIGUANG ZHAO,[1] LINA LIU,[1] XUEYING HUANG,[1] CHAO QI,[2] YANLI LIU,[2] HONGXUAN HE,[3] QIAN WANG,[1] YANG LIU,[1] and SHA CHEN[1]

[1]Key Laboratory of Pesticide and Chemical Biology (Ministry of Education), College of Chemistry, Central China Normal University, Wuhan 430079, People's Republic of China; [2]Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Science, Central China Normal University, Wuhan 430079, People's Republic of China; and [3]National Research Center for Wildlife Born Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

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Abstract—Intracellular targeted conjugates of xyloglucan and mitomycin C (MMC) were synthesized with a lysosomally degradable peptide spacer and galactosamine, a terminal moiety that can be used to target polymeric conjugates to hepatoma. The content of the MMC was about 3.5% (mol) in this conjugate. In an *in vitro* cytotoxicity experiment, the targeted prodrugs have higher cytotoxicity than free MMC against the drug resistant HepG2 cells. In a human tumor xenograft nude mouse model, the targeted prodrugs generated higher therapeutic effect than non-targeted prodrugs or free MMC. Together, these results suggest that targeted prodrugs, which have improved transfer efficiency and hepatocyte specificity, may be useful for the reversion of drug resistant HepG2 cells.

Keywords—Intracellular drug delivery, Polymeric conjugates, Hepatoma-targeting, Drug resistance.

INTRODUCTION

Hepatoma, which is the fifth most common solid tumor and the third leading cause of cancer deaths worldwide, affects more than half a million individuals per year worldwide and is responsible for over one million deaths annually worldwide.[21,22] Hepatoma is limitedly optionally treated and often display multidrug resistance, which may help to explain why so many of the clinical trials using standard cytotoxic drug therapy have been disappointing.[21] Multiple changes, such as the overexpression of P-glycoprotein (P-gp) and the alterations in lipid metabolism, often appear simultaneously in highly resistant hepatoma cell lines, consequently influencing drug uptake. This phenotype of tumor cells is mainly associated with the overexpression of a P-gp, which is a member of the ATP-binding-cassette (ABC) superfamily of membrane transporters and is encoded by the multidrug resistance (MDR) gene. It has been well recognized that P-gp is an energy-dependent drug efflux pump. A wide variety of structurally and functionally unrelated anti-cancer agents, such as adriamycin, vinblastine, teniposide, paclitaxel, and mitomycin C (MMC), have been proven to be the drugs transported by P-gp.[21,22] Therefore, anticancer drugs, even if they are located in the tumoral interstitium, can have limited efficacy against numerous tumor types.

Other major problem for treatment is the inability to deliver therapeutics to all regions of a tumor in therapeutically effective quantities without causing undue toxicity, which is able to be solved by drug delivery systems (DDS). Polymeric conjugates for DDS have attracted interest for stepping over the two obstacles mentioned above and other advantages, such as better bioavailability for poorly soluble drugs, a wide potential application spectrum (oral, dermal, and intravenous), and protection of sensitive drug molecules from the environment (water and light).[15,17] Polymeric conjugates provide a better penetration of the particles inside the body as their size allows delivery via intravenous injection or other routes than that of nanoparticle DDS.[17,18,20]

Address correspondence to Yu Cao, Key Laboratory of Pesticide and Chemical Biology (Ministry of Education), College of Chemistry, Central China Normal University, Wuhan 430079, People's Republic of China and Hongxuan He, National Research Center for Wildlife Born Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China. Electronic mail: caoyu@iccas.ac.cn, hehx@ioz.ac.cn

In recent years, the peculiar physicochemical and biological properties of natural and semisynthetic polysaccharides have been exploited to develop polymeric conjugates which can exhibit favorable biopharmaceutical properties and enforce the therapeutic performance of the parent drugs.[2,13] For the application of polysaccharides for drug carriers, issues of safety, toxicity, and availability are greatly simplified. Polysaccharide-based DDS can be designed to endow derivatives with new bioresponse, targeting, or environmental triggering properties. Xyloglucan, as a kind of polysaccharide, consists of 1,4-linked β-D-glucopyranose residues as in cellulose. The glucose in the backbone is partially substituted at the O-6 position of glucopyranosyl residues with α-D-xylopyranose. Some of the xylose residues are β-D-galactosylated at O-2. So far, xyloglucan has been widely used in pharmaceutical technology as the excipient for production of traditional formulations, because it is a water-soluble, biodegradable, and non-antigenetic natural polysaccharide. In particular, the hydroxyls of xyloglucan are anchoring points for conjugation of several anticancer drugs and/or the targeting unit, which usually suffer from poor physicochemical, biopharmaceutical, and therapeutic properties that limit their therapeutic performance and proper use in anticancer treatment.[2,13,19]

However, ideal DDS is able to direct drugs at tumor cells and to control the drug release. The aim of active targeting of internalization-prone cell-surface receptors is to improve the cellular uptake of the DDS and reduce the side effects, especially when dealing with drugs that have peripheral toxicity.[4,9,10,16] The peripheral toxicity limits the clinical use of the drugs such as MMC, which shows a valuable broad spectrum of antitumor activity. Tumor-targeting polymeric conjugates assist tumor cells in the uptake of the drug by receptor-mediated endocytosis, which is a promising approach to selective drug delivery. The asialoglycoprotein (ASGP) receptor is a well-characterized molecular target expressed on the cell surface of hepatocytes and hepatomas, which can recognize galactose or N-acetylgalactosamine residues of desialylated glycoproteins and, therefore, can be exploited for liver-specific drug delivery. The DDS with galactose residues can be targeted specifically to hepatocytes by ASGPR on cell surfaces and are delivered by receptor-mediated endocytosis into the cells, where the parent drug molecules are liberated. Delivery of drugs using substances bound to appropriate glycoproteins, lactose, or galactose in a specific manner would provide significant therapeutic benefits in hepatic disease.[5,6,12] By the means of targeting DDS, MDR may be overcome by increasing anticancer drugs based only on mass action. Polymeric conjugates, as nanomedicines, gain access to the tumor interstitium through the leaky tumor vasculature. The primary mechanism for the carrier method relies on diffusion, rather than convection.

