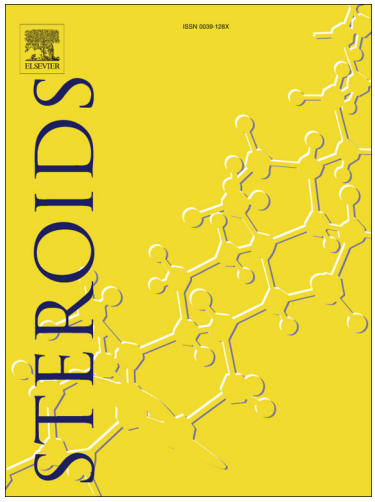

Synthesis and biological evaluation of bufalin-3-yl nitrogen-containing-carbamate derivatives as anticancer agents

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Synthesis and biological evaluation of bufalin-3-yl nitrogen-containing-carbamate derivatives as anticancer agents

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Abstract: A series of bufalin-3-yl nitrogen-containing-carbamate derivatives **3** were designed, synthesized, and evaluated for their proliferation inhibition activities against human cervical epithelial adenocarcinoma (HeLa) cell line. The structure-activity relationships (SARs) of this new series are described in this paper. Cytotoxicity data revealed that the C3 moiety had an important influence on cytotoxic activity. Compound **3i-HCl** exhibited significant *in vitro* antiproliferative activity against the ten tested tumor cell lines, with IC_{50} values ranging from 0.30 to 1.09 nM. Furthermore, **3i-HCl** can significantly inhibit tumor growth by 100% at the dose 2 mg/kg by iv, or 4 mg/kg by ig.

Keywords: Cardiac glycosides, Bufalin (BF), Natural product, Bufalin-3-yl carbamate, Cytotoxicity.

1. Introduction

Cardiac glycosides are a class of natural compounds with prominent cardiotonic effects. In clinical research, compounds such as digoxin, digitoxin, lanatosaide C, strophanthin K, and divarcosise, are used widely as drugs for treatment of heart disease. In 1960s, Shiratori [1] and Hartwell et. al. [2] reported that cardiac glycosides exhibited significant antitumor activities *in vitro*, respectively. From then on, many papers have been published on studies of cardiac glycosides as antitumor therapeutic agents [3–8]. In recent years, several cardiac glycosides, such as Anvirzel [9], PBI-05204 [10], and UNBS-1450 [11], are being tested in phase I and II clinical trials as new anti-cancer agents. Cardiac glycosides as novel drugs for treatment of tumor might be a promising research area.

Chansu is a Traditional Chinese Medicine made from the dried skin and parotid venom glands of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider. Huachansu injection, the water-soluble extract of the dried skin of *Bufo bufo gargarizans* Cantor, has been successfully used in the clinic for treatment of various cancers in China. Bufalin (Fig. 1), a cardiac glycoside, is one of the major active components of Chansu. As is known in the literature, bufalin exhibits remarkable cytotoxic activity with IC_{50} values of 10^{-8} to 10^{-9} mol/L for various tumor cells including human prostate carcinoma cells (PC3, DU145) [12], human leukemia cells (U937, HL60) [13–14], human cervical carcinoma cells (HeLa) [15] and non-small-cell lung cancer cells (A549) [16]. Although the antitumor mechanism of

bufalin has not been reported, bufalin, as with other cardiac glycosides, could bind to Na^+/K^+-ATPase and inhibit the Na^+/K^+ pump [17–20].

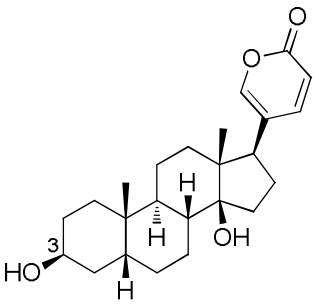


Fig. 1. The structure of bufalin (BF).

