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Synthesis and Antitumor Properties of BQC-Glucuronide, a Camptothecin Prodrug for Selective Tumor Activation

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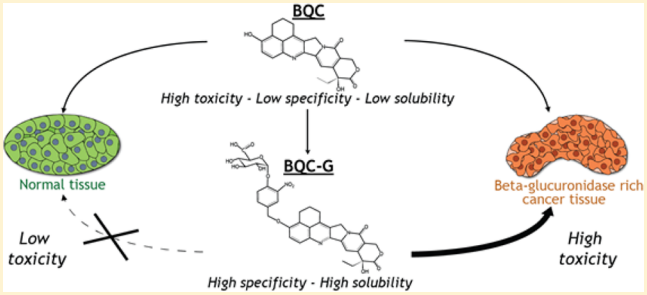
Supporting Information

ABSTRACT: Major limitations of camptothecin anticancer drugs (toxicity, nonselectivity, water insolubility, inactivation by human serum albumin) may be improved by creating glucuronide prodrugs that rely on beta-glucuronidase for their activation. We found that the camptothecin derivative 5,6-dihydro-4*H*-benzo[*de*]quinoline-camptothecin (BQC) displays greater cytotoxicity against cancer cells than the clinically used camptothecin derivatives SN-38 and topotecan even in the presence of human serum albumin. We synthesized the prodrug BQC-glucuronide (BQC-G), which was 4000 times more water soluble and 20−40 times less cytotoxic than BQC. Importantly, even in the presence of human serum albumin, BQC-G was efficiently hydrolyzed by beta-glucuronidase and produced greater cytotoxicity (IC_{50} = 13 nM) than camptothecin, 9-aminocamptothecin, SN-38, or topotecan (IC_{50} > 3000, 1370, 48, and 28 nM, respectively). BQC-G treatment of mice bearing human colon cancer xenografts with naturally or artificially elevated beta-glucuronidase activity produced significant antitumor activity, showing that BQC-G is a potent prodrug suitable for selective intratumoral drug activation.



KEYWORDS: *camptothecin, glucuronide, prodrug, cancer, targeted therapy, beta-glucuronidase, albumin influence, ADEPT*

INTRODUCTION

One of the major obstacles of cancer chemotherapy is lack of tumor specificity, which leads to life-threatening toxicity. Tumor cells secrete factors that promote an inflammatory microenvironment enriched in activated myeloid cells,[1,2] which play a crucial role in facilitating tumor progression, metastasis, and angiogenesis,[3,4] and at the same time change enzymatic patterns in the tumor tissue. Treatment of solid tumors with prodrugs that may be selectively activated by enzymes secreted by either cancer cells or tumor-infiltrating macrophages and neutrophils is under investigation to enhance the therapeutic index of cancer chemotherapy.

Human beta-glucuronidase is a lysosomal enzyme that accumulates in the tumor microenvironment due to release from necrotic tumor cells[5] and tumor-infiltrating macrophages and neutrophils.[6−11] Beta-glucuronidase activity at tumors is also enhanced by the acidic pH found in the interstitial tumor space.[12−14] Besides relying on the accumulation of beta-glucuronidase by natural mechanisms, several methods to enrich beta-glucuronidase activity in tumors are under development, including targeting beta-glucuronidase as an antibody−enzyme conjugate (ADEPT), expressing beta-glucuronidase from viral or nonviral vectors (GDEPT), selective enrichment of beta-glucuronidase expressing bacteria in tumors (Bac-DEPT), induction of beta-glucuronidase release by cytostatic agents, and use of injectable hydrogels containing beta-glucuronidase.[8,15−25] Glucuronide derivatives are generally less toxic than the parental compounds because they are water-soluble and do not enter cells. Beta-glucuronidase and glucuronides are therefore an ideal combination for the design of nontoxic and water-soluble prodrugs to take advantage of the intratumoral accumulation of macrophages and neutrophils or targeting approaches to elevate enzyme levels in the tumor microenvironment leading to local tumor-specific release of active drug.

Camptothecin (CPT, Figure 1) is a potent topoisomerase I poison which stabilizes the covalent attachment of topoisomerase I to single-strand DNA breaks, leading to S-phase specific cell death.[26] However, difficulties in formulating the highly

