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Dually Enzyme- and Acid-Triggered Self-Immolative Ketal Glycoside Nanoparticles for Effective Cancer Prodrug Monotherapy

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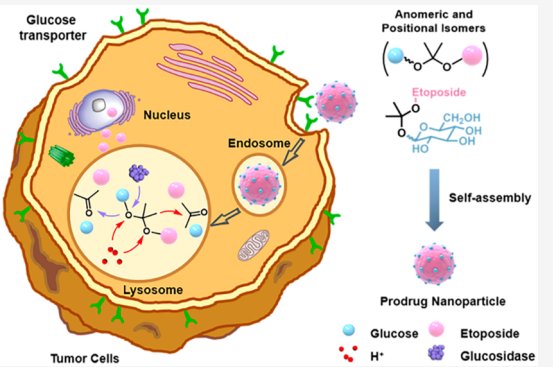
ABSTRACT: The use of glycoside prodrugs is a promising strategy for developing new targeted medicines for chemotherapy. However, the *in vivo* utility of such prodrugs is hindered by insufficient activation and the lack of convenient synthetic methods. We have developed an innovative strategy for synthesizing ketal glycoside prodrugs that are unique in being activated by a dual enzyme- and acid-triggered self-immolative mechanism. Amphiphilic glucosyl acetone-based ketal-linked etoposide glycoside prodrug isomers were synthesized and fabricated into excipient-free nanoparticles for effective cancer prodrug monotherapy. Hydrolysis of the glycosidic linkage or the ketal linkage triggered hydrolysis of the other linkage, which resulted in spontaneous self-immolative hydrolysis of the prodrugs. Nanoparticles of the prodrug isomer that was the most labile in a lysosome-mimicking environment displayed high intratumoral accumulation and strong antitumor activity in an A549 xenograft mouse model. Our strategy may be useful for the development of stimulus-responsive self-immolative prodrugs and their nanomedicines.



KEYWORDS: *Nanomedicines, Prodrugs, Glycosides, Cancer Therapy, pH-Sensitive*

INTRODUCTION

Nanomedicines are broadly employed in pharmaceutical development to treat different diseases and have achieved great success indubitably.[1−3] However, only few anticancer nanomedicines succeed so far, by solving the solubility issue for poorly soluble drugs (e.g., paclitaxel) and/or reducing adverse effects through modulating pharmacokinetics of drugs (e.g., doxorubicin).[4] The successful development of new anticancer nanomedicines has been significantly hindered by poor compatibility between nanocarriers and drugs, resulting in low drug loading, poor colloidal stability and/or premature burst release after administration, and so forth.

Prodrug strategies can improve drug performance and facilitate the design and development of new medicines, including nanomedicines.[5−15] Because of the good biocompatibility and tumor-targeting capability of various small molecules that occur naturally in the human body (e.g., monosaccharides, cholesterol, and fatty acids), they are ideal components for constructing anticancer prodrugs.[16,17] In fact, glycoside prodrugs in which monosaccharides are directly linked to the hydroxyl group of drugs via glycosidic linkages that can be activated by glycosidases have been synthesized for selective chemotherapy against malignant tumors.[18,19] One example is glufosfamide, a glycoside isophosphoramide mustard prodrug in a phase 3 clinical trial for the treatment of pancreatic cancer.[20] However, because the synthesis of glycoside prodrugs is complex and difficult, their practical utility is limited. Recently, however their synthesis has been simplified by incorporation of self-immolative linkers, which have facilitated the development of glycosidase-activatable prodrugs.[21−26] The glycosidic linkages of these prodrugs serve as the sole trigger for activation, which is thus predominantly under enzymatic control. Although the elevated glycosidase levels in necrotic tumors can activate glycoside prodrugs,[27,28] *in vivo* activation of most of these prodrugs remains insufficient when they are used as monotherapies;[29−32] thus exogenous stimulus (e.g., immunoconjugates of enzyme and tumor-targeted antibody) is often combined to achieve efficient prodrug activation. In light of these findings, the development of glycoside prodrugs that can be readily synthesized and undergo efficient *in vivo* transformation would be highly desirable.

Herein, we report a simple yet novel strategy for synthesizing glycoside prodrugs by conjugating hydroxyl-group-containing monosaccharides with hydroxyl-group-con-