Through years of research, the antitumor activity of cardiac glycosides *in vivo* and *in vitro* has been widely recognized. However, these compounds exhibite high toxicity which severely limits their clinical application. Hence, reducing toxicity, enhancing activity and improving drug-like properties of cardiac glycosides might be an important strategy for the structural modification. Bufalin, just as other cardiac glycosides, has the toxicity problems. In our previous study, we found that bufalin-3-yl piperidin-4-carboxylate (IC_{50} value on HeLa 0.76 nM) displayed significant cytotoxic potency compared to the parent bufalin (IC_{50} value on HeLa 26.3 nM) [16]. These results suggest that the hydroxyl on C3 of bufalin is a suitable site for the structural modification.

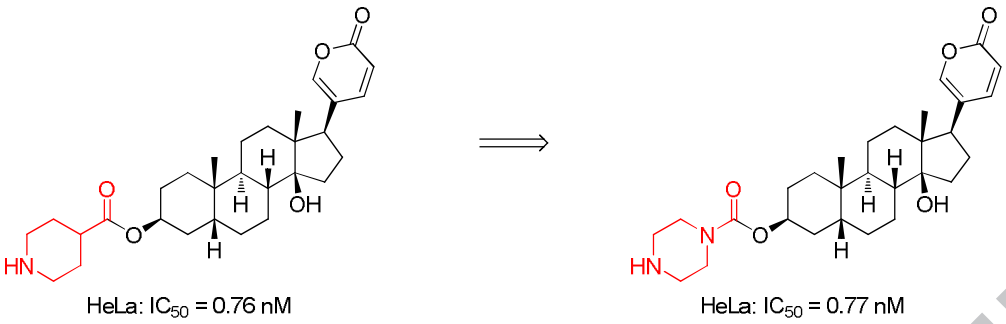


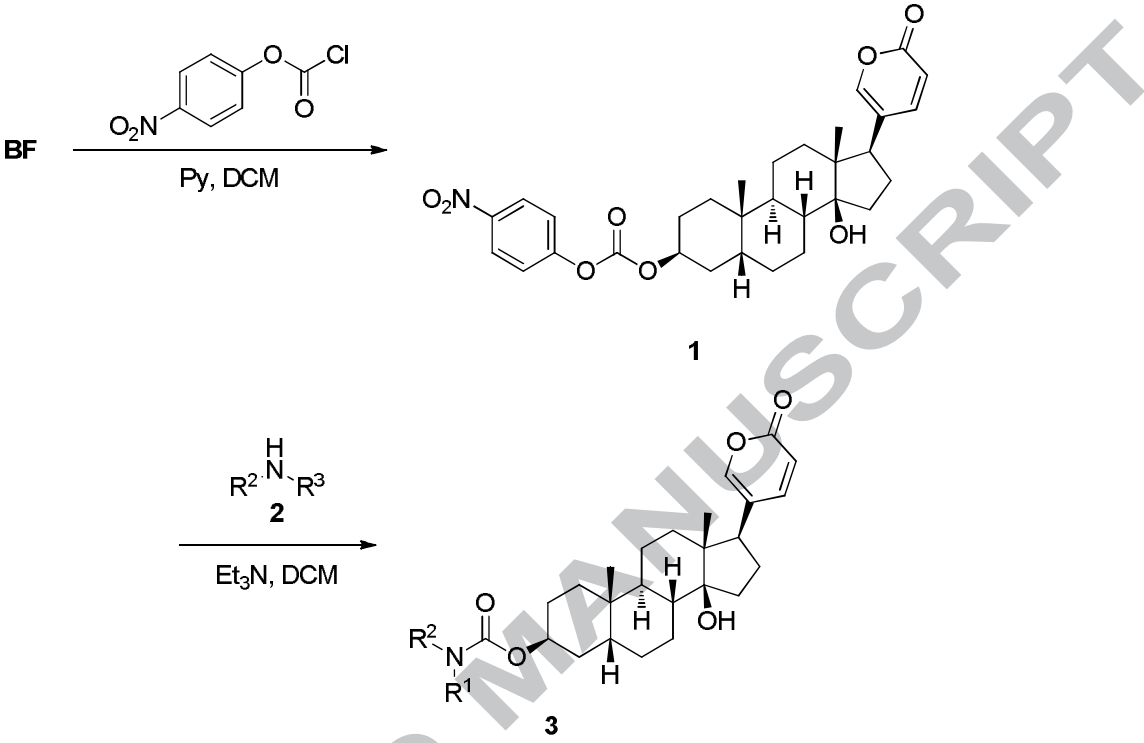
Fig. 2. The design of bufalin-3-yl carbamate derivatives.

