Letter

A Bioreductive Prodrug of Cucurbitacin B Significantly Inhibits Tumor Growth in 4T1 Xenograft Mice Model

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ABSTRACT: Cucurbitacin B (CuB), a highly cytotoxic constituent of the Cucurbitaceae plant, was identified to exhibit potent inhibitory activity against human cancer cells as well as normal cells. This disadvantage hampers the possibility of developing this compound into an anticancer drug candidate. In this work, several bioreductive prodrugs of CuB were designed to reduce toxicity to normal cells while maintaining cytotoxic effect to cancer cells. Embedded with a bioreductive delivery and cleavable system in cancer tissues, cucurbitacin B-based prodrugs **1**, **2** and **3** were synthesized and evaluated by *in vitro* and *in vivo* experiments. Compared with the parent CuB, prodrug **1** was found to significantly reduce the toxicity down to 310-fold lower against non-cancerous cells. LC-MS analyses show that prodrug **1** efficiently releases the parent compound in the reductase-overexpressed MCF-7 cells. In addition, prodrug **1** shows satisfactory and comparable effectiveness in controlling tumor growth as that by tamoxifen in the 4T1 xenograft mice model.

Development of natural products to new analogues with improved properties are highly desired in the drug discovery process.[1] Targeted prodrug design for anticancer drug is one of the interesting strategies in drug discovery.[2–5] Many anticancer natural products are not target specific, and might also quite toxic to normal cells. To lower the toxicity while keeping the therapeutic property of active natural products, proper structure modification serves as an important and useful tool in drug discovery. Prodrug design has been proven one of the workable strategies to improve the physicochemical properties of a molecule and overcome unacceptable biopharmaceutical performance. Embedment of an additional molecular device, which is cleavable by a specific enzyme expressed predominantly in tumor cells, is a commonly applied manner in contemporary drug design and development.[6] Bioreductive prodrug can be designed to target specific tumor followed by *in situ* release of the therapeutic drugs with the reductase (NAD(P)H:quinone oxidoreductase 1, NQO1) over-expressed in some tumor cells.[7–9] Fortunately, the reductases are often reported to be overexpressed in a variety of cancer cells, such as breast cancer, ovarian cancer, thyroid cancer, adrenal cancer and colon cancer.[3,10–13]

Trichosanthes cucumerina L. (Cucurbitaceae), distributed in many countries of Asia and well known for its bitter taste, is a Thai medicinal herb. Cucurbitacins, the main components of *T. cucumerina*,[14–17] exhibit a variety of pharmacological effects *in vitro* and *in vivo*, such as anticancer, hepatoprotective, cardiovascular, purgative, antiinflammatory, antimicrobial, anthelmintic, CNS effects and antifertility activities.[18–20] Our previous phytochemical study on *T. cucumerina* revealed that cucurbitacin B (CuB, Figure 1), the major constituent of the plant, shows potent inhibitory activities on human breast cancer cells (MCF-7, MDA-MB-231 and SKBR-3)[16,21] and colon cancer cells (Caco-2 and SW620).[22] Unfortunately, CuB is also highly toxic against normal cells. This disadvantage hampers the possibility of developing this compound into an anticancer drug. Lowering cytotoxic effect of CuB against normal cells while maintaining the cytotoxic potency to cancer cells keeps as a challenging task yet.

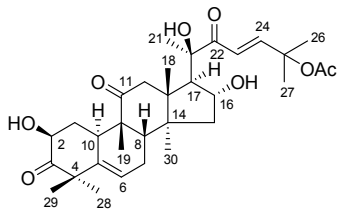


Figure 1. Structure of cucurbitacin B.

