Synthesis, Anticancer Activities, Antimicrobial Activities and Bioavailability of Berberine–Bile Acid Analogues

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Abstract: Fifteen berberine–bile acid analogues were synthesized. Anticancer activities of these analogues compared with berberine (BBR) were evaluated *in vitro*; among the analogues, **A4**, **B4**, and **B5** had higher cytotoxicity than that of BBR. Most of the analogues showed higher antimicrobial activity against *Staphylococcus aureus ATCC 25923* and *Staphylococcus albus ATCC 8799* than that of BBR, but *Bacillus subtilis AS 1.398* and *Escherichia coli ATCC 31343* were not sensitive to all of the analogues. **A4** and **B4** were stable in the serum stability assay. **B4** showed promising oral bioavailability in mice.

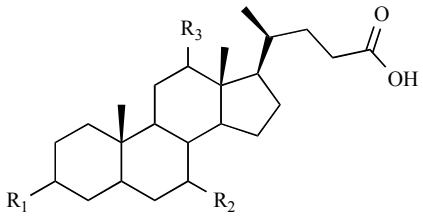
Keywords: Berberine, Bile acids, Synthesis, Anticancer, Antimicrobial, Oral bioavailability.

1. INTRODUCTION

Berberine (BBR), a plant isoquinoline alkaloid extracted from *Coptis chinensis*, has been widely used in China to treat diarrhea safely for decades [1,2]. Berberine also possesses other biological activities such as anticancer [3], antibacterial [4], anti-inflammatory [5], anti-HIV [6], and cholesterol-lowering [7] effects. Berberine is a water-soluble quaternary ammonium salt, but the absorption of berberine in the intestine is poor by oral administration [8]. Low oral bioavailability leads to poor control of plasma concentrations and therapeutic effects. The bioavailability of drugs is often severely limited due to the presence of biological barriers in the form of epithelial tight junctions, efflux proteins, and enzymatic degradation. Recently, prodrug design has shifted towards targeting specific enzymes or membrane transporters [9,10].

Bile acids (BAs, Fig. **1**), synthesized from cholesterol exclusively in hepatocytes, are facially amphipathic, having a curved profile with the angular methyl groups on the convex hydrophobic β side and the hydroxyl groups on the concave hydrophilic α side, and play an important role in enterohepatic circulation and metabolic regulation [11-13]. With versatile derivatization possibilities, a rigid steroidal backbone, enantiomeric purity, availability, and low cost combined with unique physiological properties, bile acids have been used to improve intestinal absorption and increase the metabolic stability of pharmaceuticals in organs involved in enterohepatic circulation [14,15].

In this report, we synthesized 15 berberine–bile acid analogues, assessed their cytotoxicity against SGC-7901, HCT 116, SMMC-7721, BEL-7402 cancer cell lines, and normal liver cells HL-7702 and evaluated the antimicrobial activities against *Staphylococcus aureus* ATCC 25923, *Staphylococcus albus* ATCC 8799, *Bacillus subtilis* AS 1.398, and *Escherichia coli* ATCC 31343. Among these analogues, the serum stability of **A4** and **B4** was tested *in vitro*, and the oral bioavailability of **A4** and **B4** was tested *in vivo*.



4 Bile acid: R_1=R_2=R_3= α- OH;
5 Dehydrocholic acid: R_1=R_2=R_3= carbonyl;
6 Deoxycholic acid: R_1=R_3= α- OH;
7 Chenodeoxycholic acid: R_1= R_2= α- OH;
8 Ursodeoxycholic acid:R_1= α- OH, R_2= β- OH.

Fig. (1). Structures of bile acids (**4**–**8**).

2. MATERIALS AND METHODS

2.1. Synthesis

Structural modifications at C-9 of BBR (**1**) can be generated after converting the methoxy group to hydroxyl to obtain berberrubine (**2**). In our study, **1** was converted to **2** with 74% yields by using vacuum pyrolysis [16]. Different berberine–bile acid analogues (**A1–A5**, **B1–B5**, and **C1–C5**) were prepared using different bile acids as shown in Fig. (**1**).