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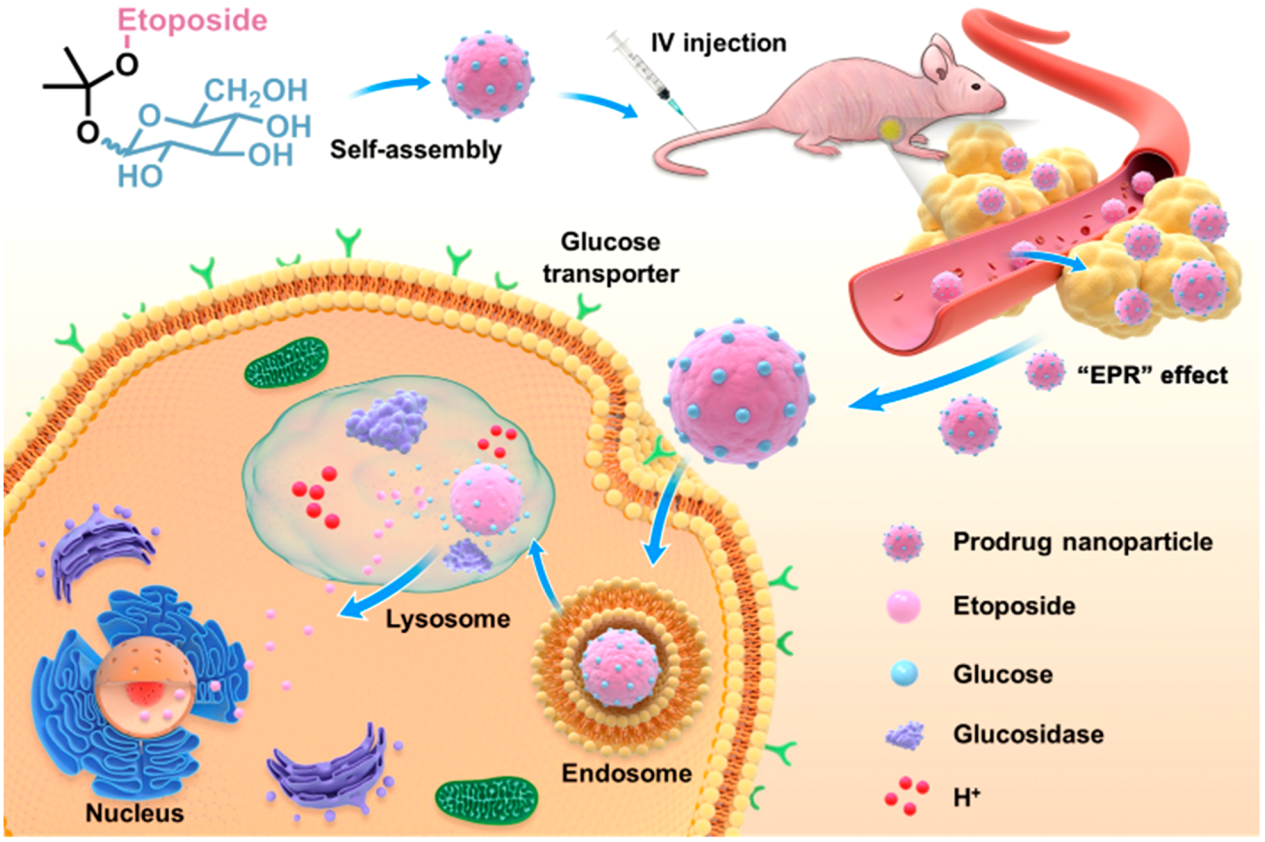
Scheme 1. Schematic Illustration[a]



[a]Schematic of self-assembly of glucosyl prodrug NPs, their glucose transporter–mediated uptake by an A549 tumor cell *in vivo*, and subsequent self-immolative activation of the prodrug by β-glucosidase and acid in a lysosome. EPR = enhanced permeability and retention.