In this work, we explored the conversion of CuB into a prodrug design to improve the toxicity/safety index between breast cancer cells and non-cancerous cells. Such a prodrug system is expected to be non-toxic or less toxic against normal cells until it reaches the tumor tissue and releases CuB. We rationally designed three prodrugs that were composed of a variety of linkers and the general quinone delivery system that

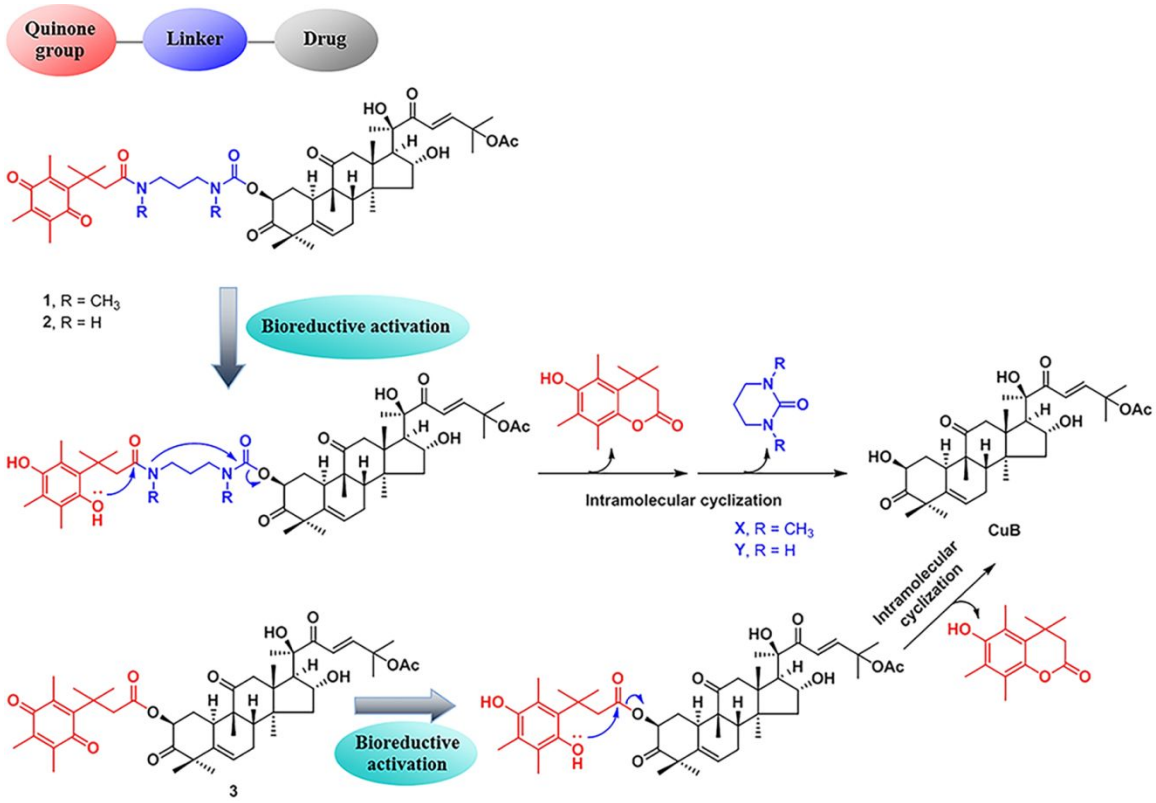


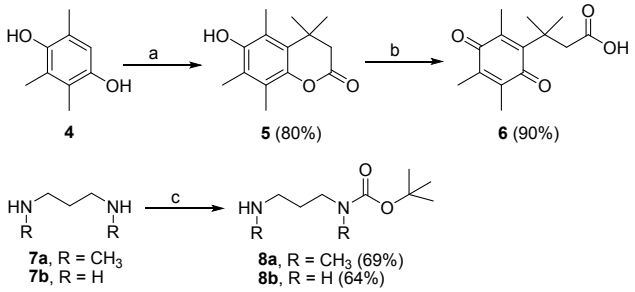
Figure 2. Rational design of CuB-based bioreductive prodrugs **1**–**3**.

has been successfully used in a number of prodrug researches.[3,12,13,23,24] Presence of the over-expressed reductase in breast tumor cells is supposed to trigger the release of CuB and cause the death of cancer cells. Three prodrugs **1**–**3** have been synthesized and their crucial properties and biological activities were evaluated and compared.