Scheme **1a** shows the synthesis of intermediates of bile acids. Scheme **1b** illustrates three synthetic methods. Route A used **2** as the starting material. Alkylation of **2** with 1, 3-dibromopropane in *N*,*N*-dimethylformamide (DMF) was stirred at 60°C for 6 h to afford **3** [17], and **3** was aminated by NH_3 in dimethylsulfoxide (DMSO) at room temperature for 30 minutes, then bile-acid active esters (**9–13**) and triethylamine (TEA) were added in the reacting solution. The

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mixture was stirred for 4 h and then suitable absolute ethyl ether was added. Filter residues were purified to obtain products **A1–A5**, whose linkers were amide bonds between BBR and bile acids.

In route B, bile acids were used as the start materials. The substitution of bile acids with 1,3-dibromopropane in DMF was catalyzed by K_2CO_3 to obtain **14–18 [**18]. Solutions containing **2** and **14–18** in DMF were stirred at 60°C for 6 h and then suitable absolute ethyl ether was added. After filtration, the crude products were purified to give products **B1–B5**.

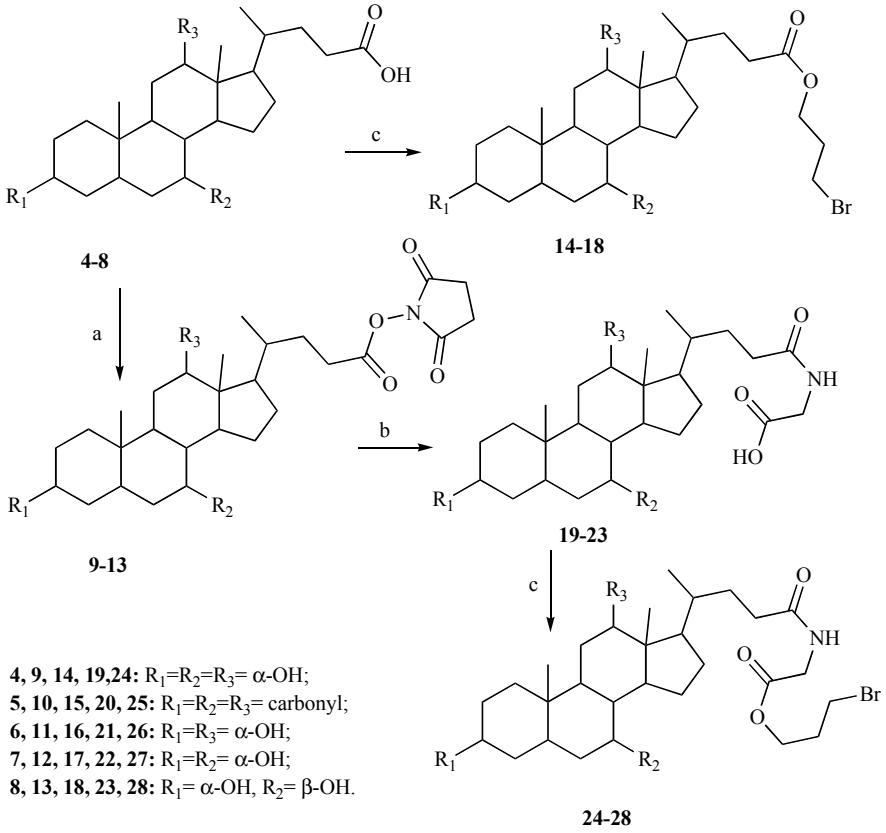
In route C, amidation of bile-acid active esters (**9–13**) with glycine in DMSO was performed using TEA as the catalyst to obtain glycocholic acids (**19–23**), then **19–23** and 1,3-dibromopropane were stirred in DMF with K_2CO_3 as the catalyst for 4 h to produce **24–28 [**18]. Subsequently, **24–28** and **2** were stirred in DMF at 60°C for 6 h, and the resultant mixture and suitable absolute ethyl ether were filtered. The crude products were purified to obtain products **C1–C5**, with linkers of amide and ester bonds between BBR and bile acids.