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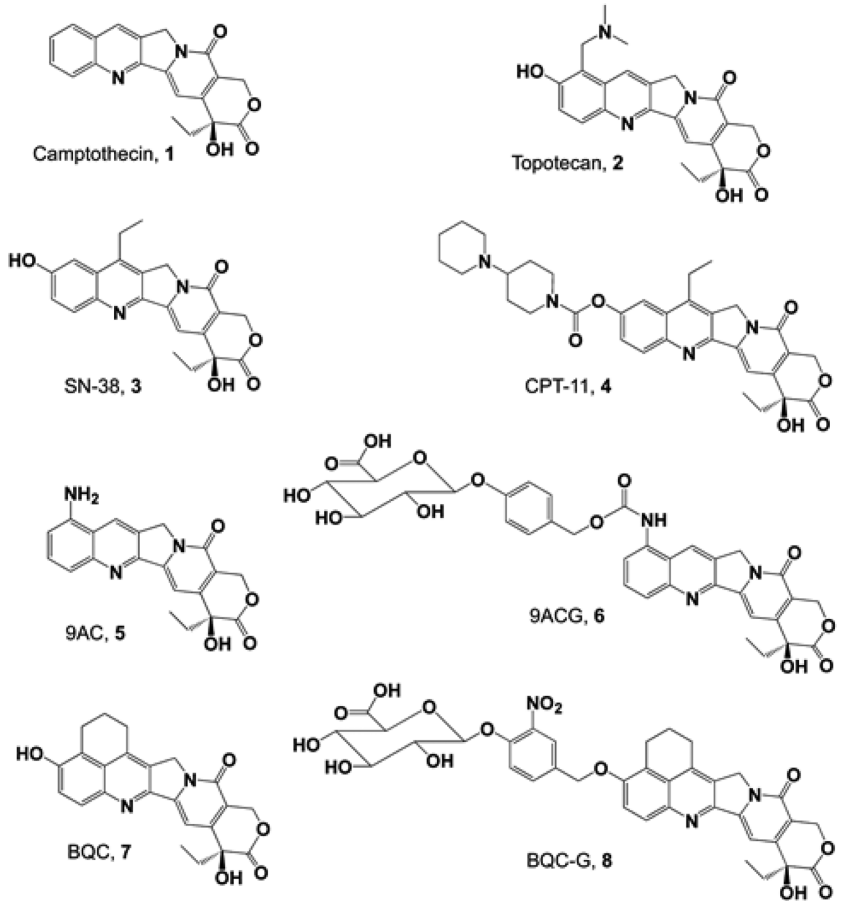

Figure 1. Structure of CPT derivatives.

water-insoluble lactone form of the drug led to the use of its salt form during human trials, which resulted in unpredictable toxicity and lack of significant antitumor activity, causing its abandonment for decades. The subsequent discovery of the mechanism of action which emphasizes the necessity of the intact lactone form of the drug and the realization that anticancer activity was greatly reduced by human serum albumin (HSA) led to the development of analogues with better activity in patients.[27,28] The camptothecin analogue, topotecan (Hycamptin), is approved for the treatment of ovarian, cervical, and small cell lung cancer while (CPT-11) (Camptosar), a prodrug of 7-ethyl-10-hydroxycamptothecin (SN-38), is approved for the therapy of colorectal carcinoma. Several other analogues are in different stages of development.

Previous studies by us and others have demonstrated that glucuronide prodrugs of antineoplastic agents can elicit selective antitumor activity in animal models of human cancer.[6,9,29,30] We previously described the conjugation of glucuronic acid to 9-aminocamptothecin (9AC) via a self-immolative carbamate linker to create the glucuronide derivative 9-aminocamptothecin-*N*-(benzyl oxycarbamate)-*β*-D-glucuronide (9ACG).[31] 9ACG displayed enhanced solubility and remarkable potency against solid human tumor xenografts in mice.[30,31] However, in common with CPT,[32] binding to HSA greatly reduced the anticancer activity of 9ACG.[33,34] By contrast, topotecan and irinotecan bind more weakly to HSA and remain active in the presence of physiological concentrations of HSA, a key property for their use in humans.[28] Development of tumor-selective camptothecin derivatives that are effective in the presence of HSA is therefore highly desirable.

Toward the goal of developing a CPT-glucuronide derivative that retains the positive attributes of 9ACG but without strong albumin binding, we turned to a novel 6-ring CPT derivative 5,6-dihydro-4*H*-benzo[*de*]quinoline-camptothecin (BQC, 7).[35] BQC is among the most active camptothecin analogues in vitro and displayed impressive antitumor activity against P388 leukemia in mice.[36] In addition, based on the limited albumin binding of SN-38 and topotecan,[28,32] which possess functional groups at the C7 and C9 positions, respectively, we hypothesized that the presence of a ring spanning the C7 to C9 position in BQC would minimize drug binding to HSA, making it an ideal candidate for generating a glucuronide prodrug. Here we describe the synthesis and antitumor activity of BQC-10-*O*-[3-nitrobenzyloxy-4-*O*-(*β*-D-glucopyranuronate)] (BQC-G), in which a glucuronide group was linked to BQC via an improved self-immolative benzyl−ether linker, aiming to generate an efficient prodrug suitable for selective cancer therapy.

MATERIALS AND METHODS

Chemicals. BQC was a generous gift from Daiichi (Tokyo, Japan). CPT, irinotecan, *p*-nitrophenyl-*β*-D-glucuronide (PNPG), 5-chloro-4-bromo-3-indolyl-*β*-D-glucuronide, and 4-methylumbelliferyl-*β*-D-glucuronide (4-MUG) were from Sigma-Aldrich (St. Louis, MO). SN-38 was from ScinoPharm (Shan-Hua, Taiwan). Topotecan hydrochloride was from SmithKline Beecham Pharmaceuticals (Philadelphia, PA).