Cucurbitacin B (CuB, Figure 1) is the major triterpenoid isolated from the fruit fibers of *T. cucumerina*. The spectroscopic (IR, ^{1}H NMR, ^{13}C NMR and mass spectra) data of CuB are consistent with those reported.[25–27] CuB showed significant cytotoxic activities against various cancer cells, such as breast cancer,[16,21] colon cancer,[22] human nasopharynx carcinoma cells,[28] non-small cell lung cancer, human hepatocellular cells,[29] HeLa cells, and HepG2 cells.[30] CuB upregulated DNA methyltransferase 1 and heavy methylation in the promoters of c-Myc, cyclin D1, and survivin, which consequently downregulated the expression of all these oncogenes, were observed.[31] However, its non-selective cytotoxic actions against both cancerous and normal cells greatly limited its further development to a potential anticancer drug. Conversion of this cytotoxic natural product to a safe prodrug is of extreme interest for potential treatment of cancers.

For bioreductive prodrug design**,** quinone delivery systems is capable to deliver drugs to target specific tumor and can be activated by overexpressed reductases in cancer cells.[3,23,32] The steric hindrance imparted by the three methyl groups on the quinone moiety (the "trimethyl lock") has been shown to induce intramolecular cyclization to release the active drugs from the prodrugs in the target sites (Figure 2).[24,33] At the beginning of the design of the prodrug(s), we found that upon acetylation of CuB, the 2-hydroxy group was more readily acetylated than the 16-hydroxy group, whereas the 20-hydroxy group was relatively inert under normal experimental condition. The result indicated that attachment of the bioreductive unit at the 16-hydroxy group would need two extra synthetic steps: protection and de-protection of the more active 2-hydroxy group. We therefore decided to attach the bioreductive unit at the 2-hydroxy position. Following such a bioreductive prodrug concept, CuB was converted into prodrug molecules **1**, **2** and **3**, in which the quinone system was introduced through the carbamate and ester linkages (Figure 2).

Scheme 1. Synthetic Procedures for Compounds 5−8[a]

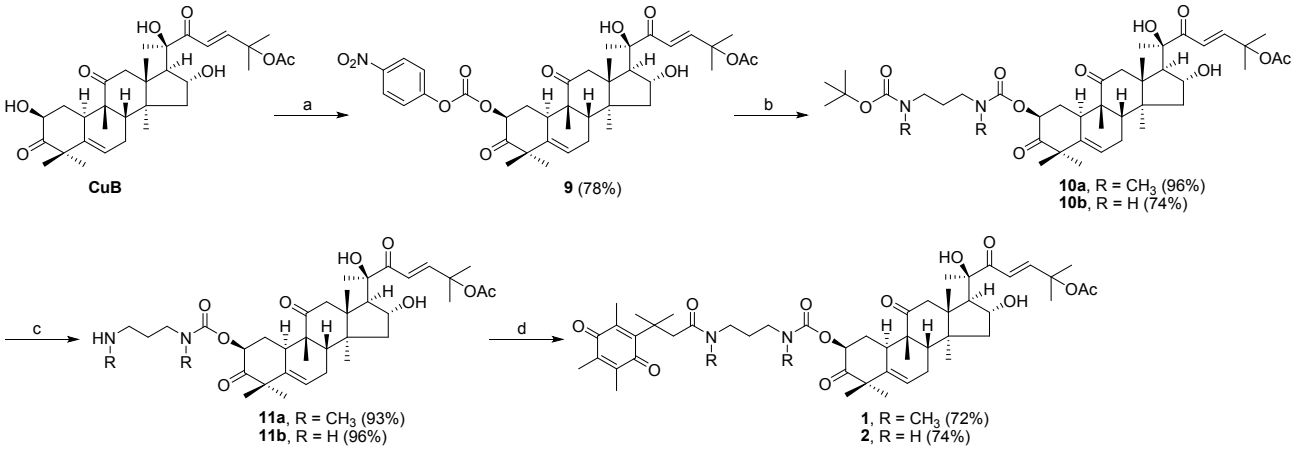


[a]Reagents and conditions: (a) 3,3-dimethylacrylic acid, CH_3SO_3H, 70 °C; (b) NBS, acetone, water; (c) Boc_2O, DCM.