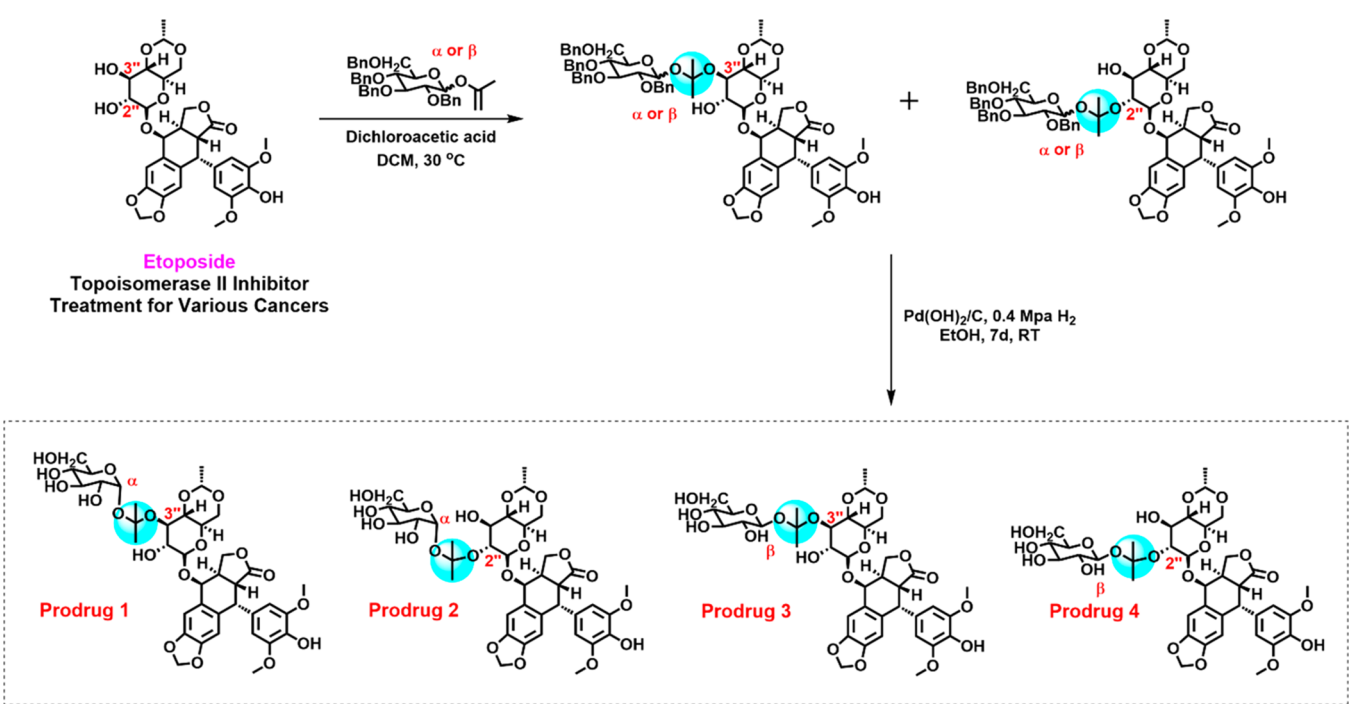


Figure 1. Syntheses of isomeric ETP glycoside prodrugs.

taining drugs via pH-sensitive asymmetrical acetone-based ketal linkages. The resulting amphiphilic ketal glycoside prodrugs self-assembled into glucose-decorated nanoparticles (NPs) that could be internalized into enzyme-rich acidic organelles of tumor cells, where they can be activated by a unique mechanism involving the dual action of glycosidase and

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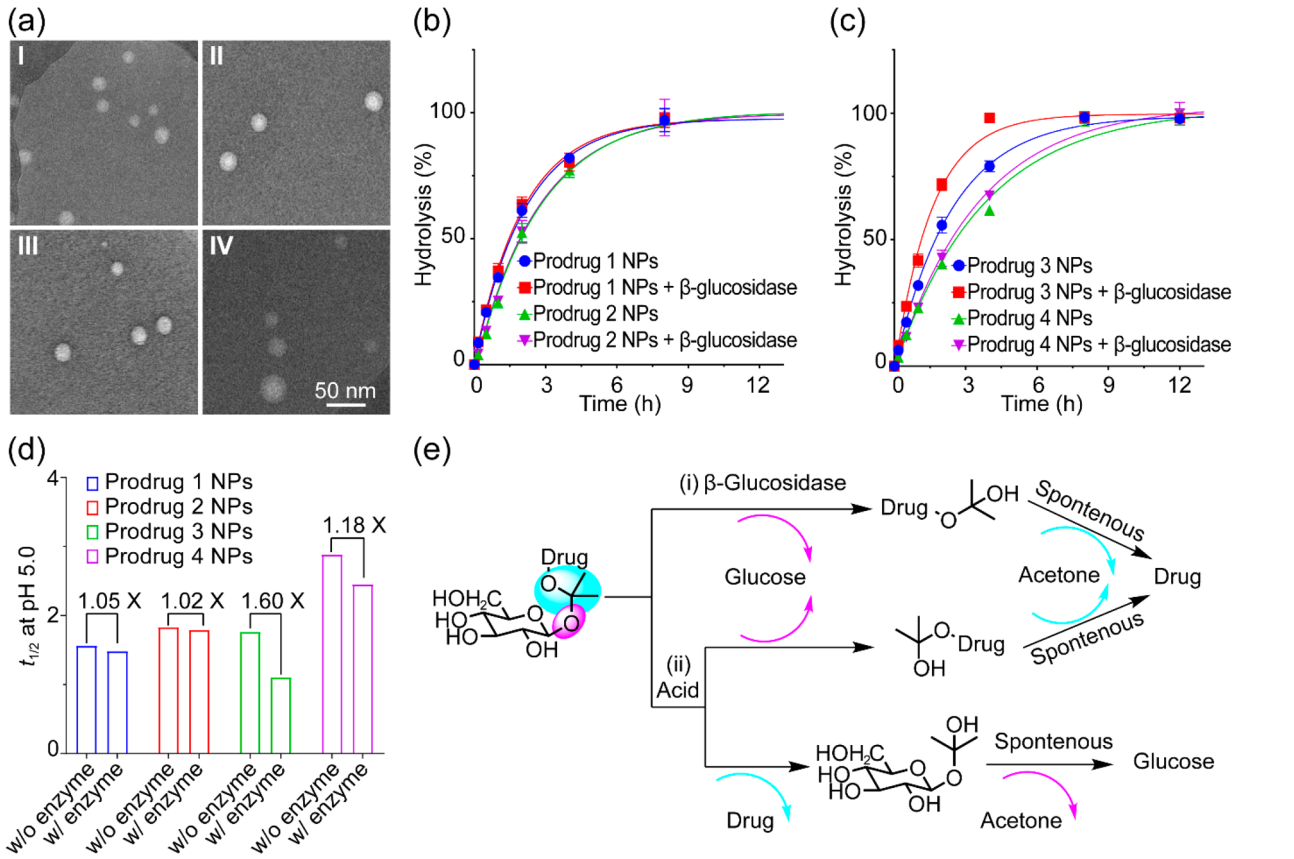