The drug is fixed via a spacer group onto the polymer backbone. Drug release can be controlled by the selection of a polymer–drug spacer offering more stability in the bloodstream with degradation in cancer cells. Targeted polymeric conjugates are transferred into the endosomal compartment of the cell, then directed into a secondary lysosomal compartment which contains a variety of lysosomal enzymes, which can degrade oligopeptide spacer.[1,3,7,8] The release rates were significantly controlled by the length of the oligopeptide spacer and the specific sequence of the oligopeptide in polymeric conjugates. Because the tripeptide Gly-Leu-Gly possesses the essential requisite that it can be degraded by lysosomal enzymes, it was used in this study as the spacer for drug binding.[1,3]

The present study aimed at design and development of MMC conjugating xyloglucan anchored with galactosamine by the spacer of tripeptide Gly-Leu-Gly. The cytotoxicity effect of the polymeric conjugates against the drug resistant variants of hepatoma cells (HepG2/DR) and the therapeutic effect on HepG2/DR tumor cells implanted in mice were also investigated.

MATERIALS AND METHODS

Materials

Xyloglucan were prepared from tamarind seed powder. About 2 wt% of tamarind kernel powder solution was prepared in distilled water. The solution was mechanically stirred in a boiling water bath for 2 h. The resulting thin clear solution was kept overnight, and the water-insoluble protein fraction was removed by centrifugation at 5000 rpm for 30 min, which was followed by suction filtration with a sand-core funnel (G4, pore size, 5–15 μm). The filtrate solution was lyophilized to obtain pure XG for further experiments. The molecular weight of xyloglucan was about 700 kDa by the measurement of GPC (Prominence GPC, Shimadzu Co., Japan).

Mitomycin C (MMC) was purchased from Chuangcheng Pharmaceutical (Wuhan) Ltd., China. *N*-t-Boc-glycyl-L-leucyl-glycine *N*-hydroxysuccinimide ester (Boc-Gly-Leu-Gly-OSu) were obtained from GL Biochem (Shanghai) Ltd., China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), collagenase IV were purchased from the Sigma-Aldrich Co, USA. All other chemicals were analytical grade unless otherwise stated. Organic solvents were purified and dried through common standard methods.

Preparation of Peptide-MMC Derivatives

0.44 g of Boc-Gly-Leu-Gly-OSu (1 mmol) and 0.37 g of MMC (1.1 mmol) were dissolved in 20 mL dry DMF, and 0.22 g of diethylphosphoryl cyanide (DEPC) was added with stirring. 0.15 mL triethylamine (TEA) was added at 0 °C and stirred for 0.5 h. After overnight reaction in the dark at room temperature, the solvent was evaporated under vacuum, and ethyl acetate was added to dissolve the dry residue. The reaction mixture was extracted with a 10% citric acid solution (3 × 5 mL) and saturated sodium bicarbonate (3 × 5 mL). The organic layer was isolated and the water layer extracted with ethyl acetate (2 × 5 mL). Ethyl acetate extracts were evaporated to dryness in vacuum, and the residue was purified by column chromatography on silica (eluent: $CHCl_3$/MeOH, 9/1). The selected fraction was dried over $MgSO_4$. After removal of the solvent, the Boc-Gly-Leu-Gly-MMC derivative was finally obtained as a blue solid.

0.1 g of Boc-Gly-Leu-Gly-MMC was dissolved in 2 mL DMF, 0.2 mL of trifluoroacetic acid (TFA) was added, and the reaction was conducted under stirring at room temperature for 1 h.[3,8] The solvent was evaporated under vacuum. The residue was dissolved in 5 mL methanol, and the solution was filtered. The Gly-Leu-Gly-MMC (1) conjugate was finally obtained after evaporation of the solvent.

Preparation of Peptide-Galactosamine Derivatives

0.24 g of galactosamine hydrochloride (1.1 mmol) was added to the solution of 0.44 g of Boc-Gly-Leu-Gly-OSu (1 mmol) in 20 mL dry DMF, and 0.22 g of DEPC was added with stirring in ice bath. 0.15 mL TEA was added to the reaction mixture and stirred overnight in the dark at room temperature. DMF was evaporated under vacuum, and ethyl acetate was added to dissolve the dry residue. The reaction mixture was extracted with a 10% citric acid solution (3 × 5 mL) and then extracted with saturated sodium bicarbonate (3 × 5 mL). The organic layer was isolated and the water layer extracted with ethyl acetate (2 × 5 mL). All ethyl acetate extracts were washed with water and then dried with MgSO4. Ethyl acetate was removed in vacuum, and oily Boc-Gly-Leu-Gly-Gal derivative was finally obtained.

0.5 g of Boc-Gly-Leu-Gly-Gal was placed into a 10-mL round-bottomed flask. 1 mL of TFA was added, and the reaction was conducted under stirring at room temperature for 1 h. The mixture was evaporated *in vacuo* to produce the oily glycyl-L-leucyl-glycine-galactosamine (Gly-Leu-Gly-Gal 2).