Although bufalin-3-yl piperidin-4-carboxylate showed significant cytotoxic potency *in vitro*, this compound was inactive *in vivo*. We speculated that the ester was prone to metabolism *in vivo* because ester groups are susceptible to hydrolysis by esterases in the blood. Therefore, we designed bufalin-3-yl piperazine-1-carbamate by bioisosteric replacement (Figure 2), and the activity test indicated that this compound could maintain cytotoxic potency (IC_{50} (HeLa) = 0.77 nM). In order to find new compounds with better cytotoxic potency, a series of bufalin-3-yl nitrogen-containing-carbamate derivatives were designed, synthesized, and evaluated for their proliferation inhibition activities.

2. Experimental

A two-step reaction route was designed for the synthesis of bufalin-3-yl carbamate derivatives as shown in Scheme 1. In the first step, bufalin was reacted with 4-nitrophenyl chloroformate in DCM using pyridine as the base to obtain the intermediate **1**. Then, **1** was reacted with amine **2** in the presence of Et_3N to obtain the

desired products **3**.



Scheme 1. The synthesis of bufalin-3-yl carbamate derivatives **3**.

All reactions were performed under a nitrogen atmosphere with dry solvents in oven-baked or flame-dried glassware, unless otherwise noted. All reagents were commercially available, and were used without further purification unless otherwise specified. All solvents were redistilled under argon atmosphere. The progress of reactions were monitored by thin layer chromatography (TLC) plates (silica gel 60GF, Yantai jiangyou company) visualized with 254-nm UV light and/or by staining with vanillin solution (2.7 g vanillin + 100 mL H_2O + 35 mL concentrated H_2SO_4 diluted

to 300 mL with 95% ethanol). Melting points were measured by a WRS-1B micromelting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury-VX300 Fourier transform spectrometer or a Bruker AM-400 spectrometer, and chemical shifts (δ) were reported in ppm; the hydrogenated residues of deuterated solvent were used as internal standard $CDCl_3$: 7.26 ppm for ^{1}H NMR and 77.00 ppm for ^{13}C NMR. ESIMS was run on a Bruker Esquire 3000 plus spectrometer in MeOH. HRESIMS were determined on a Micromass Q-TOF Global mass spectrometer.

2.1. General synthesis of bufalin-3-yl carbamate derivatives

To a stirred solution of bufalin (100 mg, 0.28 mmol) and 4-nitrophenyl chloroformate (129 mg, 0.64 mmol, 2.3 eq) in anhydrous DCM (2 mL) at room temperature under nitrogen gas was added pyridine (50 mg, 0.64 mmol, 2.3 eq), and stirring continued for 2 h. After completion (by TLC), DCM (10 mL), and H_2O (10 mL) were added. The organic phase was separated, washed with aq. Na_2CO_3, H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo to afford yellow residue **2**. To a solution of the residue in DCM (2.5 mL) was added amine (3 eq) and Et_3N (3 eq), and stirring continued at room temperature for 2 h. After completion (by TLC), DCM (15 mL) and H_2O (15 mL) were added. The organic phase was separated, washed with aq. Na_2CO_3 and brine, dried over Na_2SO_4, and concentrated in vacuo to give yellow residue, and the residue was subjected to chromatographic separation on silica gel (30

g) with petroleum ether/acetone/triethylamine (100:30:1 v/v/v).

*2.1.1. Compound **3a***

3a (77 mg, 55%) mp 199–201 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (dd, 1H, J = 9.6, 2.4), 7.22 (s, 1H), 6.25 (d, 1H, J = 9.6), 4.98 (brs, 1H), 3.27 (q, 2H, J = 6.0, 5.4), 2.75 (t, 2H, J = 6.0), 2.65 (q, 2H, J = 6.9), 1.10 (t, 3H, J = 7.2), 0.93 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.6, 156.7, 148.7, 147.0, 122.9, 115.5, 85.5, 70.8, 51.5,49.1, 48.6, 44.0, 42.5, 41.1, 40.8, 37.0, 36.0, 35.3, 33.0, 30.9, 29.9, 28.9, 26.6, 25.5, 21.6, 23.9, 21.5, 16.7, 15.4; MS (ESI) *m/z*: 501.4 $[M+H]^+$; HRMS (ESI): calcd for $C_{29}H_{45}N_2O_5$ $[M+H]^+$ 501.3323, found 501.3310.