Melting points (mp) were determined using a WRS-1B digital melting point apparatus (Shanghai, China) and uncorrected. ^{1}H NMR spectra was recorded on a Bruker Avance 500 spectrometer (500 MHz). Chemical shifts are expressed as δ values (ppm) relative to tetramethylsilane (TMS) as an internal standard (s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet and b = broad). Coupling constants (*J*) are given in Hertz (Hz). IR spectra was obtained in KBr pellets using a Nicolet Magna-560 IR Spectrometer. Electrospray ionization (ESI) mass spectrometric analysis was performed on an ABI-3000 LC-MS-MS Spectrometer. All compounds tested displayed more than 98% purity. HPLC was recorded on a Waters Alliance 2690 apparatus using a reverse-phase HiQ sil C18 (Ø 4.6 mm × 150 mm) and UV photoiode array detection at 347 nm. All reactions were monitored by thin layer chromatography (TLC) using 0.2 mm silica gel plates G (Sijiashenghua, Taizhou, China). Column chromatography was carried out using silica gel zcx.II (300–400 Mesh, Qingdao Haiyang Chemical Co. Ltd, China). Chemicals and solvents used were commercially available. Reaction components were visualized initially under UV (365 nm) or color reactions were visualized after the silica gel plates with vanillin-concentrated sulfuric acid-ethanol (10%) were heated.

The detailed data about compounds can be found in the Supplementary Materials.