4-Nitrophenyl Chloroformate Activation of Xyloglucan

Xyloglucan (2 g, 12.3 mmol unites) and 4-dimethylaminopyridine (DMAP) (0.15 g, 1.2 mmol) were dissolved in 20 mL of DMSO/pyridine solution (vol. ratio 1/1). 4-Nitrophenyl chloroformate (0.9 g, 4.4 mmol) was added at 0 °C. After 4 h reaction at 0 °C, the reaction mixture was precipitated in an anhydrous ethanol. A white precipitate was collected and washed repeatedly with the same solvent. The xyloglucan-$COO(C_6H_4)NO_2$ was finally dried *in vacuo*. The carbonate content was determined by UV analysis after activated xyloglucan hydrolysis in NaOH.

Preparation of the Xyloglucan-Gly-Leu-Gly-MMC Conjugates[3,8]

2 g of xyloglucan-$COO(C_6H_4)NO_2$ (1.3 mmol reactive groups) and 2 g of Gly-Leu-Gly-MMC (1.2 mmol) were dissolved in dry DMSO and then TEA (0.1 mL) was added. After 48 h of reaction in the dark, the conjugate was separated by precipitation in anhydrous ethanol. The product was washed and dried. Finally, the conjugate was purified by preparative GPC (Sephadex G25) with water as eluent and freeze-drying. The degree of MMC substitution in the conjugates was determined by UV analysis in water ($\lambda = 364$ nm).

Synthesis of the Galactosylated Xyloglucan-MMC (Gal-XG-MMC) Conjugate

For the conjugation of MMC and galactosamine to xyloglucan, xyloglucan-$COO(C_6H_4)NO_2$ (4 g, 2.6 mmol reactive groups), Gly-Leu-Gly-MMC (2.4 g, 1.4 mmol), and TEA (0.2 mL) have been mixed and stirred in 200 mL of dry DMSO for 6 h, gly-phe-gly-galactosamine (0.53 g, 1.2 mmol) and TEA (0.1 mL) was then added. The solution was under reaction by stirring for 3 days at room temperature. The reaction mixture was slowly dropped into anhydrous ethanol to obtain precipitate. The product was washed with a large excess of ethanol and evaporated to dryness *in vacuo*.

In Vitro Release of MMC from the Conjugates

The release study was conducted in serum, phosphate buffered saline (PBS, pH 7.4) and incubation buffer with collagenase IV (0.3 mg/mL) at 37 °C with moderate stirring.[8] The xyloglucan-MMC conjugate (1 mg/mL) was transferred in 10 mL of PBS to a dialysis tube. At different time points, the samples were analyzed by HPLC with a Shimadzu HPLC system consisted of two pumps (LC-10Avp and LC-10AS) and a SPD-10Avp ultraviolet detector (Shimadzu

Corporation, Japan) in reverse phase mode. An Extend-C18 column (4.6 × 250 mm I.D., 5 μm) was used, and the mobile phase consisted of a phosphate buffer–acetonitrile mixture (85:15) at a flow rate of 1 mL/min. The amount of MMC in the solution was determined by UV spectroscopy at 360 nm.

In Vitro Cytotoxicity Assay

The cytotoxicity of the samples was measured against human hepatoma cell line (HepG2) with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenytetrazolium) assay.[11] HepG2 cells were grown and maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum. The drug resistant HepG2 cell line was developed from HepG2 cells incubated with MMC in a stepwise increasing concentration (from 0.01 to 2 μg/mL) during a few months. The resistant cells were selected by removing the dead non-resistant cells. The drug resistance was maintained by culturing the cells at 1 μg/mL MMC. Fifty microliters of the cell suspension of 1.8×10^5 cells/mL were seeded into wells of a 96-well plate. Cells were treated with various concentrations of MMC and conjugates for 48 h at 37 °C. Cell number was measured by the MTT cell viability assay.

Safety Assessment of the Conjugates in Normal Mice

Balb/c male mice aged 4 weeks were injected at doses of 8.0, 16.0, and 24.0 mg (MMC eq.)/kg every week for four doses (days 1, 7, 14, and 21), respectively ($n = 10$ mice/group). Toxic symptoms and mortality were recorded thereafter for next 2 weeks. At the end of 5-week, blood was collected and liver samples were harvested and weighed. Serum was tested for alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), and lactate dehydrogenase (LDH) with enzymatic reagent kits.

Drug Biodistribution

Specific pathogen-free grade male BALB/c nude mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences; 4 weeks old, 20–25 g) were accommodated in a pathogen-free laboratory environment throughout the experiments and cared in accordance with the "Guidelines for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication No. 85-23, revised 1985). 10^7 drug resistant HepG2 cells in 0.1 mL RPMI-1640 medium were injected subcutaneously in BALB/c/nu mice. After 3 weeks solid tumor growth was noticeable established in most mice, a total of 20 mice bearing tumor were divided into 2 groups, with 10 mice per group. Each group was received a single i.v. dose of the following formulations on day 10 from tumor inoculation: Gal-XG-MMC conjugate or free MMC (equivalent dose of MMC = 8 mg/kg). At various time points after injection ($n = 10$ at each time point), whole blood was collected from tail veins, and plasma was prepared by centrifugation at 1200*g* for 5 min. At 6 h after the injections, the mice were killed by cervical dislocation. The livers, hearts, tumors, spleens, and kidneys of the mice were immediately removed and washed with 10 mM Na_2HPO_4 buffer and then homogenized with triple volume of ethylacetate solvent. MMC was extracted by incubation with acidic isopropanol (81 mM HCl in isopropanol) for 4 h at 4 °C. The mixture was shaken on a vortex mixer for 1 min and centrifuged at 1200*g* for 15 min. After centrifugation, MMC concentration in the superstratum layer was determined by HPLC. Free MMC or released MMC was extracted and determined without incubation by HPLC as described previously.[3]