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9ACG was synthesized as described and further purified by preparative HPLC.[31,37] Coarse (40−63 μm particle size) LiChroprep C18 for SPE was from Merck (Darmstadt, Germany). C-18 analytical column (μBondapak 10 μm, 3.9 mm × 300 mm) with a Guard-pak precolumn insert was from Waters (Milford, Massachusetts, U.S.A.).

Cell Lines and Animals. All cancer cells were from the American Type Culture Collection (Manassas, VA) except for CL1-5 human lung adenocarcinoma cells,[38] which were a generous gift from Dr. Pan-Chyr Yang (National Taiwan University, College of Medicine), and EJ human bladder carcinoma cells,[39] which were a gift from Dr. Konan Peck (IBMS, Academia Sinica, Taiwan). LS174T/mβG cells, which express membrane-tethered beta-glucuronidase, were described earlier.[24]

Eight to 10 week old female BALB/c *nu/nu* mice obtained from the National Laboratory Animal Center (Taipei, Taiwan) were maintained under specific pathogen free conditions and artificial circadian rhythm, with free access to food and water. All animal experiments were conducted in accordance with institutional guidelines and approved by the Animal Care and Use Committee of the Institute of Biomedical Sciences, Academia Sinica.

Synthetic Procedures. The detailed synthetic procedures, NMR peak assignments, and the schemes of the reactions as well as the original preparative and analytical HPLC procedures are given in the Supporting Information.

Drug Characterization. To measure solubility, BQC or BQC-G was added to water, 0.1 M acetate buffer pH 4.5, or phosphate buffer pH 7.0 and equilibrated 24 h at room temperature with shaking. Mixtures were centrifuged for 40 min at 20000*g* at room temperature. Samples were analyzed by HPLC and quantified using standard curves generated with pure BQC and BQC-G. To measure the octanol/water partition coefficient, 1 μg of either BQC or BQC-G was added to an equal volume mixture of octanol and 0.1 M acetate buffer, pH 4.5 or 0.1 M phosphate buffer, pH 7.0. After mixing for 24 h at room temperature, the samples were centrifuged for 20 min at 10000*g* before the layers were analyzed by HPLC.

Blood stability was determined by spiking normal human plasma with BQC-G to 5 μM. The mixture was incubated for 24 h at 37 °C, during which time samples were periodically removed and analyzed by SPE-HPLC. Blood compartmentalization was determined by adding drugs to fresh mouse blood for 10 min. Plasma was separated from the blood cells by centrifugation, and the concentrations of the drugs in the full blood, plasma, and the erythrocyte fractions were determined by SPE-HPLC.

Hydrolysis of BQC-G by Beta-Glucuronidase. 20 ng of recombinant *Escherichia coli* beta-glucuronidase or 2 μg of human beta-glucuronidase was added to 1 mL of BQC-G (5 μM) in 0.1 M phosphate buffer pH 7.0 (for *E. coli* beta-glucuronidase) or pH 4.5 (for human beta-glucuronidase) and incubated at 37 °C. Samples of 100 μL were periodically removed, and BQC and BQC-G were quantified by SPE-HPLC. To measure the enzyme kinetics of drug hydrolysis, defined amounts of *E. coli* beta-glucuronidase in 0.1 M phosphate buffer containing 0.1% BSA, pH 7.0 (90 μL) at 37 °C was mixed with 10 μL of substrate over a concentration range of ∼0.1 to 10 × K_m for 15 to 60 min. For PNPG and 4-MUG, the reaction was stopped by adding 100 μL of 1 M glycine/0.5 M carbonate buffer, pH 11.0 and absorbance at 405 nm or fluorescence at excitation/emission wavelengths of 365/455 nm was measured. For BQC-G and 9ACG, the reaction was stopped by addition of 100 μL of 0.2 M TCA. Samples were diluted in 200 μL of 0.1 M KH_2PO_4, pH 2.9 and analyzed by SPE-HPLC as described in the Supporting Information.

In Vitro Cancer Cell Proliferation. To compare the effects of HSA on the inhibition of cancer cell proliferation by 9AC or BQC, 5000 EJ or CL1-5 cells were cultured in each well of a 96-well plate overnight. 100 nM 9AC or BQC was added to the cells in the presence of increasing concentrations of HSA for 48 h. The cells were washed twice with PBS and incubated in fresh medium for 24 h before the cells were pulsed with 1 μCi/well of ^{3}H-thymidine for 16 h. The incorporation of ^{3}H-thymidine in newly synthesized DNA was measured on a Topcount scintillation counter.

The antiproliferative effect of selected camptothecin analogues was evaluated by adding serial dilutions of the drugs to 96-well plates containing 5000−10,000 cancer cells per well. HSA was added at concentrations of 0 and 40 mg/mL to determine the influence of serum albumin of drug activity. *E. coli* beta-glucuronidase (1 μg/well) was added in some wells to activate glucuronide prodrugs. After 48 h, the cells were washed twice with PBS, incubated in fresh medium for 24 h, and pulsed with 1 μCi/well of ^{3}H-thymidine for 16 h, and radioactivity was measured on a Topcount scintillation counter.