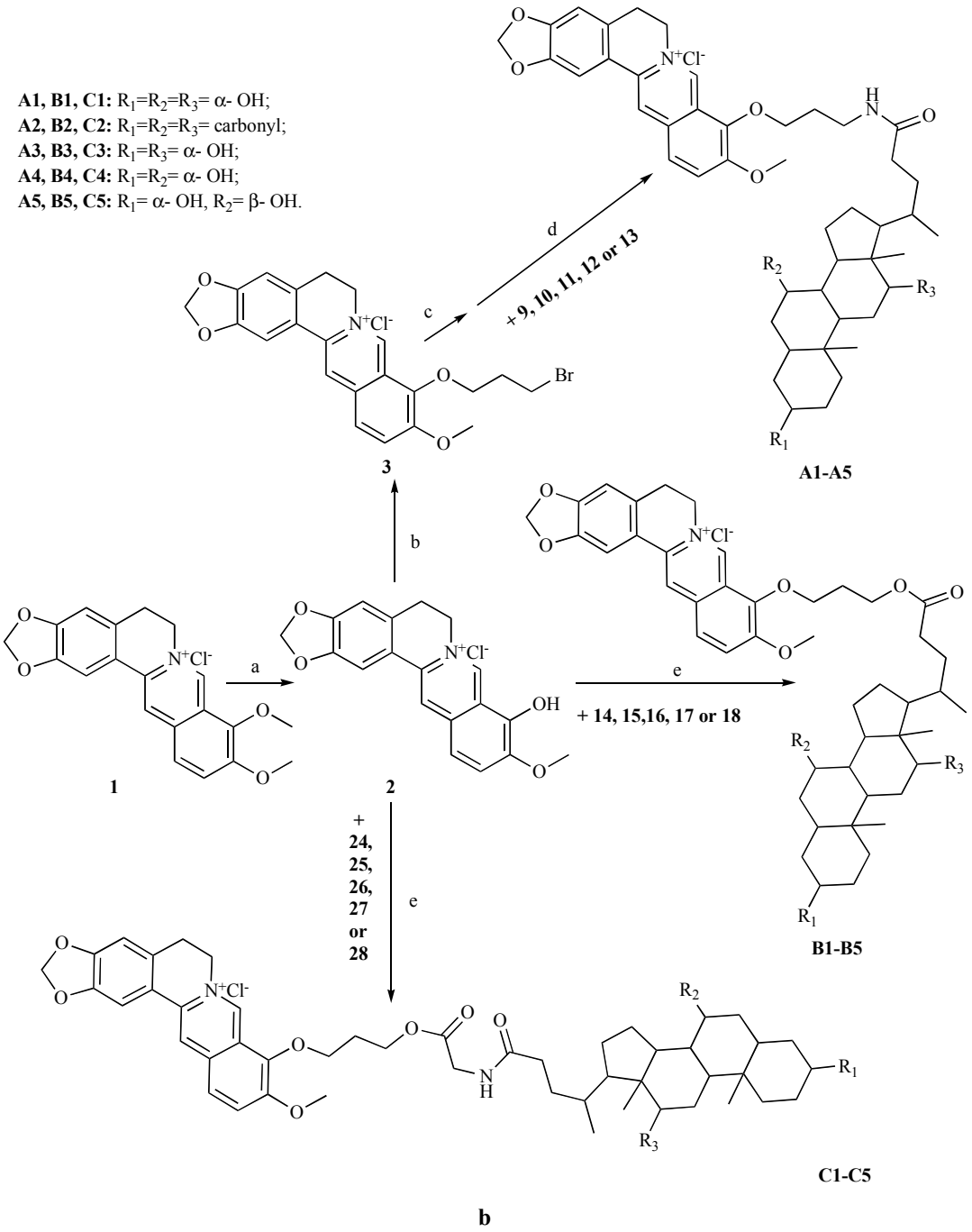
2.2. MTT Assays

MTT assays were used to assess cytotoxicity of the analogues [19]. Human hepatoma cells SMMC-7721, BEL-7402, colon cancer cells HCT 116, gastric cancer cells SGC-7901 and normal liver cell HL-7702 provided by the Chinese Academy of Sciences cell bank (Shanghai, China) were plated in 96-well plates by a density of 10^5 cells/mL for 24 h (37°C, 5% CO_2). Then cells were exposed continuously for 72 h to various concentrations of tested drugs. MTT



4, 9, 14, 19,24: R_1=R_2=R_3= α-OH;
5, 10, 15, 20, 25: R_1=R_2=R_3= carbonyl;
6, 11, 16, 21, 26: R_1=R_3= α-OH;
7, 12, 17, 22, 27: R_1=R_2= α-OH;
8, 13, 18, 23, 28: R_1= α-OH, R_2= β-OH.

a



b

Scheme 1. a. Synthesis of intermediates of bile acids. Reagents and conditions: (a) *N*-hydroxysuccinimide, 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride, CH_2Cl_2, 4 h; (b) glycin, DMSO, triethylamine, rt, 4 h; (c) 1,3-dibromopropane, DMF, K_2CO_3, 4 h.

b. Synthesis of berberine–bile acid analogues. Reagents and conditions: (a) 20–30 mmHg, 195–210°C, 30 min; (b) DMF, 1,3-dibromopropane, 60°C, 6 h; (c) NH_3, DMSO, triethylamine, 30 min; (d) DMSO, triethylamine, 4 h; (e) DMF, 60°C, 6 h.

solution (5 mg/mL in PBS) was added (20 μL/well), and the plates were incubated for another 4 h at 37°C. All media was removed and formazan crystals were dissolved with DMSO 100 μL per well. After mixing equally, the absorbance was read at 570 nm (630 nm used as a reference) on a microplate reader (Awareness Technology, Palm City, USA). Wells containing no drugs were used as blanks, and BBR was a reference. Assays were performed in three independent experiments. The IC_{50} was defined as the concentration of compound that produced a 50% reduction of surviving cells and calculated using the logit method.