*2.1.2. Compound **3b***

3b (89 mg, 60%) mp 198–200 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (dd, 1H, J = 9.9, 2.4), 7.22 (d, 1H, J = 2.4), 6.25 (d, 1H, J = 9.6), 4.98 (br s, 1H), 3.23 (br d, 2H, J = 5.4), 2.55 (m, 6H), 1.02 (t, 6H, J = 7.2), 0.94 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.6, 156.6, 148.7, 147.1 (2C), 122.9, 115.4, 85.5, 70.7, 52.1, 51.4, 48.5, 47.1, 42.5, 41.0, 38.7, 36.9, 36.0, 35.3, 32.9, 30.9, 29.9, 28.9, 26.6, 25.5, 23.9, 21.6, 21.5, 16.7, 11.8 (2C); MS (ESI) *m/z*: 529.7 $[M+H]^+$; HRMS (ESI): calcd for $C_{31}H_{49}N_2O_5$ $[M+H]^+$ 529.3636, found 529.3649.

*2.1.3. Compound **3c***

3c (89 mg, 60%) mp 202–204 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (dd, 1H, J = 9.6, 2.4), 7.23 (d, 1H, J = 2.1), 6.25 (d, 1H, J = 9.6), 5.01 (br s, 1H), 3.34 (m, 2H), 2.67 (q, 2H, J = 7.2), 1.11 (m, 6H), 0.95 (s, 3H), 0.70 (s, 3H); ^{13}C NMR (75 MHz,

$CDCl_3$): δ 162.6, 156.1, 148.7, 147.1, 122.9, 113.4, 85.4, 71.1, 51.4, 48.5, 48.1, 47.0, 44.2, 42.7, 42.5, 41.0, 37.5, 36.0, 35.4, 32.9, 31.0, 29.8, 28.9, 26.7, 25.6, 24.1, 21.6, 21.5, 16.7, 15.4, 14.0; MS (ESI) *m/z*: 529.6 $[M+H]^+$; HRMS (ESI): calcd for $C_{31}H_{49}N_2O_5$ $[M+H]^+$ 529.3636, found 529.3644.

*2.1.4. Compound **3d***

3d (102 mg, 73%) mp 205–206 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84 (dd, J = 9.8, 2.6 Hz, 1H), 7.23 (d, J = 2.3 Hz, 1H), 6.26 (dd, J = 9.9, 2.3 Hz, 1H), 5.01 (s, 1H), 3.63–3.27 (m, 4H), 3.02–2.66 (m, 4H), 2.55–2.36 (m, 2H), 0.95 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.4, 155.1, 148.4, 147.8, 123.4, 114.6, 84.7, 71.7, 50.9, 48.3, 44.9, 41.6, 40.7, 37.1, 35.6, 35.0, 32.2, 31.7, 30.6, 30.5, 29.4, 28.7, 26.3, 25.1, 23.6, 21.1, 16.4; MS (ESI) *m/z*: 499.7 $[M+H]^+$; HRMS (ESI): calcd for $C_{29}H_{43}N_2O_5$ $[M+H]^+$ 499.3166, found 499.3177.

*2.1.5 Compound **3e***

3e (108 mg, 75%) mp 203–205 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84 (dd, J = 9.8, 2.6 Hz, 1H), 7.23 (m, 1H), 6.26 (d, J = 9.7 Hz, 1H), 5.02 (s, 1H), 3.50 (dd, J = 6.4, 3.9 Hz, 4H), 2.51–2.42 (m, 1H), 2.37 (t, J = 5.0 Hz, 4H), 2.31 (s, 3H), 0.95 (s, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.6, 155.1, 148.6, 146.9, 122.8, 115.4, 85.4, 71.3, 54.9, 51.3, 48.5, 46.3, 42.5, 41.0, 37.3, 36.0, 35.3, 32.9, 30.9, 30.8, 29.8, 28.9, 26.6, 25.5, 24.1, 21.6, 21.5, 16.7; MS (ESI) *m/z*: 513.8 $[M+H]^+$; HRMS (ESI): calcd for $C_{30}H_{45}N_2O_5$ $[M+H]^+$ 513.3323, found 513.3311.