In Vivo Cytotoxicity of Xyloglucan-MMC Conjugates Against Drug Resistant HepG2 Cells in Mice

Specific pathogen-free grade male BALB/c/nu naked mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences; 4 weeks old, 20–30 g) were inoculated subcutaneously with drug resistant HepG2 cells (1×10^7 cells/animal). After 3 weeks solid tumor growth was noticeable established in most mice, Gal-XG-MMC conjugate, xyloglucan-MMC conjugate, or free MMC (equivalent dose of MMC = 8 mg/kg) suspended in PBS were injected to tail veins of animals every week for four doses (days 1, 7, 14, and 21). A major axis and a minor axis of tumors were measured using the calipers. Tumor volume was then determined. The survival time and number of long-term survivors (LTS) until day 50 were monitored.

Statistical Analysis

Data was expressed as means ± standard deviations (SD) of multi replicated determinations. Results were analyzed by one-way analysis of variance (ANOVA) with the Student–Newman–Keuls multiple comparisons or *t* test when comparing the differences between the means of two groups at the same time point. Differences were considered to be statistically significant if $p < 0.05$.

RESULTS

Preparation and Characterization of the Gal-XG-MMC Conjugate

As a polymeric carrier in DDS, xyloglucan is a water-soluble, biodegradable, and non-antigenetic

natural polysaccharide. However, the hydroxyls of polymer cannot connect with drug directly, which is required to replace with modifiable carboxylic derivatives before being coupled with the drug. In this study, the 4-nitrophenyl chloroformate activation is selected for the activation of the hydroxyl groups of xyloglucan, in order to introduce the prepared peptide-MMC derivatives into the polymeric carrier. The absorption of the carbonate residue was determined by UV–visible spectroscopy at 402 nm. The amount of carbonate in xyloglucan, calculated with Beer's law, was about 12 mol%. As an alkylating cytostatic antibiotic, mitomycin C is one of the most powerful antitumor drugs in the field of cancer chemotherapy and has a broad spectrum of activity against several solid tumors of different organs. However, its use in chemotherapy is limited by the manifestation of severe bone marrow depression and gastrointestinal damage. The amino groups of MMC were highly reactive and suitable for grafting the drug to carriers by amide bond in order to overcome these side effects and to enhance the therapeutic efficiency. The attachment of MMC and galactosamine to the xyloglucan was accomplished using tripeptide glycyl-L-leucyl-glycine as spacer. In Fig. 1, the strong absorptions of the methyl and methylene groups of xyloglucan between 2850 and 2930 cm^{-1} are different to the absorption peaks groups of the Gal-XG-MMC conjugate due to the tripeptide spacer residue. The peaks about 1660 and 1550 cm^{-1} might be the absorption of the amides groups in the tripeptide spacer and the conjugated MMC.

The content of MMC, determined by detection and calculation via UV spectra, was about 3.5% (mol). The drug load in the polymeric conjugate is relative low, which is major limitation of the application about the peptide spacer.

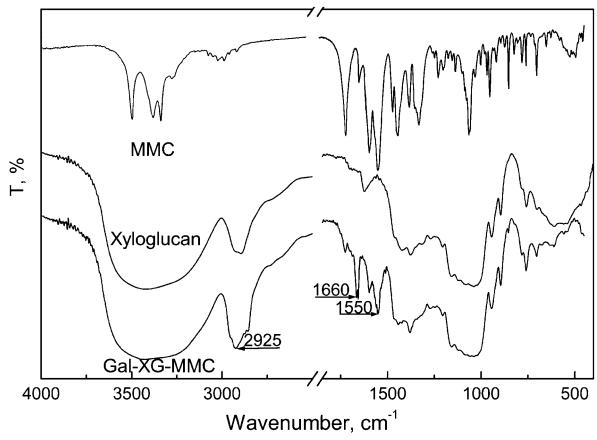


FIGURE 1. FTIR spectra of mitomycin C (MMC), xyloglucan (XG), and galactosylated xyloglucan-MMC (Gal-XG-MMC) conjugate.

The molecular weight of the conjugate was about 630 kDa and its polydispersity was 1.4 by the measurement of GPC (Prominence GPC, Shimadzu Co., Japan). The conjugates had a mean diameter of 36.5 nm by the measurement of dynamic light scattering (Zetasizer Nano 90, Malvern Instruments LTD, UK).

Release of MMC from the Gal-XG-MMC Conjugate

The hydrolytic stability of the conjugates was tested in serum or buffers both at extracellular and lysosomal pH. The hydrolytic release of MMC can be controlled by the amide groups in the spacer at lysosomal pH 5.5. When treated with PBS at pH 7.4, the release of MMC was almost negligible. The stability of the conjugates in serum indicates that the conjugates are stable during plasma circulation. The rate of MMC release in native serum was always higher than in buffer pH 7.4. This may indicate the presence of little peptidase activity in serum that can affect the release of MMC.