Figure 2. (a) Transmission electron microscopy images of (I) prodrug 1, (II) prodrug 2, (III) prodrug 3, and (IV) prodrug 4 NPs. Hydrolysis curves of NPs of (b) α-anomeric and (c) β-anomeric prodrugs in the presence or absence of β-glucosidase (10 U/mL) at 37 °C at pH 5.0. Data are means ± SD, $n = 4$. (d) Prodrug $t_{1/2}$ values were derived from the data in panels (b,c). The numbers denote fold increases in hydrolysis rate in the presence of β-glucosidase. (e) Dual glycosidase- and acid-catalyzed self-immolative hydrolysis of ketal glycoside prodrugs; hydrolysis of the glycosidic or ketal linkage triggers hydrolysis of the other linkage, resulting in spontaneous self-immolation.

acid; that is, hydrolysis of either the glycosidic linkage or the ketal linkage triggered spontaneous self-immolative hydrolysis of the other linkage, efficiently releasing the drug, along with acetone and glucose.

RESULTS AND DISCUSSION

As a proof of concept, glucosyl acetone-based ketal-linked glycoside prodrugs of etoposide (ETP, a topoisomerase II inhibitor for the treatment of various cancers[33]) were synthesized and fabricated into prodrug NPs (Scheme 1). These NPs exhibited glucose transporter-mediated uptake by tumor cells, where they might undergo dual β-glucosidase- and acid-triggered self-immolative hydrolysis in lysosomes or undergo acid-triggered ketal hydrolysis in endosomes. In an A549 xenograft mouse model, they displayed high intratumoral accumulation and strong antitumor activity. The use of excipient-free NPs of amphiphilic prodrugs that can be internalized into enzyme-rich acidic organelles represents a novel strategy for overcoming the problems caused by insufficient acidity and low enzyme levels outside tumor cells. This feature is particularly important in situations in which the extracellular acidity and enzyme levels in tumors are too low for glycoside prodrug activation in traditional glycoside prodrug monotherapies. The structure of ketal glycoside prodrugs reported herein, the method by which they were synthesized, and the unique mechanism by which they are released (self-immolation dually triggered by an enzyme and by acid) are novel features that have rarely been reported previously.

Synthesis of Ketal Glycoside Prodrugs. Given acetone-based ketal-linked prodrugs can be obtained from acid-catalyzed reactions of alcohols and isopropenyl ethers[34−36] and that substitution at the 1-position of glucose does not affect the affinity of glucose for its transporters,[37,38] we began by carrying out reactions of ETP with isopropenyl 2,3,4,6-tetra-O-benzyl-α- or β-D-glucopyranoside and subsequent hydrogenolysis to remove the benzyl protecting groups (Figure 1, Schemes S1−S4). For most ETP prodrugs, nucleophilic substitution under basic conditions proceeds at the phenol group of the ETP molecule. However, in our case, the reaction between isopropenyl 2,3,4,6-tetra-O-benzyl-D-glucopyranoside and the ETP phenol group was inhibited, even when an excess of the isopropenyl ether was used and the reaction time was prolonged. We speculate that previously reported syntheses of ETP glycoside prodrugs at the phenol position succeeded owing to the use of a longer spacer and/or the use of a less bulky protecting group (e.g., acetyl). The reactions occurred instead at the 2″- and 3″-hydroxyl groups. The similar polarities of the two resulting isomers impaired their separation, however they could be isolated by using preparative high-performance liquid chromatography. Prodrugs 1 and 2 were synthesized from the α-anomeric isopropenyl ethers, and prodrugs 3 and 4 were synthesized from the β-anomeric ethers. The 2″ and 3″ isomers were unambiguously confirmed on the