2.3. Antimicrobial Testing

The microorganisms used in this study were *S. aureus* ATCC 25923, *S. albus* ATCC 8799, and *E. coli* ATCC 31343 provided by the American Type Culture Collection (Maryland, America) and *B. subtilis* AS 1.398 provided by

the China General Microbiological Culture Collection Center (Beijing, China). MIC was determined by the meams of a serial 2-fold dilution following the recommendations of the Clinical and Laboratory Standards Institute (CLSI). Bacterial cells (10^5 CFU/mL) were inoculated into a nutrient broth at 0.1 mL per well of 96-well microtiter plates, in the presence of derivates and BBR dissolved in sterilized physiological saline solution (0.9%) at a final concentration (0, 3.9, 7.8, 15.7, 31.3, 62.5, 125, 250, 500 μg/mL). The plate was incubated under anaerobic conditions at 37°C for 24 h. After incubation, the wells were examined for growth of microorganisms and the MIC was defined as the minimal concentration of derivates that yielded no visible growth. [20] MIC values were determined by three independent assays. Subculturing (10 μL) from each well from the MIC assays was reinoculated on nutrient agar plates. The plates were incubated at 37°C for 24 h. The MBC was defined as the minimal concentration of samples at which all inoculated microorganisms were killed. [21] All determinations were performed in duplicate.

2.4. Serum Stability Studies

Serum stability assays were carried out on mouse serum to measure the stability of **A4**, **B4**, and BBR. [22] The compounds were dissolved in 100 μL DMSO solution (4 mmol/L), then incubated in 4 mL rat blood serum at 37°C. Aliquots were taken at different time points (0, 1, 2, 4, 6, 8, 10, 12, and 24 h). Samples (200 μL) with chromatographic-grade methanol (600 μL) were centrifuged at 12 000 r/min for 10 min. Liquid supernatant was analyzed by HPLC. Deionized water with 2% triethylamine was adjusted to a pH of 3 by phosphoric acid. The separation of **A4** and **B4** was carried out with a mobile phase of a (25:75) water/methanol mixture, and the separation of BBR was carried out with a mobile phase of a (55:45) water/methanol mixture. The flow rate was 1.0 mL/min and the elution profile was monitored by recording the UV absorbance at 347 nm. Assays were performed in three independent experiments.

2.5. Calibration Curve

The stock solutions (3 000 ng/mL) of **A4**, **B4**, and BBR were dissolved in methanol and diluted to give concentrations of 3–1500 ng/mL. The control plasma samples (200 μL) with a range of drug concentrations (20 μL) and the internal standard (20 μL) were diluted with 2-fold methanol and then centrifuged at 12 000 r/min for 10 min. A 10 μL volume of each sample supernatant was injected into LC-MS-MS for analysis.

2.6. Oral Bioavailability

Twelve female Wistar mice (180–220 g) were from the Institute of Laboratory Animal Science (Shanghai, China), and housed with 3 mice per cage to evaluate the oral bioavailability of **A4**, **B4**, and BBR. [23] The drugs were dissolved and diluted in physiological saline including DMSO (3%) and ethanol (10%). The drug was administered by oral gavage at a dose of 62.2mmol/kg. Blood was collected from the orbital sinus at 0, 15, 30, 60, 120, 180, 240, 360, 480, and 720 min and centrifuged at 4000 r/min for 10 min. 10-Hydroxycamptothecin was used as the internal standard of BBR, then **A4** and **B4** were used as the internal