The *in vitro* release behaviors of MMC from Gal-XG-MMC conjugate were examined by incubating the conjugate with collagenase IV (or PBS) at 37 °C, and the result was displayed in Fig. 2. As time proceeded, MMC was released under treatment with collagenase IV; the total release was approximately 57% at 8 h and then the release of MMC slowed down. Half-maximal release time ($t_{1/2}$) that freed 50% of loaded MMC from conjugates was 8.5 h in buffer with collagenase IV. In comparison with the release from the conjugate incubated with collagenase IV, the release from the conjugate treated with buffer at pH 5.5 was much slower and the apparent release did not exceed 30% after 36 h.

In Vitro Cytotoxicity of Gal-XG-MMC Conjugate Against Tumor Cells

The cytotoxicity of Gal-XG-MMC conjugate compared with that of free MMC and xyloglucan-MMC

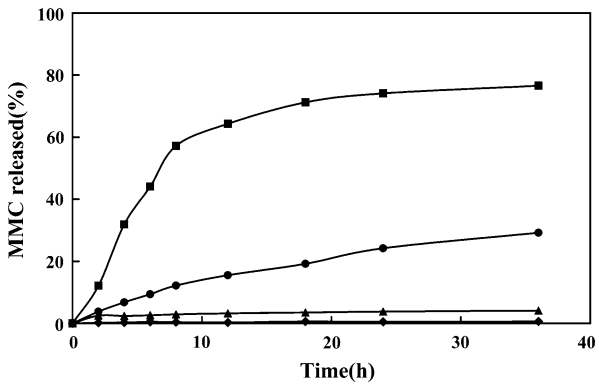


FIGURE 2. Release profiles of galactosylated xyloglucan-MMC conjugate [incubating with collagenase IV (■), pH 5.5 buffer (●), serum (▲), pH 7.4 buffer (♦)].

conjugate was determined by the cell growth inhibition assay of the drug resistant HepG2 cells. The results for MTT assay are shown in Fig. 3. The MMC formulated in conjugate showed equivalent or better effects against the cancer cells than free MMC, while Gal-XG-MMC conjugate achieved even better therapeutic effect than xyloglucan-MMC conjugate and free MMC. Compared with targeting conjugate, xyloglucan-MMC conjugate shows low cytotoxicity against the drug resistant HepG2 cells in experiment concentration. The MMC concentrations that kill 50% of cells were measured as 0.328 and 3.29 μg/mL for Gal-XG-MMC conjugate and xyloglucan-MMC conjugate, respectively.

Toxicological Study

Administration of Gal-XG-MMC conjugate caused no mortality at all doses, indicating that the median lethal dose (LD50) was up to 57.3 mg (MMC eq.)/kg. Compared with MMC (LD50 of MMC: 13.4 mg/kg), the safety effect of Gal-XG-MMC was improved.

An important indicator of a non-specific toxic effect following treatments with anticancer chemotherapy is body weight decrease. Therefore, we monitored the mice body weights following treatments with the conjugate. Mice treated with the conjugate did not produce any observable side effect and gained weight similar to the control group. Their body weight increased during the course of the treatment.

The alleviative effect of the conjugates on heart was further supported by that no significant increase of the CK or LDH enzyme level happened at all doses (Table 1). Hepatotoxicity of the conjugates was evaluated by the serum biochemical parameters and relative liver weight reported in Table 1. The conjugate did not produce significant change in AST, ALT, LDH, and liver weight, even though the doses used were as high as 24.0 mg (MMC eq.)/kg for continuously four times.

In Vivo Pharmacokinetic Studies of Free MMC and Gal-XG-MMC Conjugate in Tumor-Bearing Mice

In plasma, Gal-XG-MMC administration produced a little more total DOX concentration than that of XG-MMC, but much higher than that of free MMC which was rapidly eliminated from the circulation (Fig. 4). Formulating MMC in conjugate extended its retention in circulation, substantially by the Gal-XG-MMC conjugate (Fig. 4). The area under the concentration versus time curve (AUC) ratios Gal-XG-MMC/free MMC was over 14.

As expected, compared to free drug, conjugation in the Gal-XG-MMC lowered MMC uptake significantly in heart, lung, kidney, and spleen, and the released

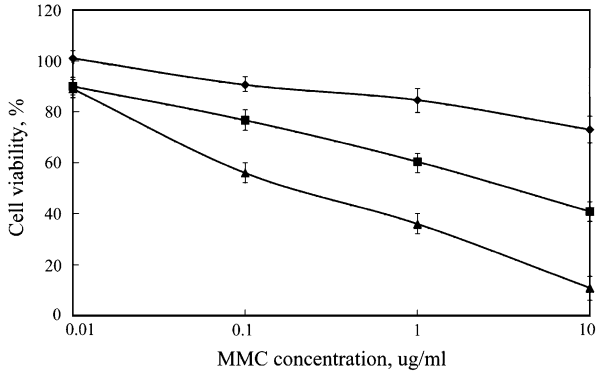


FIGURE 3. Antitumor activity *in vitro* of free MMC (♦), xyloglucan-MMC conjugate (■), and galactosylated xyloglucan-MMC conjugate (▲) against the drug resistant HepG2 cells. Data were given as mean ± SD ($p < 0.05$).

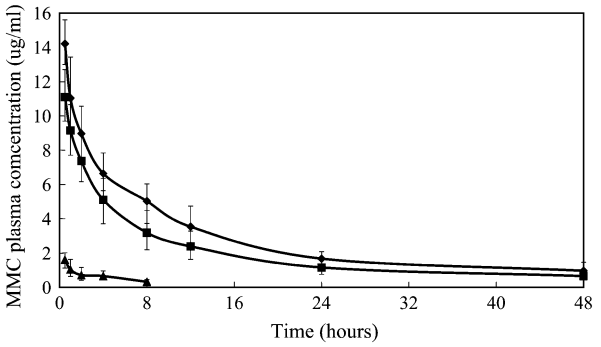


FIGURE 4. Concentrations of MMC in plasma of free MMC (▲), XG-MMC conjugate (■), and Gal-XG-MMC conjugate (♦) administered i.v. [8 (MMC eq.) mg/kg] in tumor-bearing mice. Data were given as mean ± SD ($p < 0.05$).