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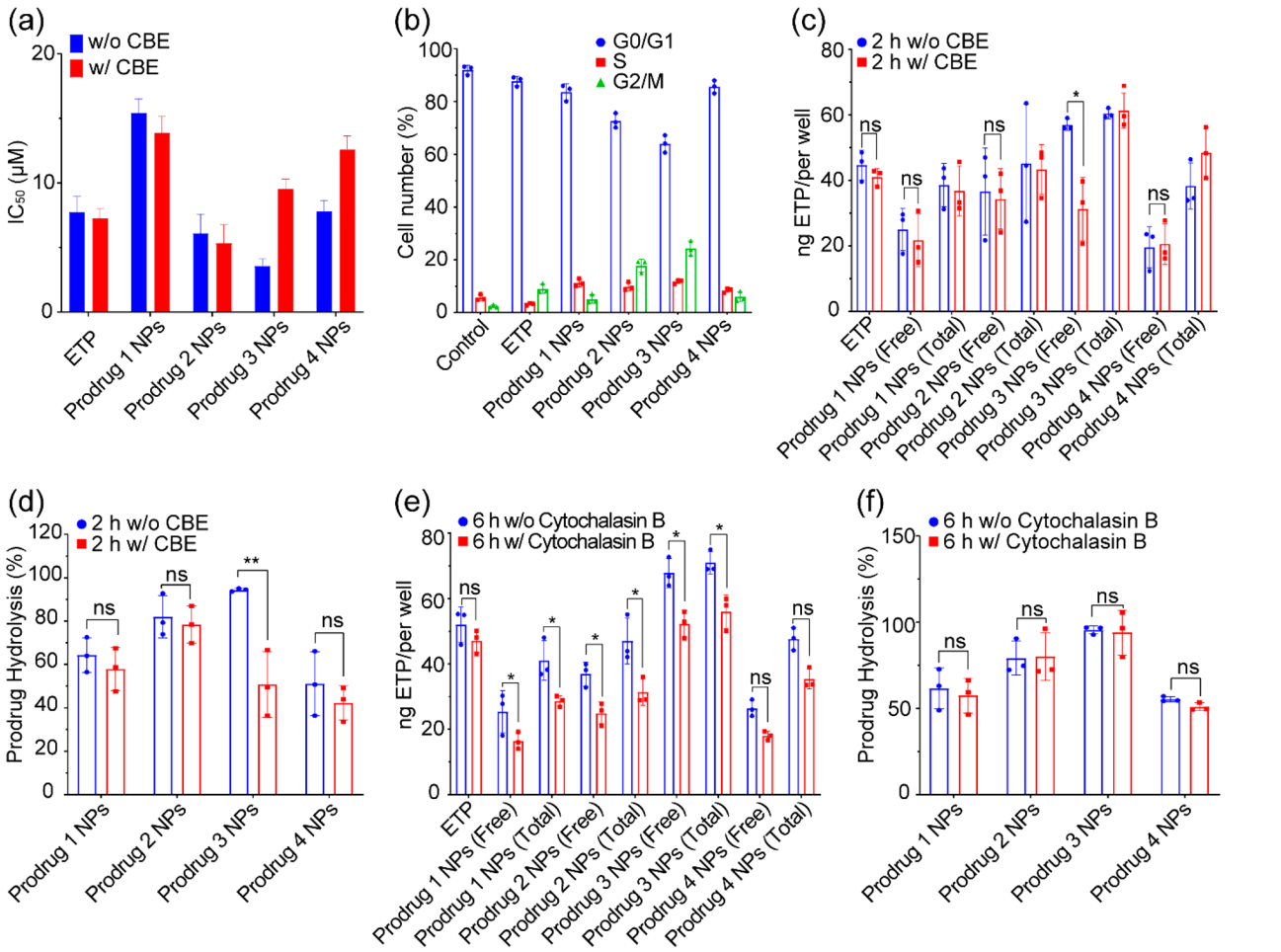

Figure 3. (a) IC_{50} of ETP formulations in A549 cells. (b) Cell cycle arrest by flow cytometry. Cells were treated with 20 μM ETP formulations for 12 h. Data are means ± SD; $n = 3$. (c,e) Cellular uptake and (d,f) intracellular hydrolysis (%) of prodrugs. In c–f, cells were incubated with ETP formulations at 20 μM; data are means ± SD; $n = 4$; * indicates $p < 0.05$, and ** indicates $p < 0.01$; ns = not significant. Ten micromolar conduritol B epoxide (CBE) and 50 μM cytochalasin B were used. "Prodrug NPs (Free)" means free etoposide hydrolyzed from prodrugs, and "Prodrug NPs (Total)" means total etoposide in prodrugs including those hydrolyzed and unhydrolyzed forms.

basis of their ^{1}H- and ^{13}C NMR (including DEPT-135) and 2D-NMR (HSQC and HMBC) spectroscopy (Figures S1–S24). The overall yield of each prodrug was approximately 35%, and HPLC confirmed them to be highly pure (Figure S25). Note that there has been only one report of the synthesis of a ketal glycoside prodrug by Tietze et al., who used a multistep approach involving a TMSOTf-catalyzed nucleophilic substitution reaction.[39] However, this approach is limited in that it releases carbonyl-group-containing drug derivatives rather than native drugs. In contrast, our simple yet novel approach, which features mild reaction conditions and high yields, can release native drugs rather than derivatives and may boost the development of ketal glycosides.