The proliferation of cancer cells exposed to drugs was compared to untreated control cancer cells by the following formula: cell proliferation (% control) = 100 × (drug treated cells cpm)/(untreated cells cpm). IC_{50} values were calculated by fitting the data to a sigmoid dose/response equation.

Drug Pharmacokinetics. Mice were injected iv with 10 mg/kg BQC or 25 mg/kg BQC-G. Blood samples were collected in heparinized hematocrit glass capillary tubes and spun down for 3 min at 3000*g*. The recovered plasma was diluted 2-fold with 20% acetonitrile in 20 mM potassium-phosphate buffer containing 0.2 M TCA, vortex-mixed, and then spun down for 5 min at 20000*g*. The clear supernatant was analyzed by SPE-HPLC as described above.

Flow Cytometry. LS174T and LS174T/mβG cells were stained with a rat antibody against beta-glucuronidase (7G7)[16] followed by a goat antibody against rat IgG (H+L) conjugated with FITC (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cell viability was confirmed by propidium iodide staining. The surface immunofluorescence of 10,000 viable cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, PA), and fluorescence intensities were analyzed with Flowjo V7.2.5 (Tree Star Inc., Ashland, OR).

In Vivo Antitumor Activity. BALB/c *nu/nu* female mice were sc injected in the right flank with 3 × 10^6 LS174T or LS174T/mβG human colon cancer cells. Mice bearing established tumors were iv injected with vehicle only (20% DMSO, 2% mannitol, 0.5% citric acid, and 0.1% Tween 20 in water), 10 mg/kg BQC, or 25 mg/kg BQC-G on day 10 when average tumor size was 125−150 mm^3. The treatment was repeated on day 20 for all of the groups except for the LS174T/mβG BQC-G group, which was not treated a second time due to the limited amounts of BQC-G available. The tumor size and body mass were followed every 2−3 days for 3 weeks. The experiment was terminated when the first mouse in the control group died.

Statistical Analysis. Statistical significance of differences between mean values was estimated with GraphPad Prism Version 5 using the unpaired (independent) *t* test with Welch's

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Table 1. Solubility and Partition Coefficients of BQC and BQC-G[a]

	BQC			BQC-G		
	water	buffer pH 4.5	buffer pH 7.4	water	buffer pH 4.5	buffer pH 7.4
solubility (μg/mL)	0.200 ± 0.02	0.125 ± 0.003	0.200 ± 0.005	nd	nd	775 ± 50
Po/w	1.91 ± 0.02	1.67 ± 0.01	1.46 ± 0.01	−0.44 ± 0.01	−0.44 ± 0.02	−1.01 ± 0.04

[a]Mean values and standard deviations of three independent measurements are shown; nd, not determined.

correction without assuming equal variances. P values of ≤0.05 were considered statistically significant.

RESULTS

BQC-G Synthesis and Characterization. BQC-G was synthesized by linking BQC to glucuronic acid via a self-immolative benzyl-ether spacer (Supporting Information Schemes I–III). Self-immolative linkers facilitate chemical synthesis of glucuronide prodrugs and can enhance the rate of hydrolysis by beta-glucuronidase.[31,37] To maximize the usefulness of the new prodrug, we used a new and improved benzyl-ether linker rather than the classical carbamate linker used for 9-ACG. Highly purified BQC-G (>99.90%) was obtained by preparative HPLC on a reverse phase C18 column.

BQC was poorly soluble in water-based buffers (125 ng/mL at pH 4.5 and 200 ng/mL at pH = 7.0, Table 1). The octanol/water partition coefficient was 1.67 and 1.46 at pH 4.5 and 7.0, respectively, indicating a strong preference for the octanol phase. The greater solubility of BQC at pH 7.0 is probably due to spontaneous, pH-mediated opening of the lactone ring to the carboxy form. The low aqueous solubility of BCQ may cause problems in drug formulation for intravenous administration. BQC-G, by contrast, was almost 4000-fold more soluble in water based buffers (775 μg/mL at pH = 7.0) with an octanol/water partition coefficient of −0.44 and −1.01 at pH 4.5 and 7.0, respectively. BQC-G was stable in PBS and human serum for at least 24 h at 37 °C (Figure 2A).

An important criterion for effective cancer therapy with glucuronide prodrugs is efficient generation of active anticancer drug by hydrolysis of the glucuronide moiety by limiting amounts of beta-glucuronidase in the tumor microenvironment. BQC-G was an excellent substrate for *E. coli* and human beta-glucuronidase and could be completely hydrolyzed by low concentrations of the enzymes to BQC (Figure 2B,C). The kinetic parameters for hydrolysis of BQC-G by beta-glucuronidase compared favorably to 9ACG as well as the widely used beta-glucuronidase substrates PNPG and 4-MUG (Table 2).

In Vitro Inhibition of Cancer Cell Proliferation. A major goal of the current research is to develop a glucuronide derivative of a camptothecin analogue that would display better activity than 9AC in the presence of human serum albumin. We therefore first compared the antiproliferative activity of 9AC and BQC in the presence of increasing concentrations of HSA. Increasing the concentration of HSA from 0 to 50 mg/mL (the approximate concentration of HSA in human serum) resulted in progressive neutralization of the antiproliferative activity of 9AC to EJ human bladder carcinoma cells and CL1-5 human lung cancer cells (Figure 3A). By contrast, even 50 mg/mL HSA did not appreciable neutralize the activity of BQC to the same cells. These results indicate that BQC displays less sensitivity to HSA as compared to 9AC.