TABLE 1. Serum biochemical parameters and relative liver weight at 2-week after administration of different doses of the conjugates to mice.

Dose (mg/kg)	ALT (U/l)	AST (U/l)	AST/ALT	LDH (U/l)	CK (U/ml)	Liver/Body (wt%)
Control (0)	26.6 ± 3.6	32.0 ± 3.5	1.20	480.2 ± 68.8	0.25 ± 0.17	6.27 ± 0.42
8	27.9 ± 3.9	33.4 ± 5.2	1.20	478.1 ± 53.8	0.21 ± 0.11	6.22 ± 0.47
16	27.6 ± 3.0	32.7 ± 4.6	1.18	472.6 ± 61.3	0.31 ± 0.18	6.41 ± 0.39
24	26.4 ± 4.6	32.9 ± 3.9	1.25	488.2 ± 46.4	0.34 ± 0.21	6.34 ± 0.26

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *LDH* lactate dehydrogenase, *CK* creatine kinase.

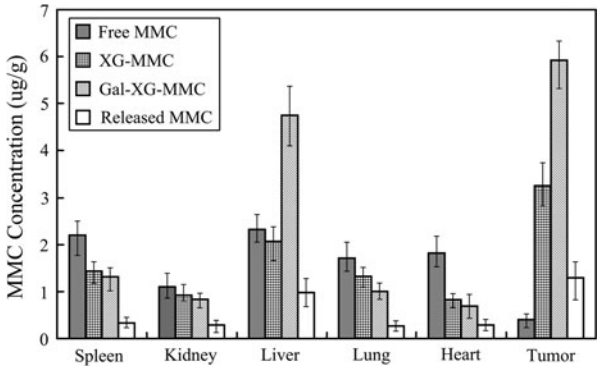


FIGURE 5. Drug biodistribution in tumor-bearing mice (n = 10 per group, equivalent dose of MMC = 8 mg/kg). Data were given as mean ± SD ($p < 0.05$).

MMC was much lower than free drug in these tissue. No drug was detected in brain tissue of mice due to the blood–brain barrier. In contrast, the liver uptake of Gal-XG-MMC was much higher than that of the non-galactosylated conjugate as well as free DOX (Fig. 5). However, the released MMC was not up to the half of free drug in liver. Meanwhile, conjugated by the polymeric prodrug, drug uptake into tumor was 14-fold higher than free drug, and the released MMC was 3-fold higher than free MMC (Fig. 5).

In Vivo Anti-Tumor Activity of Gal-XG-MMC Conjugate

The *in vivo* cytotoxicity effect of free MMC, xyloglucan-MMC conjugate, and Gal-XG-MMC conjugate was examined in order to compare their ability to suppress the growth of drug resistant HepG2 cells in Balb-C nude mice. The changes of tumor size are shown in Fig. 6. Overall, these conjugates demonstrated improved therapeutic profile in terms of tumor growth inhibition compared to free MMC in animal models. Tumor volumes increased rapidly and exponentially when animals were treated with saline alone or free MMC, with little difference among these two treatment groups. At a similar dose, Gal-XG-MMC conjugate was found to be more effective than free MMC and xyloglucan-MMC conjugates in inhibiting the growth of the drug resistant HepG2 cells xenografts. Gal-XG-MMC conjugate-treated groups were associated with more alleviated piloerection, adynamia, and hypodynamia than MMC-treated groups. These results were also mirrored by the survival data (Fig. 7). Treatment with Gal-XG-MMC conjugate was distinctly different than the other three groups, resulting in highly prolonged survival (47 days) compared to free drug (20.6 days) and to xyloglucan-MMC conjugate (33.4 days), respectively (Fig. 7). As normal mice treated with the conjugate did not

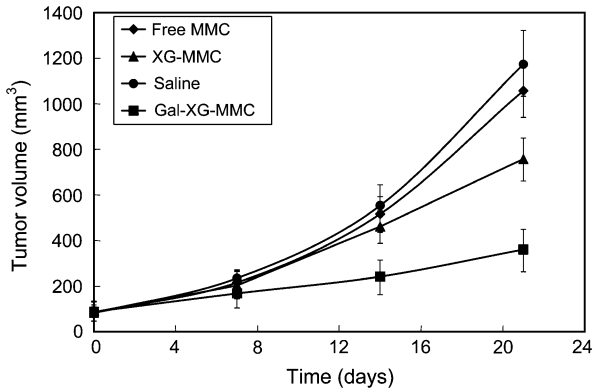


FIGURE 6. Tumor size changes of the treated xenograft nude mice bearing the drug resistant HepG2 tumors [saline (●), free MMC (♦), xyloglucan-MMC conjugate (▲), and galactosylated xyloglucan-MMC conjugate (■)]. After injected HepG2/DR cells for 3 weeks, these drug resistant HepG2 tumor-bearing mice were treated with drugs (8 mg MMC/kg) by tail vein injection every week for four doses (days 1, 7, 14, and 21). Data were given as mean ± SD ($p < 0.05$).