Preparation and Characterizations of Ketal Glycoside Prodrug NPs. To elucidate the relationship between prodrug structure and properties, we carried out various experiments with the above-mentioned isomeric ETP prodrugs, which had differing anomeric and positional configurations. Given that prodrug-containing NPs can be internalized by tumor cells into enzyme-rich acidic organelles and then efficiently activated by enzymes and acid, we fabricated excipient-free NPs from the amphiphilic ETP glycoside prodrugs by means of a nanoprecipitation method. Transmission electron microscopy showed that the NPs were uniform spheres with diameters of 20–30 nm (Figure 2a), a result that was confirmed by dynamic light scattering (Figure S26, Table S1). The zeta potentials of the glucose-decorated NPs were approximately −14 mV and their ETP content was 72.8 wt %, which is much higher than that of previously reported nanoparticulate ETP formulations.[40,41] Moreover, we quantified the number concentrations of prodrug NPs (1 mg/mL) by the NP tracking analysis (NTA) system and calculated the average number of prodrug compound molecules in each NP (Figure S27). As a result, the number concentrations of NPs ranged from 5.68×10^8 to 6.65×10^8 particles/mL, and each prodrug NP contained approximately 1.12×10^9 to 1.31×10^9 molecules.

For practical use, the prodrug in the NPs must undergo acid- and enzyme-catalyzed transformation in lysosomal environments. Therefore, we investigated the kinetics of hydrolysis of the prodrug NPs at lysosomal pH (5.0)[42] and physiological pH (7.4) by using HPLC. Note that the acetal linkage in the glucose moiety is stable at weakly acidic pH,[43] and thus any pH sensitivity exhibited by the prodrugs can be attributed to the acetone-based ketal linkages. In all cases, prodrug hydrolysis followed pseudo-first-order kinetics (Figures 2b,c, S28, and S29 and Table S2). Calculation of the half-lives ($t_{1/2}$) of the prodrugs at pH 5.0, ln $2/k_{obs}$, where k_{obs} is the hydrolytic

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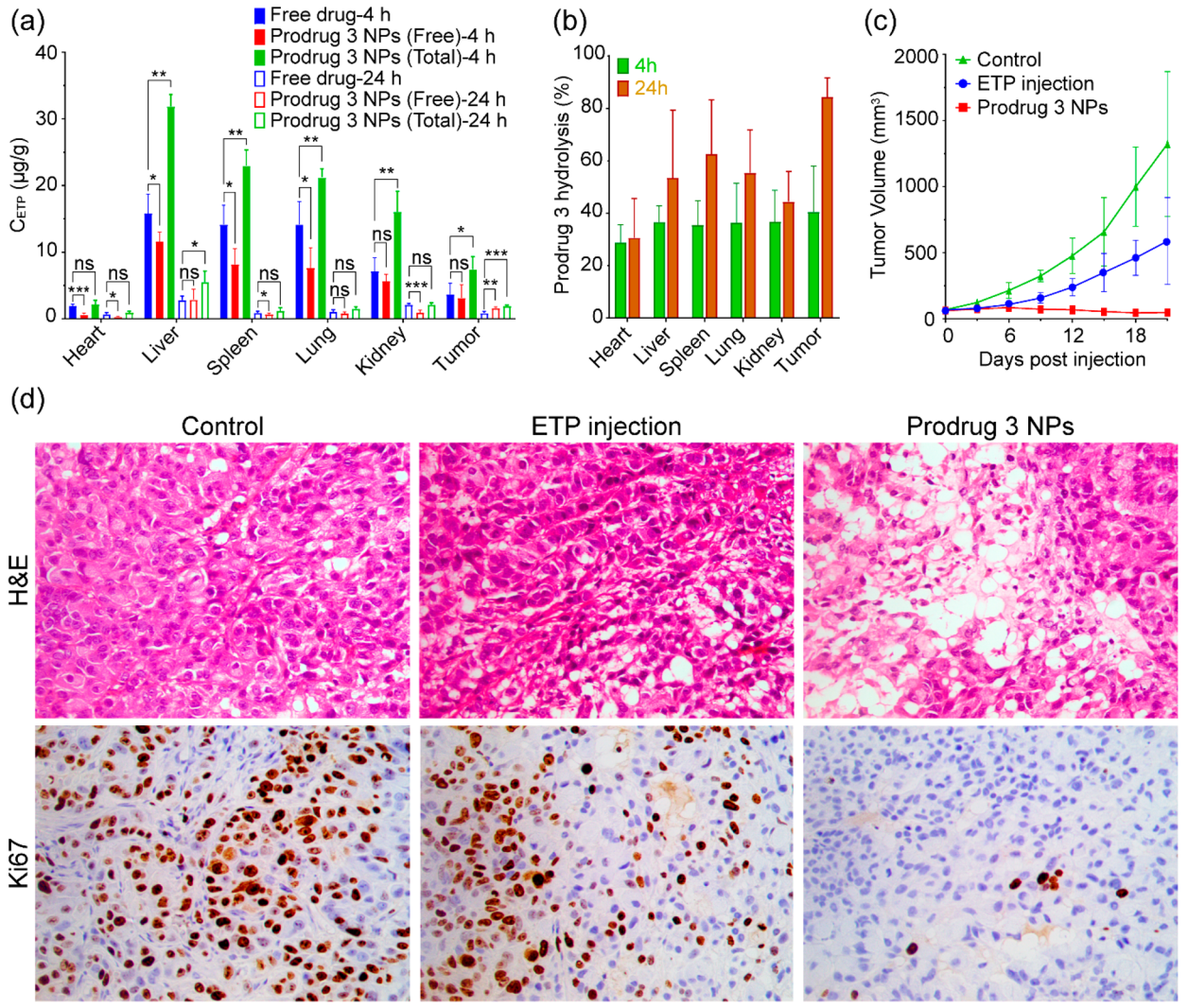