Another important characteristic for an anticancer prodrug is reduced cytotoxicity as compared to the parental drug. BQC-G

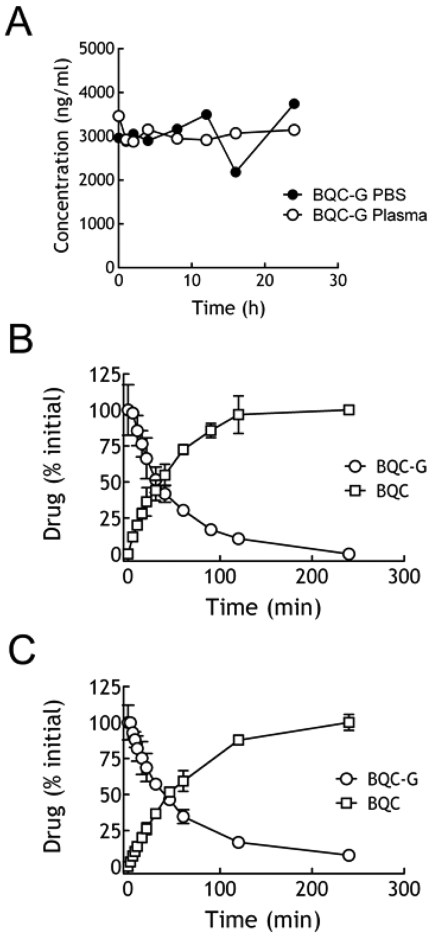

Figure 2. Stability and enzymatic hydrolysis of BQC-G. (A) The concentrations of BQC-G in PBS (●) or human plasma (○) at 37 °C were monitored over 24 h. (B, C) Disappearance of 5 μM BQC-G (○) and appearance of BQC (□) were monitored during incubation with 20 ng/mL *E. coli* (B) or 2 μg/mL human (C) beta-glucuronidase. Mean and standard deviations of triplicate determinations are depicted.

Table 2. Kinetic Parameters for Glucuronide Hydrolysis by Beta-Glucuronidase[a]

compound	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})
BQC-G	11.7 ± 2.0	152	12.9
9ACG	29.5 ± 4.0	59.5	2.0
4-MUG	80.2 ± 12.4	22.2	0.28
PNPG	490 ± 84	173	0.35

[a]Kinetic parameters were determined for hydrolysis of the indicated glucuronides by *E. coli* beta-glucuronidase at pH 7.0.

and BQC were incubated with CL1-5 human lung cancer cells for 48 h, fresh medium was added for another 24 h, and then the incorporation of ^{3}H-thymidine into newly synthesized DNA was measured. The antiproliferative activity of BQC-G to

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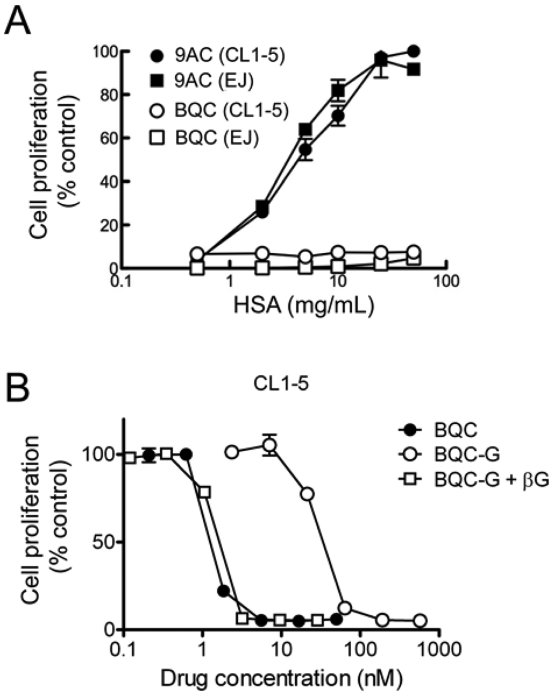

Figure 3. Antiproliferative activity of drugs to human cancer cells. (A) 100 nM 9AC or BQC was added to CL1-5 or EJ cancer cells in triplicate in the presence of increasing concentrations of HSA for 48 h. (B) CL1-5 cells were exposed for 48 h to the indicated concentrations of drugs. ^{3}H-Thymidine incorporation into cellular DNA was measured 24 h later. Results show the percentage of ^{3}H-thymidine incorporated into cellular DNA relative to untreated control cells (n = 3). Bars, SD.