Table 1. Anticancer Activities of A1–A5, B1–B5, and C1–C5

	Cell lines/IC_{50} (μM)[a]			
Compd	**SGC-7901[b]**	**HCT 116**	**SMMC-7721**	**BEL-7402**
BBR	3.33±0.26	4.16±0.14	4.55±0.15	28.06±0.34
A1	16.58±0.39	20.19±0.40	12.40±0.25	29.78±0.40
A2	52.45±0.35	NA[c]	43.33±0.57	47.63±0.80
A3	7.64±0.38	19.33±0.44	14.38±0.65	28.10±0.16
A4	3.13±0.29	3.33±0.51	1.44±0.08	10.38±0.45
A5	7.17±0.53	6.52±0.39	3.60±0.09	14.00±0.10
B1	4.52±0.59	6.73±0.61	2.97±0.09	11.18±0.32
B2	7.10±0.38	7.74±0.49	6.26±0.33	5.66±0.32
B3	3.78±0.54	6.27±0.35	2.74±0.07	25.91±0.64
B4	1.13±0.08	1.52±0.43	0.50±0.02	24.28±0.35
B5	2.08±0.13	4.14±0.35	1.62±0.10	5.82±0.13
C1	23.07±0.34	19.60±0.41	6.05±0.28	31.20±0.52
C2	48.36±0.35	42.48±0.39	27.86±0.50	47.42±0.54
C3	35.79±0.26	31.39±0.12	13.13±0.26	12.11±0.23
C4	8.84±0.24	17.47±0.22	25.24±0.38	17.62±0.41
C5	28.32±0.43	40.25±0.21	34.92±0.33	46.32±0.93

[a]Effects on cancer cell line replication were determined using a standard method. Anticancer activities are expressed as IC_{50} values for each cell line, the concentration of compound that caused 50% inhibition.
[b]SGC-7901, human gastric cancer cells; HCT 116, human colon cancer cells; SMMC-7721, human hepatocellular cells; Bel-7402, human hepatocellular cancer cells.
[c]NA, not active.

Table 2. Cytotoxicity of A4, B4, C4 and BBR Against a Normal Liver Cell Line HL-7702 with the Concentration of 2.000, 0.400, 0.080 and 0.016(μM)

	cell viability(% of control)			
c(μM)	BBR	A4	B4	C4
2.000	69.44±0.44	101.82±0.23	72.28±0.62	74.50±0.36
0.400	70.38±0.35	102.47±0.97	76.71±0.68	84.38±0.21
0.080	85.79±0.18	104.24±0.35	85.47±0.41	96.42±0.77
0.016	92.76±0.67	105.55±0.49	90.12±0.70	101.60±0.50

standard for each other. A liquid supernatant/methanol (1:2) mixture was centrifuged at 12 000 r/min for 10 min after blending. The sample (10 μL) was injected into LC-MS-MS. The pharmacokinetic parameters were analyzed by noncompartmental analysis using PKSolver 2.0.

3. RESULTS AND CONCLUSIONS

3.1. Synthesis

The yields of **B1–B5**, whose linkers were ester bonds, were higher than those of **A1–A5** and **C1–C5**. It was presumed that the lipophilicity of **B1–B5** was higher than that of the others. **A1–A5** and **C1–C5** were not only quaternary ammonium salts but also linked by amide bonding and, therefore, might be more easily adsorbed by silica gel and more difficult to elute.

3.2. Anticancer Activities

All new analogues were evaluated against 4 human tumor cell lines (SGC-7901, HCT 116, SMMC-7721, and BEL-7402) by using an MTT assay [19]. Table **1** lists the values of IC_{50} of **A1–A5**, **B1–B5**, and **C1–C5** with BBR as the positive control. Table **1** indicated that most of **B1–B5** compounds with ester linkers showed lower values of IC_{50} and better anticancer activities than that of BBR. However, **A1**, **A2**, **A3**, **A5** and **C1–C5** displayed inferior anticancer activities compared with BBR. **A4**, **B4** and **B5** showed higher cytotoxicities against the tested cancer cell lines than BBR.