The synthesis of prodrugs **1**, **2** and **3** is depicted in Schemes 1–3. First of all, reaction between trimethylhydroquinone (**4**) and 3,3-dimethylacrylic acid in the presence of dry methanesulfonic acid provided **5**, which was further treated with *N*-bromosuccinimide (NBS) in aqueous acetone to afford

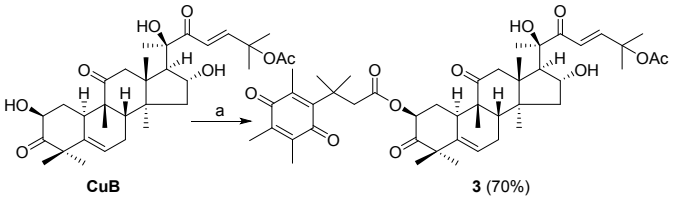
6. The 1,3-diaminopropanes **7a** and **7b** were treated with di-*tert*-butyldicarbonate (Boc_2O) in DCM, yielding the mono-Boc-protected linkers **8a** and **8b** (Scheme 1).

Scheme 2. Synthesis of Prodrugs 1 and 2[a]



[a]Reagents and conditions: (a) 4-nitrophenyl chloroformate, Et_3N, DCM, 0 °C; (b) compounds **8a** and **8b**, Et_3N, DCM; (c) 10% TFA, DCM; (d) compound **6**, EDCI, DMAP, DCM.

Scheme 3. Synthesis of Prodrug 3[a]



[a]Reagents and condition: (a) **6**, EDCI, cat. DMAP, DCM.

Connection of the quinone bioreductive unit **6** to CuB is summarized in Scheme 2. CuB was treated with 4-nitrophenyl chloroformate in DCM in the presence of Et_3N to furnish compound **9**. The attachment of the leaving group at the 2-position was evident from large down-field shifts of the NMR chemical shifts from CuB (δ_H 4.39 and δ_C 71.6) to **9** (δ_H 5.39 and δ_C 77.7) and was further confirmed by HMBC experiments (see Supporting Information). Parallel condensation of **9** with the linkers **8a** and **8b**, respectively, provided **10a** and **10b**. Treatment of **10a** and **10b** with 10% TFA gave amine intermediates **11a** and **11b**, in parallel. Finally, quinone unit **6** was coupled to amines **11a** and **11b** using EDCI and a catalytic amount of DMAP to yield the prodrugs **1** and **2**, respectively (Scheme 2). Following similar procedure, quinone **6** was directly conjugated to CuB to afford prodrug **3** (Scheme 3). The purity of the prodrugs **1**–**3** was checked by HPLC (see Figure S1).

Stability of the prodrug is an important parameter for further bioassays *in vitro* and *in vivo*. The stability of prodrugs **1**, **2** and **3** was examined in the cell culture medium, DMEM with 10% FBS for a period of 72 h by using HPLC. The results show that all prodrugs were reasonably stable under the experiment conditions, and more than 90% of the tested molecules remained after 24 h. Chemically, the carbamate functionality applied in the prodrug linkage of **1** and **2** is expected to be more stable than the corresponding ester functionality of prodrug **3** to bind CuB with the quinone moiety. The experiment confirmed that prodrugs **1** and **2** were more stable than prodrug **3** (see Figure S2). In addition, it was also observed that the 1,3-di-*N*-methylaminopropyl linkage of **1** showed better stability than the unsubstituted 1,3-diaminopropyl linker employed in compound **2**.

CuB and the prodrugs **1**, **2** and **3** were evaluated for their cytotoxicity against breast cancer (MCF-7) and non-cancerous Vero (African green monkey kidney) cells using the MTT assay. Tamoxifen (TAM), a well established breast cancer drug,[34] was used as a positive control. As shown in Table 1, CuB exhibited potent activity toward MCF-7 cells (IC_{50} 12.0 μM) and it was highly toxic toward the noncancerous Vero cells (IC_{50} 0.04 μM). The prodrugs **1**, **2** and **3** exhibited better cytotoxicity over TAM (IC_{50} 22.6 μM) against MCF-7 cells with IC_{50} values of 18.1, 15.4 and 16.6 μM, respectively. Compared with CuB, the prodrugs **1**, **2** and **3** showed lower cytotoxic activity towards the Vero cells, with an IC_{50} values of 12.4, 1.87 and 0.07 μM, respectively. They are approximately 310-, 47- and 2-fold less toxic than CuB, respectively. Based on the above, significant decrease of the cytotoxic action against the normal Vero cells while keeping its cytotoxic effect against the cancerous MCF-7 cells mentions that the bioreductive prodrug design of CuB works by aid of the reductase overexpressed in the cancer cells. To further verify the role of NQO1 in the drug release process in the cells, we also performed MTT assays for MCF-7 cells pretreated with dicoumarol (DIC), an inhibitor of NQO1.[35] Upon pretreatment with DIC, the cell inhibition decreased significantly (Table 1), suggesting that the prodrugs exerted its cytotoxicity depending on NQO1 bioreductive activation. We also evaluated whether the prodrugs could be efficiently reduced by NQO1 and could specifically release CuB[3] (see Figure S3).