*2.1.6. Compound **3f***

3f (95 mg, 66%) mp 203–205 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (m, 1H), 7.23 (s, 1H), 6.26 (d, J = 9.7 Hz, 1H), 5.02 (s, 1H), 3.69–3.29 (m, 4H), 2.94 (t, J = 5.3 Hz, 2H), 2.91–2.85 (m, 2H), 2.59–2.36 (m, 1H), 0.94 (m, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 155.9, 148.6, 147.3, 123.1, 115.2, 85.2, 71.4, 51.2, 49.0, 48.8, 47.9, 47.5, 45.7, 42.2, 40.9, 37.4, 35.9, 35.2, 32.7, 30.9, 30.8, 29.8, 28.8, 26.6, 25.4, 24.0, 21.5, 21.4, 16.6; MS (ESI) *m/z*: 513.8 $[M+H]^+$; HRMS (ESI): calcd for $C_{30}H_{45}N_2O_5$ $[M+H]^+$ 513.3323, found 513.3316.

*2.1.7. Compound **3g***

3g (44 mg, 30%) mp 209–211 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (d, J = 9.7 Hz, 1H), 7.22 (s, 1H), 6.25 (d, J = 9.8 Hz, 1H), 4.99 (s, 1H), 4.07 (br s, 2H), 2.86 (m, 2H), 2.60–2.47 (m, 2H), 2.44 (s, 3H), 2.26–2.10 (m, 1H), 2.04–1.11 (m, 26H), 0.94 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.6, 155.1, 148.6, 147.0, 122.9, 115.4, 85.4, 71.2, 58.4, 56.7, 51.3, 48.5, 42.7, 42.4, 41.0, 37.3, 35.9, 35.3, 33.3, 32.9, 32.0, 30.9, 30.8, 28.9, 26.6, 25.4, 24.1, 21.5, 21.5, 18.5, 16.7; MS (ESI) *m/z*: 527.5 $[M+H]^+$; HRMS (ESI): calcd for $C_{31}H_{47}N_2O_5$ $[M+H]^+$ 527.3479, found 527.3488.

*2.1.8. Compound **3h***

3h (98 mg, 65%) mp 210–212 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (dd, J = 9.8, 2.2 Hz, 1H), 7.23 (s, 1H), 6.26 (d, J = 9.6 Hz, 1H), 5.01 (s, 1H), 4.27–4.07 (m, 2H), 2.94–2.63 (m, 2H), 2.54–2.38 (m, 1H), 2.28 (s, 6H), 2.20–1.12 (m, 27H), 0.95 (s, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.6, 155.0, 148.6, 147.0, 122.9, 115.4, 85.4, 71.1, 62.2, 51.3, 48.5, 43.2, 42.4, 41.6, 41.0, 37.3, 35.9, 35.3, 32.9, 30.9, 30.8,

28.9, 26.6, 25.4, 24.1, 21.5, 21.5, 16.7; MS (ESI) *m/z*: 541.9 $[M+H]^+$; HRMS (ESI): calcd for $C_{32}H_{49}N_2O_5$ $[M+H]^+$ 541.3636, found 541.3626.