CL1-5 cells was reduced by about 40-fold as compared to BQC, indicating that the glucuronide moiety successfully decreased the activity of BQC-G (Figure 3B). Addition of 1 μg of beta-glucuronidase with BQC-G restored the antiproliferative activity of BQC-G to CL1-5 cells, reflecting effective conversion of BQC-G back to BQC. Similar results were observed with a range of cancer cell lines with BQC displaying IC_{50} values ranging from 1.3 nM to 1.8 nM and BQC-G exhibiting IC_{50} values ranging from 24.6 nM to 58.7 nM (Table 3). Addition of beta-glucuronidase with BQC-G resulted in restoration of drug cytotoxicity, consistent with complete hydrolysis of BQC-G to BQC.

We next compared the effect of HSA on the in vitro activity of BQC-G and 9ACG. Addition of 40 mg/mL HSA with 9ACG to CL1-5 cancer cells reduced the IC_{50} of the prodrug from 650 nM to over 10,000 nM (Table 4). More importantly, HSA also dramatically reduced the IC_{50} of 9ACG incubated with beta-glucuronidase from 26.1 nM to 2170 nM (Table 4). BQC-G was also partially neutralized by HSA, but the IC_{50} of BQC-G activated with beta-glucuronidase was still 13.3 nM even in the presence of 40 mg/mL HSA. These results demonstrate that BQC-G is far less affected than 9ACG by human serum albumin.

We also compared the effect of HSA to the antiproliferative activity of several camptothecin analogues. All camptothecin drugs (BQC, 9AC, CPT, SN-38, and topotecan) displayed similar IC_{50} values ranging from 1.37 to 27.8 nM to CL1-5 human lung cancer cells (Table 4). On the other hand, these drugs displayed up to 150-fold reduced IC_{50} values in the presence of 40 mg/mL HSA. In particular, CPT and 9AC displayed IC_{50} values of over 3000 and 1370 nM, respectively. BQC was the most potent analogue in the presence of HSA with an IC_{50} value of 13 nM followed by the commercially used camptothecin analogues topotecan (28.4 nM) and SN-38 (47.9 nM), the active metabolite of CPT-11. Interestingly, topotecan was the only camptothecin analogue that was unaffected by HSA.

In Vivo Pharmacokinetic and Blood Compartmentalization. The pharmacokinetics of BQC fit a biphase elimination model with an initial half-life of 0.16 h and a terminal half-life of 4.3 h whereas BQC-G was eliminated with an initial half-life of 0.31 h and a terminal half-life of 2.5 h (Figure 4A). The interpolated concentration of BQC-G at t = 0 gave a volume of distribution of about 2 mL, indicating that the drug was slowly distributed from the blood to the body compartments. The distribution volume of BQC was significantly higher (about 10 times) than that of BQC-G. BQC added to whole blood predominately distributed into the blood cell fraction, a behavior noticed with other hydrophobic camptothecin drugs (Figure 4B). By contrast, the more hydrophilic BQC-G was almost entirely located in the plasma fraction.

Table 3. Cytotoxicity of Drugs to Cancer Cells[a]

cell line	IC_{50} (nM)[b] BQC	BQC-G	BQC-G + βG[c]	QIC_{50}[d]
CL1-5	1.37 ± 0.01	58.7 ± 2.4	1.33 ± 0.03	44.1
LS174T	1.31 ± 0.03	24.6 ± 2.8	1.35 ± 0.01	18.2
CaSki	1.25 ± 0.05	31.2 ± 0.5	1.44 ± 0.09	21.7
EJ	1.78 ± 0.08	44.8 ± 0.7	2.0 ± 0.1	22.4

[a]Drugs were incubated with the indicated human cancer cells for 48 h. Results represent mean IC_{50} values ± SD of triplicate determinations. [b]IC_{50} is the concentration of test compound that inhibits incorporation of ^{3}H-thymidine into cellular DNA by 50%. [c]A sample of 1 μg of *E. coli* beta-glucuronidase was added with BQC-G to each well. [d]QIC_{50} is equal to IC_{50} of prodrug/IC_{50} of prodrug in the presence of beta-glucuronidase.

Table 4. Comparison of Prodrug Antiproliferative Activities with or without Human Serum Albumin[a]

compound	IC_{50} (nM)[b] −HSA	+ HSA[c]	HSA cytotoxicity ratio[d]
BQC	1.37 ± 0.01	13.0 ± 0.3	9.5
BQC-G	58.7 ± 2.4	1080 ± 60	18.4
BQC-G + βG[e]	1.33 ± 0.03	13.3 ± 0.03	10
9AC	9.8 ± 0.3	1370 ± 70	140
9ACG	650 ± 40	≫10000	≫15
9ACG + βG	26.1 ± 0.4	2170 ± 50	83
CPT	20.5 ± 1.3	>3000	>146
SN-38	5.25 ± 0.08	47.9 ± 1.9	9.1
topotecan	27.8 ± 0.3	28.4 ± 0.7	1.0

[a]Drugs were incubated with CL1-5 human lung cancer cells for 48 h. Results represent mean IC_{50} values ± SD of triplicate determinations. [b]IC_{50} is the concentration of test compound that inhibits incorporation of ^{3}H-thymidine into cellular DNA by 50%. [c]HSA (40 mg/mL) was added with the test compound to each well. [d]Ratio of IC_{50} value in the presence of HSA to the IC_{50} value without HSA. [e]A sample of 1 μg of *E. coli* beta-glucuronidase was added with prodrug to each well.