The complexes of bile acid could interreact with DNA to inhibit DNA synthesis and to reduce cell proliferation [24]. But the anticancer effect of these analogues were diferent. It might be accounted for by the structure/activity relationship. It was presumed that the bile acid lipophilicity influenced cytotoxicity and that bile acids linked to BBR might produce appropriate lipophilicity and polarity for absorption [25]. The hydroxyl functionality at the C_7' position of the steroid nucleus may be crucial for the cytotoxic activity. Moreover, the hydroxyl functionality at C_{12}' may suppress the activity even in the presence of the hydroxyl group at C_7' [26,27]. **B4** modified by chenodeoxycholic acid exhibited higher cytotoxicty than **B5** modified by ursodeoxycholic acid, that is consistent with the result the hydroxyl at the C_7' position in the stereochemistry alpha was suggested to enhance the anticancer activity [26]. It suggests that bile acids play an important role in entry into cancer cells [28].

3.3. Cytotoxicity Assays on Liver Cells

The cytotoxicity against the liver cells HL-7702 of **A4, B4, C4,** and BBR was mesured. The results in Table **2** indicated that **A4, B4,** and **C4** showed lower cytotoxicity than BBR at the same concentration. The specific carrier proteins responsible for liver bile acid uptake might carry compouds through the cell membrane [29].

3.4. Antimicrobial Activities

The antimicrobial activities of these new compounds were evaluated by MIC [20] and the MBC [21] against the gram-positive bacteria *S. aureus* ATCC 25923, *S. albus* ATCC 8799, and *B. subtilis* AS 1.398 and the gram-negative bacterium *E. coli* ATCC 31343 *in vitro*. BBR was used as a positive control. MIC and MBC values are shown in Table **3**. The new compounds displayed superior activity against *S. aureus ATCC 25923* and *S. albus ATCC 8799*, and most of the compounds displayed lower MIC, MBC values and better antimicrobial activity than that of BBR. **A3**, **B3**, and **C3** connected with deoxycholic acid or **A4**, **B4**, and **C4** connected with chenodeoxycholic acid showed better inhibitory activity than that of **A1**, **B1**, and **C1** connected with bile acid. **A2**, **B2**, and **C2** with poor activity might be due to the connection with dehydrocholic acid.

The structure of the quaternary ammonium salt in berberine analogues potentially destroys the cell membrane of gram-positive bacteria due to disturbing the charge balances of the cell membrane. [30] BBR modified by bile acids acquires facial amphiphiles, which may improve uptake by bacterial cell membranes. The substituents of the bile acid steroid nucleus were also important to the affinity and uptake rates of the bile acid conjugates. [31] Two hydroxyls at C_3', C_7' or C_{12}' on the steroid might be optimal for antibacterial activity, but three hydroxyl groups could decrease the activity. The α-hydroxyl groups at C_7' or C_{12}' acted as hydrogen bond donors, and the hydroxylation degree of the steroid skeleton had a significant effect on the transport activity.[15,32,33] Chenodeoxycholic acid conjugates exhibited greater inhibitory potency than ursodeoxycholic acid conjugates. This suggests that the α-OH is favored over the β-OH at C_7' [15,34].

3.5. Serum Stability Assays

The ideal prodrug should withstand the rigors of the gastrointestinal environment with physiochemical and

Table 3. Antimicrobial Activities of A1–A5, B1–B5, and C1–C5 (μg/mL)

Compd	*S. aereus* ATCC 25923		*S. albus* ATCC 8799		*B. subtilis* AS 1.398		*E. coli* ATCC 31343	
	MIC[a]	MBC[b]	MIC	MBC	MIC	MBC	MIC	MBC
BBR	125	>500	125	>500	250	>500	500	>500
A1	125	125	125	125	>500	>500	>500	>500
A2	>500	>500	>500	>500	>500	>500	>500	>500
A3	7.8	31.3	7.8	31.3	>500	>500	>500	>500
A4	7.8	31.3	7.8	31.3	>500	>500	>500	>500
A5	125	125	125	125	>500	>500	>500	>500
B1	7.8	15.7	7.8	31.3	>500	>500	>500	>500
B2	>500	>500	>500	>500	>500	>500	>500	>500
B3	7.8	15.7	7.8	15.7	>500	>500	>500	>500
B4	7.8	15.7	62.5	250	>500	>500	>500	>500
B5	7.8	15.7	3.9	7.8	>500	>500	>500	>500
C1	62.5	62.5	62.5	62.5	>500	>500	>500	>500
C2	>500	>500	>500	>500	>500	>500	>500	>500
C3	15.7	62.5	15.7	250	>500	>500	>500	>500
C4	15.7	62.5	15.7	31.3	>500	>500	>500	>500
C5	250	500	250	500	>500	>500	>500	>500