Figure 4. (a) *In vivo* biodistribution of ETP formulations in an A549 xenograft tumor model and (b) hydrolysis (%) of prodrug 3 NPs. Data are means ± SD, $n = 4$; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$; ns = not significant. (c) Temporal dependence of tumor volume after IV injection of ETP formulations (15 mg/kg ETP equivalent) on days 0, 3, and 6. Data are means ± SD, $n = 7$. (d) Representative H&E staining and Ki67 staining of tumor sections.

constant, showed that the α-anomeric prodrugs hydrolyzed faster than the β-anomeric prodrugs; the 3″-isomer of each anomer hydrolyzed faster than the 2″-isomer. The $t_{1/2}$ values for NPs of prodrugs 1−4 at pH 5.0 were 1.56, 1.83, 1.76, and 2.88 h, respectively. However, after 24 h at pH 7.4, less than 5% of the prodrugs had hydrolyzed (Figure S29).

We also evaluated enzymatic hydrolysis by incubating the prodrug NPs with 10 U/mL β-glucosidase at pH 5.0 and pH 7.4 (Figures 2b,c, S28, and S29 and Table S2). At pH 5.0, the hydrolysis rates for β-anomeric prodrugs 3 and 4 in the presence of the enzyme were 1.6 and 1.18 times the values in its absence, respectively (Figure 2d); whereas, the enzyme had a negligible effect on the rate of hydrolysis of the α-anomeric prodrugs. At pH 7.4, no significant difference in prodrug hydrolysis in the absence and presence of β-glycosidase was observed (Figure S29), which may have been due to the low activity of the enzyme at pH 7.4. However, it must be noted the effect of β-glycosidase on prodrug activation may be different *in vivo* than in buffers. HPLC analysis revealed that the only degradation products of the prodrugs were native ETP, acetone (a metabolite), and glucose (which is biocompatible) (Figure S30). These results indicate that our rationally designed ketal-linked β-anomeric glycoside ETP prodrugs exhibited β-glucosidase- and acid-catalyzed self-immolative hydrolysis by the mechanism depicted in Figure 2e, which is different from that for previously reported self-immolative glycoside prodrugs.

***In Vitro* Cell Studies.** Modification of NPs with glucose has been demonstrated to enhance their intratumoral accumulation by glucose transporters present in endothelial cells and tumor cells.[44,45] Therefore, we used A549 cells, which highly express glucose transporters,[46,47] to assess the cytotoxicity and therapeutic efficacy of the ETP prodrug NPs. Compared with free ETP (IC_{50} = 7.7 μM), prodrug 2 (IC_{50} = 4.4 μM) and prodrug 3 (IC_{50} = 3.66 μM) were more toxic, whereas prodrugs 1 and 4 were less toxic (IC_{50} = 14.2 and 7.95 μM, respectively) (Figures 3a and S31). The presence of conduritol B epoxide (a β-glucosidase inhibitor) decreased the cytotoxicities of β-anomeric prodrugs 3 and 4 (IC_{50} = 9.56 and 12.1 μM, respectively), but the toxicities were still comparable to that of free ETP. This result demonstrates the stereoselectivity of the interaction between β-glucosidase and

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the glycoside prodrugs and strong acid-activation of the glycoside prodrugs in cells. A cell cycle arrest assay demonstrated that the arrest ability of prodrug 3 in the S phase and the early G2/M phase was higher than the arrest abilities of the other drugs in these two phases (Figures 3b and S32), in accordance with the cytotoxicity results. Among the prodrug NPs, the prodrug 3 NPs showed the highest cellular uptake, which exceeded that of free ETP at 2 h (Figure 3c). However, the uptake of ETP was similar to or better than other prodrug NPs, indicating that the glucose transporter-mediated endocytosis for prodrug NPs was markedly affected by the prodrug structure and other pathways might also be involved in cellular uptake of prodrug NPs. The intracellular hydrolysis percentages of the prodrugs exceeded 50%, and intracellular hydrolysis of prodrug 3 reached 94% at 2 h (Figure 3d). However, in the presence of conduritol B epoxide, the intracellular hydrolysis percentage of prodrug 3 at 2 h was only 51%. In contrast, the presence of the β-glucosidase inhibitor had no statistically significant effect on the hydrolysis percentage for the other prodrugs. We also found that the presence of cytochalasin B, an inhibitor of glucose transporter type I, reduced the cellular uptake of all four prodrugs (Figure 3e) without affecting their intracellular hydrolysis (Figure 3f), indicating that this transporter facilitated the cellular uptake of the glucose-decorated NPs.