*2.1.9. Compound **3i***

3i (79 mg, 55%) mp 210–212 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (d, J = 9.6 Hz, 1H), 7.23 (d, J = 1.5 Hz, 1H), 6.25 (d, J = 9.6 Hz, 1H), 5.00 (s, 1H), 4.09 (br s, 2H), 2.89–2.75 (m, 3H), 2.51–2.40 (m, 1H), 2.23–1.12 (m, 25H), 0.95 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.7, 156.4, 150.5, 149.4, 125.0, 115.4, 86.0, 73.5, 68.8, 52.2, 49.7, 49.6, 43.0, 41.8, 38.8, 36.9, 36.3, 33.2, 31.9, 31.8, 30.9, 29.9, 27.7, 26.2, 24.4, 22.6, 22.5, 17.4; MS (ESI) *m/z*: 513.5 $[M+H]^+$; HRMS (ESI): calcd for $C_{30}H_{45}N_2O_5$ $[M+H]^+$ 513.3323, found 513.3320.

*2.1.10. Compound **3j***

3j (92 mg, 58%) mp 202–204 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (d, J = 9.8 Hz, 1H), 7.23 (s, 1H), 6.26 (d, J = 9.8 Hz, 1H), 4.99 (s, 1H), 4.43 (d, J = 8.8 Hz, 1H), 3.95–3.91 (m, 1H), 2.54–2.39 (m, 1H), 2.25–1.02 (m, 26H), 1.24 (s, 6H), 1.12 (s, 6H), 0.94 (s, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.6, 155.6, 148.5, 147.1, 122.9, 115.2, 85.2, 70.5, 51.3, 51.2, 48.4, 45.5, 44.0, 42.2, 40.8, 36.8, 35.8, 35.1, 34.8, 32.7, 30.7, 30.5, 28.8, 28.3, 26.4, 25.3, 23.8, 21.4, 21.3, 16.6; MS (ESI) *m/z*: 569.1 $[M+H]^+$; HRMS (ESI): calcd for $C_{34}H_{53}N_2O_5$ $[M+H]^+$ 569.3949, found 569.3940.

*2.1.11. Compound **3k***

3k (37 mg, 25%) mp 204–206 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (dd, J = 9.7, 2.6 Hz, 1H), 7.22 (s, 1H), 6.25 (d, J = 9.6 Hz, 1H), 5.02 (s, 1H), 3.16 (d, J = 11.7 Hz,

2H), 2.79 (s, 3H), 2.73–2.55 (m, 2H), 2.21–1.12 (m, 26H), 0.94 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.6, 148.7, 146.9, 122.8, 115.5, 85.5, 71.1, 51.3, 48.5, 42.5, 41.0, 36.0, 35.3, 32.9, 30.9, 28.9, 26.6, 25.5, 21.6, 21.5, 16.7; MS (ESI) *m/z*: 527.9 $[M+H]^+$; HRMS (ESI): calcd for $C_{31}H_{47}N_2O_5$ $[M+H]^+$ 527.3479, found 527.3470.

*2.1.12. Compound **3l***

3l (103 mg, 70%) mp 200–202 ^{o}C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (dd, J = 9.7, 2.6 Hz, 1H), 7.22 (d, J = 2.5 Hz, 1H), 6.25 (d, J = 9.7 Hz, 1H), 4.99 (d, J = 5.0 Hz, 1H), 4.14 (br s, 2H), 2.74 (br s, 2H), 2.62–2.54 (m, 1H), 2.51–2.38 (m, 1H), 2.21–1.13 (m, 26H), 0.94 (s, 3H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.4, 155.1, 148.4, 147.8, 123.5, 114.6, 84.7, 71.3, 51.0, 46.8, 43.5, 41.7, 40.7, 38.4, 37.1, 35.6, 35.0, 32.2, 30.6, 30.5, 29.4, 28.6, 26.3, 25.1, 23.6, 21.3, 21.1, 16.4; MS (ESI) *m/z*: 527.9 $[M+H]^+$; HRMS (ESI): calcd for $C_{31}H_{47}N_2O_5$ $[M+H]^+$ 527.3479, found 527.3474.

*2.2. General procedure for synthesis of compound **3i-HCl***

To a stirred solution of 1% HCl (5 mL) was added compound **3i** (0.51 g, 1 mmol). The mixture was stirred at rt for 15 min. Then, the precipitate was collected by filtration and washed by cold 50% aq EtOH (5 mL) to afford **3i-HCl**.