To get an insight into how the prodrug releases drug (CuB) or other active species with the reductases in cancer cells, cellular uptake study was performed using MCF-7 cells, a typical reductase-overexpressing cancer cells.[12] First, MCF-7 cells were treated with 25 μM prodrugs, and the cells were lysed after 24 h incubation. The concentrations of prodrug in

the culture medium and cell lysate were determined by HPLC with a calibration curve.[2] As shown in Figure 3A, the concentrations of **1** and **3** in culture medium decreased to 8.39 and 6.21 μM after 24 h incubation, respectively. However, the concentration of **2** in the culture medium was not detected. This might be caused by better cell permeability of prodrug **2** compared with those of prodrugs **1** and **3**.

Table 1. *In Vitro* Cytotoxicity Activity of CuB and Prodrugs

Compound	IC_{50}[a] (μM)			CI[c]
	MCF-7	Vero[b]	MCF-7+DIC	
CuB	12.0 ± 0.4	0.04 ± 0.02	9.72 ± 1.08	–
1	18.1 ± 0.4	12.4 ± 0.5	>100	310
2	15.4 ± 0.2	1.87 ± 0.24	>100	46.8
3	16.6 ± 0.9	0.07 ± 0.02	>100	1.75
TAM	22.6 ± 0.3	–	–	–
Ellipticine	–	4.06 ± 0.58	–	–

[a]Each value was reproduced in three experiments. [b]African green monkey kidney. [c]Cytotoxicity index, IC_{50} of prodrug in Vero compared with IC_{50} of CuB parent in Vero.