***In Vivo* Ketal Glycoside Prodrug NPs Monotherapy.** Because prodrug 3 had the lowest IC_{50}, the highest cellular uptake, and the most efficient hydrolysis in A549 cells, we used it for *in vivo* studies. Specifically, we evaluated the biodistributions and antitumor efficacies of ETP formulations in an A549 xenograft tumor model (Figure 4). Free ETP injection prepared according to the method used for the commercial formulation Toposar (which is more efficacious than Etopophos[25]) was used for comparison. Like other kinds of NPs, prodrug 3 NPs were cleared mainly by the liver and spleen. Four hours after administration, the accumulations (μg/g) of prodrug 3 NPs in the liver, spleen, lungs, and kidneys were higher than the accumulation in tumor tissue; whereas 24 h after administration, the accumulations in these organs were significantly decreased (Figure 4a). Note that the ratio of intratumoral prodrug accumulation to prodrug accumulation in organs increased over time, indicating the enhanced permeability and retention of NPs[48−50] and GLUT-mediated transport of the glucose-decorated prodrug NPs. Compared with free ETP injection, prodrug 3 NPs exhibited higher accumulation in tumor tissue (Figure 4a). Furthermore, prodrug 3 was efficiently transformed to native ETP to a greater extent in tumor tissue than in the organs (Figure 4b); intratumoral hydrolysis of prodrug 3 was about 85% at 24 h. The efficient transformation of prodrug 3 in tumor tissue was probably due to enhanced cellular uptake mediated by the high levels of the GLUT1 transporter on tumor cells, the high abundance of β-glucosidase in the tumor cells, and the acidic tumor microenvironment. Prodrug 3 NPs exhibited much better inhibitory effects on A549 tumor than free ETP injection (Figures 4c and S33), and there was no obvious change in body weight in mice treated with the NPs (Figure S34). Hematoxylin and eosin (H&E) and Ki67 immunohistochemical staining confirmed that prodrug 3 NPs significantly inhibited the growth of tumor cells (Figure 4d). In addition, H&E staining of organs and blood biochemistry analysis showed no obvious abnormalities in the treatment groups (Figures S35 and S36).

Although many glycoside prodrugs have been synthesized, the mechanism of their activation in cells and animals has not been investigated in detail, and the resulting lack of information is partially responsible for the fact that glycoside prodrugs have not advanced into practical use. Here, we comprehensively investigated the characteristics of the ketal glycoside prodrugs and their NPs in cells and animal models. Glucosyl prodrug NPs were internalized by tumor cells into enzyme-rich acidic organelles, where they can be efficiently activated by enzymes and acid. Our findings provide important insight into the mechanism of action of ketal glycoside prodrug formulations and should facilitate the design of more-efficient glucoside prodrugs.

The development of more-efficacious glycoside prodrugs and prodrug formulations is an important goal of work that is ongoing in our group and the groups of others in the field. The strategy reported herein, which combines rational chemical design and nanotechnology for the development of efficacious glycoside prodrug monotherapy, is an important one.

CONCLUSIONS

In summary, we have developed a simple yet novel method for synthesizing ketal glycoside prodrugs in which a monosaccharide moiety is directly conjugated to the drug *via* an acetone-based ketal linkage, and we evaluated the activities of self-assembled NPs of isomeric ETP glycoside prodrugs in an A549 xenograft tumor model. The pH sensitivity and β-glucosidase sensitivity of the prodrug NPs depended on the anomer of the prodrug: specifically, NPs of α-anomeric prodrugs underwent only acid-activated hydrolysis, whereas hydrolysis of β-anomeric prodrug NPs was activated by both β-glycosidase and acid. Of all the prodrugs, the 3″-positional β-anomeric prodrug (i.e., prodrug 3) was the most labile toward both acid and β-glucosidase, and the prodrug 3 NPs exhibited the highest cytotoxicity against A549 cells. The presence of a GLUT1 inhibitor reduced uptake of the NPs by tumor cells, and the presence of a glucosidase inhibitor downregulated the extent of β-anomeric prodrug hydrolysis. In a biodistribution study, prodrug 3 NPs showed notably higher accumulation than free ETP injection; the NPs were effectively transformed in tumor tissue and showed much better efficacy in reducing tumor volume than did free ETP injection. Thus, they have the potential to be effective as a prodrug monotherapy, which has been difficult to achieve with previously reported glycoside prodrugs. Our strategy of combining rational chemical design and nanotechnology for the development of efficacious glycoside prodrug monotherapy may be useful for the synthesis of stimulus-responsive self-immolative prodrugs and may facilitate the development of targeted chemotherapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.0c01973.

Detailed methods, synthesis procedures of ketal glycoside prodrugs, NMR spectra, hydrolysis parameters, DLS, NanoSight characterization, HPLC chromatograms, cell toxicities, cell cycle arrest by flow cytometry, H&E staining of organs, weight of resected tumors, blood biochemistry analysis and change in body weight of mice treated with the NPs (PDF)

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Notes

The authors declare no competing financial interest.

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