2.3. In vitro cytotoxic assay

HeLa, HepG2, Daudi, MV-4-11, Jurkat, HT1080, PC-3, AGS, MCF-7 and NCI-H2228 were obtained from American Type Culture Collection (Rockville, MD), and were used for the cytotoxicity assay in vitro by the MTT assay method as reported before [21].

2.4. In vivo antitumor assay

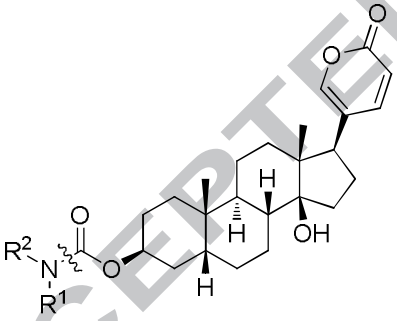
Female 7-week-old specific pathogen free (SPF) BALB/cA-nude mice (weight, 25–27 g) were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. MV-4-11 cells (7 × 107) were subcutaneously implanted into the axilla region of mice. After tumors reached 100~200 mm^3 in size, mice were randomly divided into 5 groups (8–16 mice in each group) and started treatment of vehicle control (iv., QOD), positive control (sunitinib, 40 mg/kg, ig., QD), bufalin (1 mg/kg, ip., QOD), **3i-HCl** (1 mg/kg, iv., QOD), **3i-HCl** (2 mg/kg, iv., QOD), **3i-HCl** (2 mg/kg, ig., QOD), **3i-HCl** (4 mg/kg, ig., QOD). Tumor sizes were measured every 3–4 days using calipers and their volumes were calculated using a standard formula: $width^2 \times length/2$. Body weight was measured daily. The weights of tumors were measured at the end of the experiment and the inhibition rates of drugs were calculated.

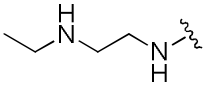
3. Results and discussion

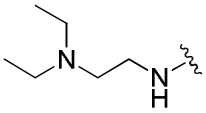
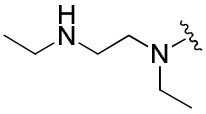
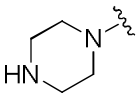
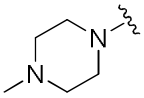
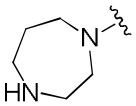
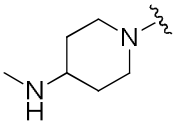
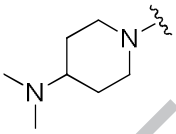
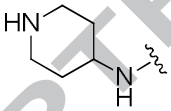
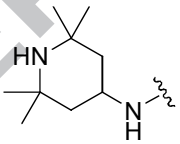
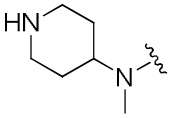
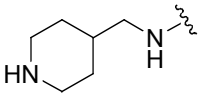
In our previous study, we found that bufalin-3-yl nitrogen-containing-ester

derivatives displayed a significant cytotoxic potency compared to the parent compound bufalin [16]. However, these compounds showed low activities *in vivo*. In order to maintain the activities *in vitro* and improve the activities *in vivo*, a series of bufalin-3-yl nitrogen-containing-carbamate derivatives **3** were designed and synthesized (Table 1). Of the examples listed in Table 1, the yields of **3g** and **3k** were much lower than the others, because when *N*-methylpiperidin-4-amine was used as the substrate, both **3g** and **3k** were formed in 30% and 25% yields, respectively. As shown in Table 1, although asymmetric diamines were used as substrates (Tabel 1, entries 9, 10 and 12), the compounds mentioned in Table 1 were the main products, and the yields of the another isomers were very low.

Table 1. Synthesis of bufalin 3-carbamate derivatives **3**.



Entry		Product **3**	Yield (%)[a]
1		**3a**	55

2		**3b**	60
3		**3c**	60
4		**3d**	73
5		**3e**	75
6		**3f**	66
7		**3g**	30
8		**3h**	65
9		**3i**	70
10		**3j**	58
11		**3k**	25
12		**3l**	70

[a] Isolated yields based on bufalin.