[a]The MIC (μg/mL) was defined as the concentration of the agent that completely inhibited cell growth during a 24 h incubation at 37 °C.
[b]The MBC (μg/mL) was defined as the concentration of the agent that sterilized the cells.

biological properties permitting good absorption and efficient conversion to the active parent compound once absorbed. **A4** and **B4**, conjugated with chenodeoxycholic acid *via* different bonds, had significant effects in anticancer and antimicrobial activities. To obtain further information on bioavailability, **A4**, **B4**, and BBR were incubated in mouse serum to determine the stability by HPLC. These compounds did not degrade over a 24-hour period. **A4** and **B4** showed good solubility and chemical stability *in vitro*.

3.6. Oral Bioavailability Studies

The oral bioavailability of **A4** and **B4** was evaluated in female Wistar mice compared with that of BBR. The drug was administered by oral gavage at a dose of 62.2mmol/kg. Blood was collected at various time points and plasma samples were analyzed by LC-MS-MS. The pharmacokinetic parameters are shown in Table **4**. The time (t_{max}) taken to reach the maximum plasma concentration (c_{max}) of **B4** was shorter than that of BBR. Compared with BBR, the AUC of **B4** was significantly higher. Conversely, the AUC of oral **A4** was less than that of BBR. The clearance of **B4** presented group was significantly less than that of the other groups. Therefore, **B4** is more easily absorbed by the gastrointestinal system. For enterohepatic cycling of bile acids, both the hepatocyte and the enterocyte must efficiently transport bile acids. The major site of bile acid absorption from the small intestine is in the terminal ileum. [35] However, the ester-linked berberine derivative enhanced oral bioavailability; therefore, the linking of the prodrug may have a determinant effect in the oral bioavailability in mice.

In summary, we have designed and synthesized a novel series of BBR analogues conjugated with bile acids to increase the oral bioavailability of BBR. **A4**, **B4**, and **B5** showed superior cytotoxity against the tested cancer cell lines, but showed lower cytotoxity against the normal liver cells HL-7702. All of the analogues except for **A2**, **B2**, and **C2** displayed significantly greater antimicrobial activity than that of BBR against *S. aureus* ATCC 25923 and *S. albus* ATCC 8799. **A4** and **B4** were stable in serum. **B4** showed superior oral bioavailability *in vivo*. The oral bioavailability of BBR was improved by chenodeoxycholic acid *via* ester bonding. Reports from our backup program where efforts are focused on berberine–bile acid analogues with improved pharmacokinetic properties will be forthcoming.

Table 4. Oral Bioavailability of A4, B4, and BBR

Compd	c_{max} (nM)	t_{max} (min)	$AUC_{0\to t}$ (nM/ mL*min)[a]	Clearance (mmol/kg)/(nmol/ml)/min
BBR	0.0086	180	1.424	27.15
A4	0.0017	180	0.3549	154.17
B4	0.0324	60	6.755	6.99

[a]$AUC_{0\to t}$ was defined as the area under curve from 0 min to t min.

CONFLICT OF INTEREST

Declared none.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BBR = berberine

BA = bile acid

DMF = *N*,*N*-dimethylformamide

DMSO = dimethylsulfoxide

TEA = triethylamine

MIC = the minimum inhibitory concentration

MBC = the minimum bactericidal concentration

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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