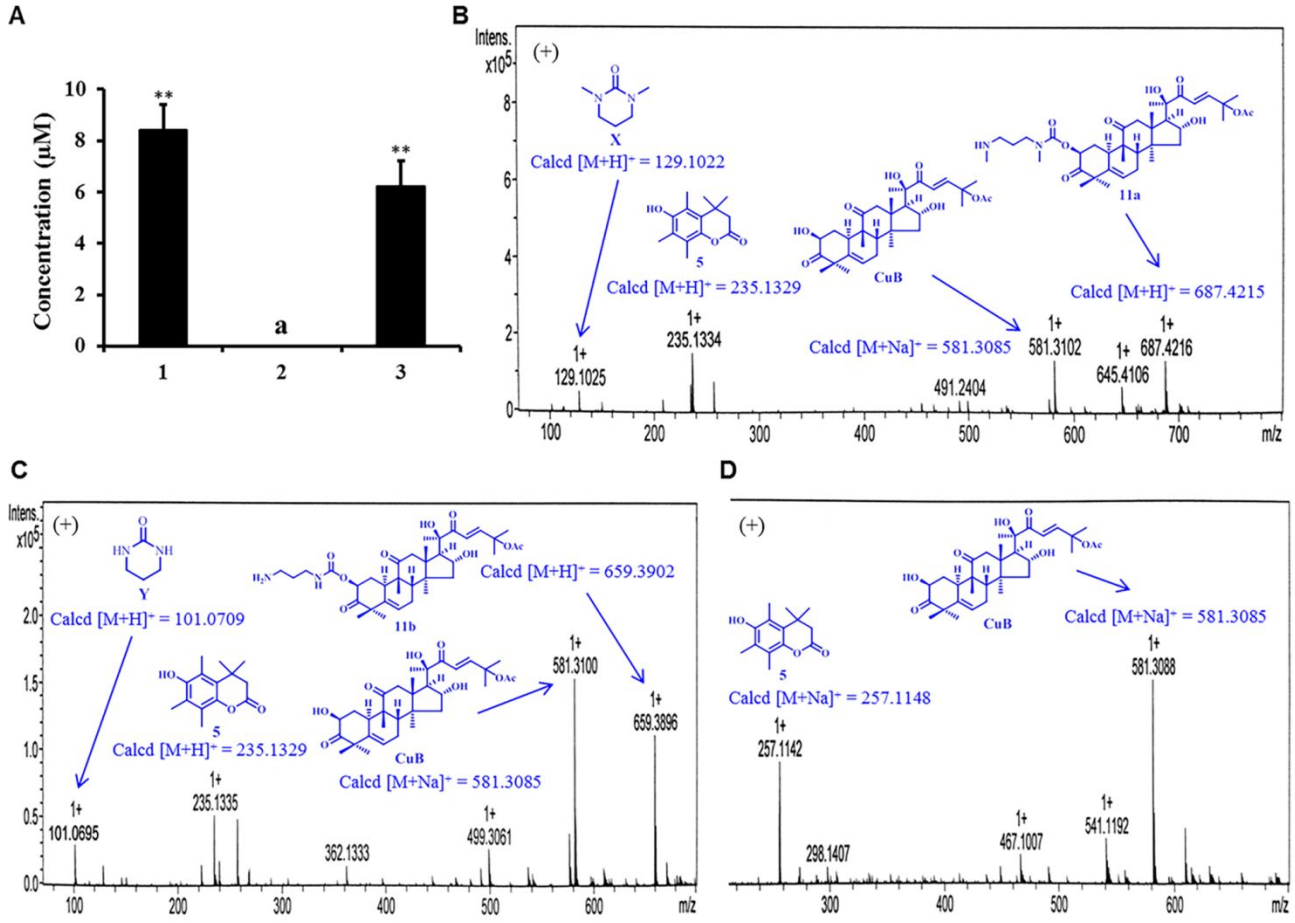


Figure 3. Cellular uptake experiments of prodrugs **1**, **2** and **3**. (A) Concentration of-prodrugs **1** and **3** in cell culture media after 24 h incubation. [a]Not detected by HPLC. ** $P < 0.01$ (n = 3). (B), (C), (D) HRMS analyses for prodrugs **1**, **2** and **3** in cell lysate after 24 h incubation, respectively.

Next, we examined whether the prodrugs could release therapeutic drug CuB in the cells. As shown in Figure 3, prodrugs **1**, **2** and **3** efficiently released CuB in the cell lysate, while the intermediates **11a** and **11b** were also detected in the cell lysate

of **1** and **2**, respectively. Very interestingly, **1**, **2** and **3** in the cell lysate were not detected by HPLC. This highly mentions that the transformation of prodrugs to CuB is very efficient in the cancer cells by aid of the corresponding reductases. Furthermore, observation of the anticipated compounds or intermediates released from the prodrugs by high resolution ESI-TOFMS analysis confirmed such a conclusion. For the prodrug **1**, the corresponding ions including CuB ($[M + Na]^+$ at m/z = 581.3102), **11a** ($[M + H]^+$ at m/z = 687.4216) and the lactone **5** ($[M + H]^+$ at m/z = 235.1334) were detected (Figure 3B). In addition, the ions of CuB and the lactone **5** were also detected by HRMS for prodrugs **2** and **3** (Figure 3C and 3D, respectively). Moreover, the $[M + H]^+$ ion at m/z 659.3896 indicated the presence of intermediate **11b** in the cell lysate of **2** (Figure 3C). It is noteworthy that the byproducts cyclic ureas **X** and **Y** (see Figure 2) were undetectable in the cell lysate under the HPLC conditions, because they are not UV-detectable. However, both compounds showed low-intensity ion peaks $[M + H]^+$ ion at m/z 129.1025 and $[M + H]^+$ ion at m/z 101.0695 in the HRMS analysis (see Figures 3B and 3C, respectively). It should also be noted that the ion peaks of the prodrugs **1**, **2** and **3** at the extended m/z range to 1000 were not detected (see Figure S4). The cytotoxicity of the lactone **5** and cyclic ureas **X** and **Y** against MCF-7 and Vero cells using the MTT assay was evaluated and they were found to be nontoxic (see Table S1).