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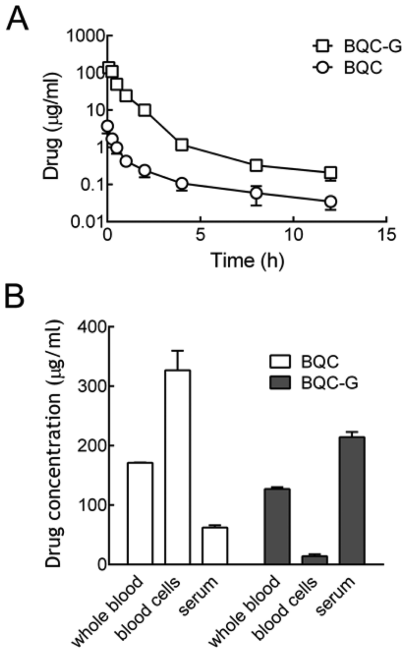

Figure 4. Pharmacokinetics of BQC and BQC-G in mice. (A) Mice were iv injected with 10 mg/kg BQC or 25 mg/kg BQC-G, and drug concentrations in serum samples were determined by SPE-HPLC. (B) Fresh mouse blood was spiked for 10 min with BQC or BQC-G before drug concentrations in whole blood, plasma, and blood cell fractions were measured.

BQC-G Activity against Colon Cancer Xenografts. To examine the suitability of BQC-G for tumor therapy, we employed LS174T human colon cancer xenografts as a model with natural levels of extracellular beta-glucuronidase (probably from tumor cell necrosis[5] and immune cell involvement)[6−11] and LS174T/mβG xenografts, in which beta-glucuronidase is anchored on the surface of cancer cells, as a model of elevated extracellular beta-glucuronidase levels (i.e., by ADEPT, GDEPT, BacDEPT, etc.). Relatively large tumor growth before the treatment (125−150 mm^3) was chosen to allow development of necrosis and immune cell infiltration. LS174T/mβG cells are equally sensitive to camptothecin drugs including BQC-G and display a similar doubling time as parental LS174T cells in vitro.[24]

Both BQC and BQC-G displayed strong antitumor activity and inhibition of tumor growth (Figure 5). The mean tumor size in mice bearing LS174T/mβG tumors treated with BQC or BQC-G was significantly smaller than that in control mice from day 2 after initiation of therapy, while the mean size of LS174T tumors in mice treated with BQC-G was significantly ($p < 0.05$) smaller than that of control tumors from day 4 after initiation of treatment. A modest steady loss of body mass of about 10% over 3 weeks was observed even for the control groups during therapy. Body mass loss for the treated groups did not exceed more than 5% of the control groups, probably caused by reduction of the tumor burden, indicating acceptable treatment toxicity. No signs of diarrhea were observed.

DISCUSSION

Camptothecins have been of great interest for development of potent and more efficient anticancer drugs. However, despite potencies in the low nM range, just two camptothecin analogues (topotecan and irinotecan) are in clinical use. High toxicity, low selectivity, and poor water solubility are major limitations which have hindered the translation of many camptothecin drugs to clinical practice. Additionally, strong binding by HSA neutralizes the antitumor activity of many camptothecins.[28,34,40] In the present study, we sought to develop a new camptothecin-based prodrug that retains activity in the presence of HSA with potential for human clinical use.

We demonstrate here that BQC is highly active against a panel of several cancer cell lines in vitro, being more potent even than SN-38, the active metabolite of the camptothecin