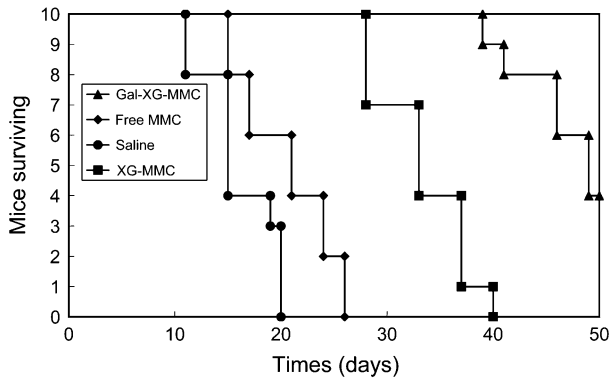


FIGURE 7. Kaplan–Meier survival curve of the treated xenograft nude mice bearing the drug resistant HepG2 tumors [saline (●), free MMC (♦), xyloglucan-MMC conjugate (■), and galactosylated xyloglucan-MMC conjugate (▲)]. After injected HepG2/DR cells for 3 weeks, these drug resistant HepG2 tumor-bearing mice were treated with drugs (8 mg MMC/kg) by tail vein injection every week for four doses (days 1, 7, 14, and 21). The survival time and number of long-term survivors (LTS) until day 50 were monitored ($p < 0.05$).

produce any observable side effect, reason of death of mice is due to the tumor. Treatment with free MMC produced obvious side effects such as reduced activities and weight loss in animals. In contrast, obvious side effects were not observed in mice treated with Gal-XG-MMC conjugate, indicating that higher dose of Gal-XG-MMC conjugate can be used to achieve a better therapeutic efficacy in future study.

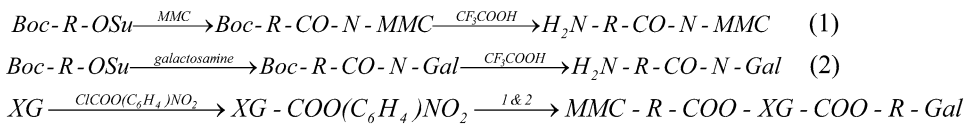
DISCUSSION

The objective of this study was to assess the feasibility of multifunctional polymeric prodrug containing

peptide spacers and, at the same time, decorated with galactose ligands to macromolecular chain for enhanced anticancer drug delivery to metastatic cancer cells. The therapeutic potential of MMC led us to use it as the drug of choice with which to pursue our working hypothesis that tumor-specific polymer–drug conjugates can also be created by adding specific targeting moieties to the polymer to aid in treatment of specific tumors. In this case, the selective delivery of anticancer drug (MMC) was hypothesized to enhance to tumor cells through integration of two targeting strategies: (1) first, active targeting of galactosylated carrier to the endosome of hepatoma cells that overexpress ASGP receptor and (2) second, enzymatic peptide spacers degradation and enhanced release of drug or active drug derivatives, exclusively, in the lysosome of cells. For the purpose of intracellular drug delivery, xyloglucan chemically conjugated MMC by the tripeptide and modified with galactosamine was prepared (Scheme 1). The potential of this architecture *in vitro* and *in vivo* through combination of aforementioned targeting strategies was investigated. A major motivation to pursue carrier-mediated MMC formulations was the potential to reduce the toxicity of free MMC, but without introducing new, carrier-derived, risks of toxicity. Due to their adjustable water solubility, excellent biodegradability and biocompatibility, polysaccharides represented one of the most promising carriers for macromolecular drug delivery.[13,16] Research on cancer-targeted polymeric prodrug has been greatly advanced in the past decade.[1,7,13,16] However, the application of multifunctional polysaccharide DDS against the drug resistant tumor cell has not been explored sufficiently. To the best of our knowledge, this is the first report on the successful preparation of Gal-XG-MMC conjugate and its application as multifunctional polymeric conjugate with controlled drug release properties to overcome MDR in a human cancer cell.

All polymeric carriers[7,16,20] ultimately arrive in the lysosomal compartment of the cell following their pinocytic capture. The spacer between drug and macromolecule is of utmost importance for targetable lysosomotropic DDS. Drug–polymer spacers are designed to be hydrolyzed by lysosomal enzymes and to be resistant to attack in other body compartments. Collagenase is a highly specific collagen-degrading enzyme which is secreted by many tumors. There is a substantial body of evidence which supports a positive correlation between type IV collagenase activity and tumor cell invasion. Highly aggressive human tumors all show elevated levels of type IV collagenase activity.[1,3,7] In the present study, we introduced a tripeptide chain into the conjugate as the lysosomal enzyme degradable spacer. The tripeptide spacer Gly-Leu-Gly has sufficient length and the sequence followed the rules of oligopeptide design. Therefore, conjugate released MMC by the specific hydrolysis of collagenase IV but did not release MMC in pH 7.4 buffer and serum. Stability studies of the MMC conjugates in buffer lysosomal pH 5.5 demonstrated low stability and conversion to free MMC. Release of MMC from its carrier is dependent on the degradation rate of the tripeptide spacers.