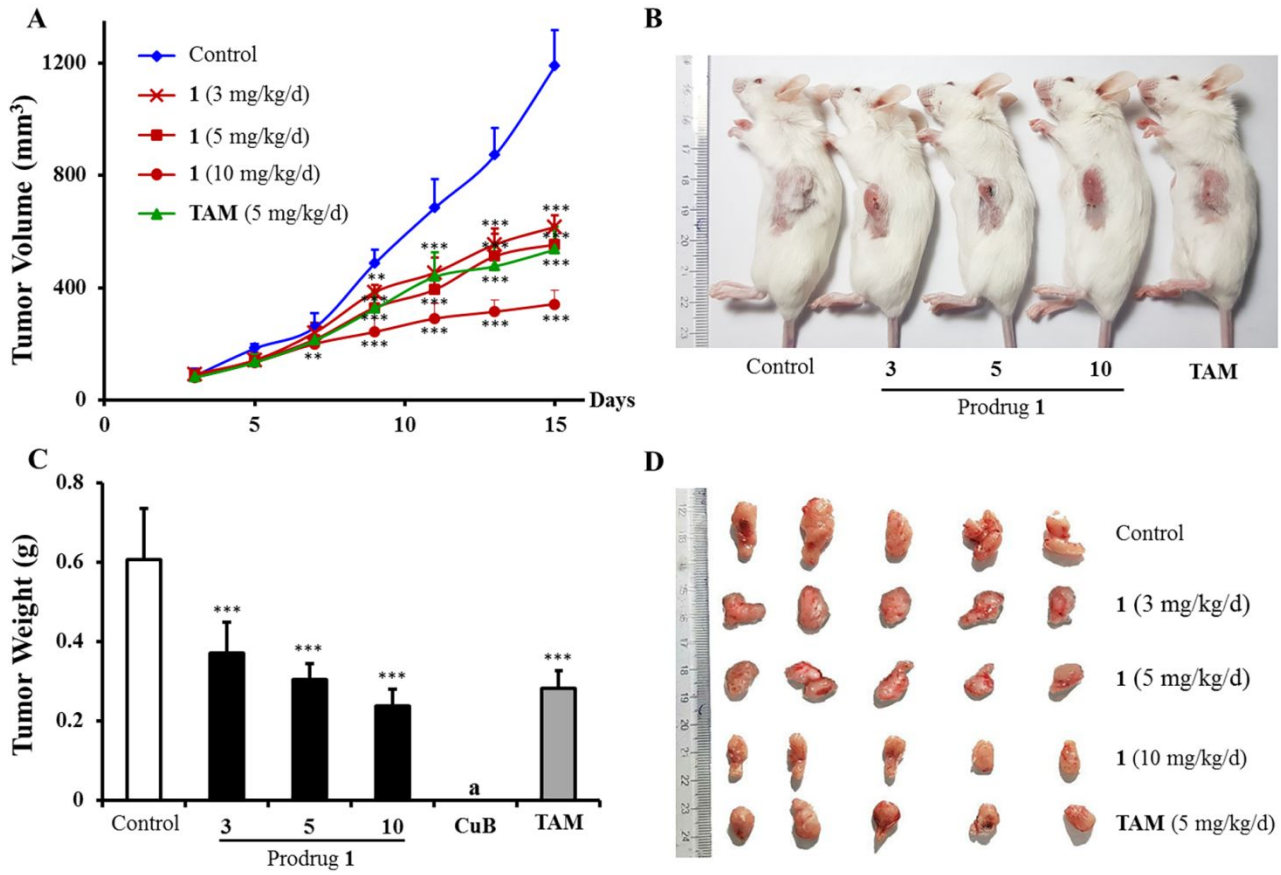


Figure 4. *In vivo* antitumor activity of prodrug **1** on tumor growth against 4T1 xenograft in BALB/c mice. (A) Growth curves of tumor volume and days were plotted. (B) xenografted mice after being sacrificed. (C) Mean tumor weight, and (D) tumor photographs after mice were sacrificed. [a]All mice died, and the tumor weight of the CuB (3 mg/kg/d) treated group was not determinable. ** $P < 0.01$, *** $P < 0.001$ vs Control group; Student's t test (n = 6).