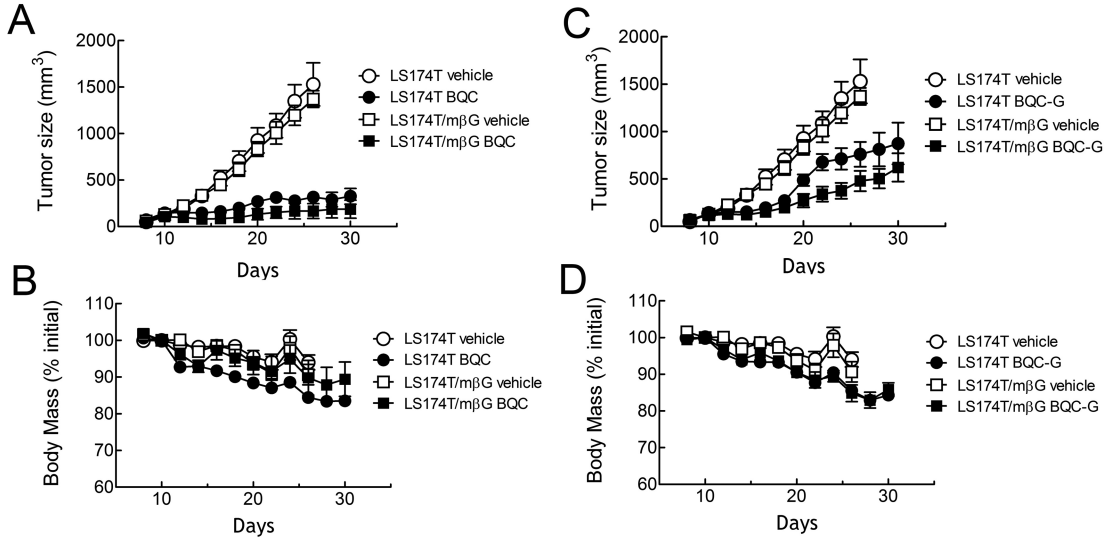

Figure 5. Antitumor activity of BQC-G. BALB/c *nu/nu* female mice were implanted with LS174T or LS174T/mβG human colon cancer cells on day 1. Groups of 6−7 mice were iv injected with vehicle, 10 mg/kg BQC (A, B) or 25 mg/kg BQC-G (C, D) on day 10. The same therapy was repeated on day 20 for all groups except the LS174T/mβG group treated with BQC-G, which did not receive a second dose of prodrug. Results show mean tumor sizes ± SEM (A, C) and mean body weights ± SEM (% initial) (B, D). Significant differences ($p < 0.05$) were observed from day 12 for all the treated groups compared to groups treated with vehicle while highly significant differences ($p < 0.005$) were observed after day 14 for all the groups except for the LS174T/BQC-G group.

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prodrug irinotecan (CPT-11), the most active clinically used camptothecin derivative. Moreover, BQC retained low nM potency even in the presence of physiological concentrations (40 mg/mL) of HSA and was more active than topotecan, the most HSA-resistant camptothecin derivative. However, in common with many camptothecins, BQC is almost insoluble in aqueous solutions, making formulation difficult. Also, it does not possess selectivity or ability to be targeted.

Glucuronidation of camptothecins can improve their solubility, decrease toxicity, and enhance selectivity by tumor-located activation in beta-glucuronidase-rich tumors, as we previously demonstrated with 9ACG.[9,41] The newly synthesized glucuronide prodrug BQC-G was about 4000 times more water-soluble than BQC, facilitating formulation in water-based systems, yet was substantially less toxic than BQC in vitro. Interestingly, BQC-G displayed even less toxicity in the presence of physiological concentrations of HSA, an important property suggesting less systemic toxicity if applied in humans. BQC-G is also an excellent substrate for beta-glucuronidase with a higher K_{cat}/K_m value than commonly used glucuronide substrates such as PNPG and 4-MUG. The self-immolative benzyl-ether linker present in BQC-G contains a nitro group in the ortho position, which might promote binding of these prodrugs to beta-glucuronidase, resulting in lower K_m values. This facilitates rapid conversion to BQC, resulting in full restoration of antitumor activity in vitro. BQC-G also remained a good substrate in the presence of HSA and was completely hydrolyzed to BQC, retaining low nM cytotoxicity, suggesting that it may be efficiently activated locally if used clinically in humans.

Even though it possesses relatively low toxicity, BQC-G displayed good antitumor activity against LS174T xenografts, comparable with results we previously observed with CPT-11 in the same tumor model.[24] Additionally, a single dose of BQC-G exhibited the same or better antitumor activity against LS174T/mβG tumors, which express beta-glucuronidase on their surface, as did two doses of BQC-G against LS174T tumors, consistent with intratumoral activation of BQC-G to BQC by beta-glucuronidase. Both BQC and BQC-G displayed minimal toxicity with no signs of diarrhea. This is important because diarrhea is one of the dose-limiting complications of irinotecan therapy. This is caused by glucuronidation of the active metabolite SN-38 in the liver followed by hydrolysis of the glucuronide back to SN-38 by microbial beta-glucuronidase in the gut, causing intestinal wall damage and diarrhea.[42−45]

BQC-G is a better substrate for beta-glucuronidase than 9ACG and releases a product that is more potent than topotecan or SN-38, suggesting that this prodrug is an excellent candidate for cancer therapy in tumors with elevated beta-glucuronidase levels. BQC-G may also be a good choice in therapies where the enzyme levels are artificially elevated (ADEPT, GDEPT, BacDEPT, etc.). Although targeting strategies such as ADEPT, GDEPT, and BacDEPT are more complex, they offer the possibility of extending glucuronide prodrug treatment to smaller tumors without necrotic regions and to tumors that are not heavily infiltrated with monocytes.

Both BQC and BQC-G displayed good antitumor activity, but BQC-G may have some advantages. The greater water solubility of BQC-G makes formulation easier. BQC-G also preferentially remains in serum for greater drug availability as compared to BQC, which partitions into erythrocytes, a property described for other camptothecins. The lower toxicity of BQC-G in the presence of HSA may result in safer application with reduced systemic side effects. Finally, activation by intratumoral beta-glucuronidase may allow more selective tumor therapy, further reducing systemic toxicity.

Taken together, these results indicate that BQC-G is a good candidate to increase the therapeutic index and selectivity of cancer chemotherapy and may benefit the clinical treatment of tumors that display elevated levels of beta-glucuronidase due to necrotic release of the enzyme from intracellular stores or due to an inflammatory microenvironment as well as strategies that target beta-glucuronidase to cancer cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00771.

Experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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