In addition, drugs in conjugates could enter tumor cells by endocytosis, depending on the conjugates and cell characteristics,[1,7,16] which express the receptors at high levels on the surface to recognize specifically a targeting moiety of the conjugate. It was well known as an ASGP biomimetic with the aim of targeting the hepatocyte and hepatoma ASGP receptor (ASGPR) for the treatment of liver cancer. ASGPR internalizes the glycoprotein by receptor-mediated endocytosis (RME), by which cells bind macromolecules through receptor recognition.[5,14] The drug resistant HepG2 cells are hepatoma cells and overexpress ASGP receptors on their surface. Endocytic uptake by hepatocytes after interaction with the ASGPR leads to lysosomal trafficking and their subsequent degradation. The targeting conjugates were able to enhance the uptake of entrapped MMC into the tumor cells *in vitro* compared to non-modified ones. Therefore, galactosylated conjugates enhanced uptake of MMC compared to their counterpart conjugates by HepG2/DR cells. The cellular uptake for the conjugates led to their localization in lysosomes where MMC rapidly released from its multifunctional polymeric carriers. The higher cytotoxicity effect of targeting DDS than xyloglucan-MMC conjugates are due to their faster rate of cellular uptake. It is suggested that conjugates without an internalizing ligand have rare chance to be endocytosed by tumor cells. The results indicated that



SCHEME 1. Reaction sequence for the synthesis of the xyloglucan-MMC conjugates (Boc-R-OSu: *N*-t-Boc-glycyl-L-leucyl-glycine *N*-hydroxysuccinimide ester; XG: Xyloglucan).

endocytosis was involved in the drug uptake and mainly responsible for the improved intracellular uptake of MMC for Gal-XG-MMC compared to XG-MMC. Chemotherapy enhanced via polymeric DDS has a promising potential as a strategy to overcome MDR in a human cancer cell. With polymeric DDS, the resistant cells can be sensitized to antitumor drug. The ability of polymeric DDS to overcome MDR is that the targeting conjugates could enter the cells by an endocytotic pathway,[1,16,20] thus bypassing the P-gp dependent efflux, leading to an increased intracellular drug concentration and drug cytotoxicity.

MMC pharmacokinetics, as function of formulation species, was followed *in vivo* to evaluate one part of the design, that the many hydroxyl residues present in carbohydrate would provide the conjugates with the hydrophilic coat needed for longterm retention in circulation. MMC in the conjugate already enhanced retention in circulation compared to free drug (Fig. 4), probably due to the small size of these conjugates. Adding the carbohydrate was the critical factor. It turned these macromolecules into long-circulating species. Drug biodistribution studies were carried out to test the second part of the design, which the galactose group positioned covalently on the chain and endowed these prodrugs with the ability to truly mediate drug targeting to HepG2 tumors. These expectations, affirming the design, were clearly met. When delivered via the targeting conjugates, MMC accumulation in the tumor was 15-fold higher than when the drug was administered in free form (Fig. 5). Tumor-selectivity is also seen from the distribution to other organs in the tumor-bearing mice. MMC uptake into kidneys was not very sensitive to the nature of drug formulation, and spleen and heart uptake was significantly reduced when the drug was delivered via the targeting conjugates (Fig. 5). The high MMC uptake by spleen for free drug can explain the rapid clearance of MMC from the circulation as compared to conjugates. The adverse effect is a major concern for the Gal-XG-MMC as a potential DDS, because ASGPR could also be found in hepatocytes. Thus, the intracellular MMC uptake by hepatocytes and the cytotoxicity on these cells might be enhanced by conjugates. MMC uptake into livers and HepG2 tumors was very similar. Though the conjugates uptake into livers was higher than free drug, the released MMC was much lower than free drug. It is the interpretation of the improvement in the safety of the conjugates. Meanwhile, no significant signs of toxicity were observed with the mice treated with different MMC formulations when the weight loss was used as the indicator for toxicity measurement and systemic side effects of this conjugate was explored the in normal mice. Due to sustainedly release of MMC from the conjugate and relatively low concentration in various tissue, highly prolonged mice life time as well as much reduced cardiotoxicity and hepatotoxity was achieved when MMC was given in the conjugate form (Table 1). These indicate that the conjugate can be administrated at a much elevating dose in chemotherapy as compared with free drug.

Similar to the *in vitro* results, much more relevant trends were found when the targeting DDS were subjected to *in vivo* testing. Taking together the measures of tumor response to treatment (Figs. 6, 7), tumor size and survival treatments with free MMC were not much different than no treatment. The responses to treatment with xyloglucan-MMC conjugates were only a slight improvement over free drug or no treatment. On this low background, we find the highly positive responses to treatment with targeting conjugates, quite striking. The targeting DDS slowed-down tumor progression with concomitant smaller tumors and increased life span (compared to treatment with free drug). The combined effect of passive accumulating and enhanced intracellular delivery should be responsible for the improved therapeutic efficacy of Gal-XG-MMC over XG-MMC or free MMC. Conjugates can passively accumulate into the tumor tissues by the effect of EPR. Conjugates diffusion into the interstitial fluid of the tumor was heavily dependent on the AUC in the blood stream. In addition, as mentioned above, Gal-XG-MMC can efficiently deliver the drugs into the tumor cells by the receptor-mediated endocytosis and lead to a high concentration of MMC in the tumor cells. By the means of targeting conjugates, MDR may overcome by increasing anticancer drugs based only on mass action. We suggest that the encouraging results reported here stem from combination of all attributes of this formulation: sustained release, long retention in circulation, and high affinity binding to the HepG2 tumor, allowing the polymeric DDS to act there as sustained-release drug depots against MDR tumor.

CONCLUSION

The objectives of the current study were to develop a targeting macromolecular DDS for overcoming drug resistant cancers. The results of this study show a great potential for galactosylated xyloglucan based MMC conjugates and spacers for lysosome triggered enhanced intracellular drug delivery to tumor cells against MDR. The targeting conjugates emerge as a valid option for MDR tumor chemotherapy, worthy of further pursuit, with distinct advantages: intracellular drug delivery, long circulation, and high affinity to the target.

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