The possibility of deactivation of prodrugs by thiol-containing species[36] was also investigated. The prodrugs **1**–**3** was treated with glutathione (GSH), but no reaction was observed (Supporting Information).

Since the prodrug **1** exhibited significantly decreased toxicity against normal cells, it was therefore selected for *in vivo* antitumor study using the BALB/c mice model with breast cancer cells (4T1),[37,38] and CuB and TAM were used as comparison controls. The antitumor efficacy of different dosages of the prodrug **1** (3, 5 and 10 mg/kg/d), CuB (3 mg/kg/d) and TAM (5 mg/kg/d) were tested in the animal model. As shown in Figure 4A, all concentrations of **1** showed significant inhibitory effects on tumor growth. The tumor growth inhibition (TGI) rate of **1** at dose of 5 mg/kg/d was 53.8%, which was comparable to that of TAM (55.0%, 5 mg/kg/d) (Table S2). The excised tumors from the control animals ranged from 500 to 700 mg, while all concentrations of the prodrug **1**-treated tumors weighed less than 400 mg (Figure 4B, 4C and 4D). In addition, both dosages of **1** (3 and 5 mg/kg/d) showed increase in body weight (6.46 and 5.27%, respectively) compared with the control group (−1.48%) and TAM (4.99%). The prodrug **1** at 10 mg/kg/d group exhibited significant body weight loss −2.49% (Figure S5 and Table S2), which could mainly ascribe to the tumor weight being

inhibited. It is noteworthy that, when a dose of 3.0 mg/kg/d of CuB was used *in vivo*, all treated mice died after one day of CuB administration for its high *in vitro* cytotoxicity. These data clearly mention that the prodrug **1** is a successful design to reduce the *in vivo* toxicity of CuB, and it might be a therapeutically promising and less toxic agent for potential cancer treatment.

In summary, we present a new successful example to convert a highly toxic natural product into potentially useful and less toxic anticancer compounds using cellular degradable prodrug design. Three bioreductive prodrugs (**1**, **2** and **3**) were synthesized from CuB, the major constituent of *T. cucumerina*. Our study showed that these prodrugs significantly reduced toxicity against non-cancerous cells than CuB and maintained the original actions against cancer cells. The experiments also confirmed that the prodrugs could efficiently release CuB in the reductase-overexpressing MCF-7 cells. Among them, the prodrug **1** exhibited significant toxicity reduction in both *in vitro* and *in vivo* studies, and showed a comparable tumor growth inhibition in the 4T1 xenograft mice model at a dose of 5 mg/kg/d by comparison with tamoxifen (TAM).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental section, HPLC purity analyses of prodrugs **1**, **2** and **3**; Stability examination of prodrugs **1**, **2** and **3** in culture medium; In vitro cytotoxic activity of lactone **5**, cyclic ureas **X** and **Y**; HPLC assay for drug release studies; Drug release studies as a function of time in the presence of NQO1 and NADPH in PBS; Stability of prodrugs under the GSH activation; The original HRMS analysis of prodrugs **1**, **2** and **3** in cell lysate after 24 h incubation; *In vivo* antitumor activity of prodrug **1** on mice body weight; *In vivo* effects of prodrug **1** and CuB. General; Isolation of cucurbitacin B; Synthesis of compounds **5**, **6**, **8a**, **8b**, **9**, **10a**, **10b**, **11a** and **11b** and spectroscopic data; Synthesis of prodrugs **1**, **2** and **3** and spectroscopic data; and NMR spectral copies of all the compounds shown in Schemes 1–3 (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CuB, cucurbitacin B; TAM, tamoxifen; NBS, *N*-bromosuccinimide; Boc_2O, di-*tert*-butyldicarbonate; DCM, dichloromethane; TFA, trifluoroacetic acid; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, *N,N*-dimethyl-4-aminopyridine; DIC, dicoumarol; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline

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For Table of Contents