All the bufalin-3-yl nitrogen-containing-carbamate derivatives **3** were evaluated *in vitro* for their cytotoxicities against the human cervical epithelial adenocarcinoma (HeLa) cell line, and the results are presented in Table 2. The screening results suggested a rough SAR: among the nitrogen-contained carbocycle carbamates, the piperazinyl, homopiperazinyl and piperidinyl moieties were effective in increasing cytotoxicity with the IC_{50} values of 0.59–0.85 nM, which were 8–28 fold more potent than bufalin. Compounds **3d**, **3f** and **3i** which contained piperazinyl, homopiperazinyl and piperidinyl moieties displayed the most significant activity. The compounds with N-methyl substituents (**3e**, **3g**, **3h**, **3j** and **3k**) at the nitrogen-contained carbocycle demonstrated lower activities.

Table 2. Cytotoxic activity of bufalin-3-yl carbamate derivatives **3**.

Compound	IC_{50} (nM)[a] HeLa	Compound	IC_{50} (nM)[a] HeLa
BF	7.27 ± 1.00	**3g**	16.50 ± 3.25
3a	3.26 ± 0.45	**3h**	9.88 ± 1.00
3b	2.19 ± 0.40	**3i**	0.59 ± 0.04
3c	5.99 ± 0.92	**3i-HCl**	0.54 ± 0.04
3d	0.77 ± 0.11	**3j**	15.50 ± 1.70
3e	21.70 ± 5.50	**3k**	22.33 ± 3.16
3f	0.85 ± 0.17	**3l**	> 200

[a] IC_{50}: 50% inhibitory concentration. Values are an average of three separate experiments.

The results showed that both **3i** and **3i-HCl** displayed strong anti-proliferative activity, therefore, **3i** and **3i-HCl** were chosen for further evaluation against a panel of human tumor cell lines, including HeLa, HepG2, Daudi, MV-4-11, Jurkat, HT1080, PC-3, AGS, MCF-7 and NCI-H2228. Bufalin was used as a positive control and the antiproliferative activities of **3i** and **3i-HCl** are shown in Table 3. As shown in Table 3, **3i** and **3i-HCl** exhibited significant *in vitro* anti-proliferative activity against the ten tested tumor cell lines, with IC_{50} values ranging from 0.30 to 10.78 nM, and were more potent than bufalin. Remarkably, **3i-HCl** showed the greatest cytotoxicity against MV-4-11 cell line with the IC_{50} value of 0.30 nM.

Table 3. Cytotoxic activity of bufalin, **3i** and **3i-HCl** towards 10 human tumor cell lines.

Cell line	IC_{50} (nM)[a]		
	BF	**3i**	**3i-HCl**
HeLa	7.27 ± 1.00	0.59 ± 0.04	0.54 ± 0.04
HepG2	6.02 ± 0.71	0.51 ± 0.04	0.33 ± 0.02
Daudi	12.56 ± 0.76	2.86 ± 0.21	2.34 ± 0.22
MV-4-11	6.26 ± 2.22	0.75 ± 0.19	0.30 ± 0.11
Jurkat	8.79 ± 2.48	1.09 ± 0.26	0.67 ± 0.10
HT1080	11.31 ± 0.38	2.90 ± 0.33	2.34 ± 0.10
PC-3	6.00 ± 0.45	0.55 ± 0.10	0.38 ± 0.08
AGS	51.80 ± 1.34	9.62 ± 0.69	9.35 ± 0.53
MCF-7	45.87 ± 5.18	9.97 ± 1.45	10.78 ± 1.08
NCI-H2228	16.06 ± 2.51	0.56 ± 0.05	0.67 ± 0.03

[a] IC_{50}: 50% inhibitory concentration. Values are an average of three separate experiments.

The water solubility of bufalin, **3i** and **3i-HCl** were tested, and the solubilities were 0.04, 0.10, and 2.0 mg/mL, respectively. The result indicated that the solubility of **3i-HCl** was about 50 times greater than that of bufalin.

On the basis of its potent anti-proliferative effect, bufalin and **3i-HCl** were selected for *in vivo* studies using sunitinib as a positive control. Xenograft tumors were generated by subcutaneous implantation of MV-4-11 cells into nude mice. Mice were treated ig once a day with sunitinib, or ip once in two days with bufalin, or iv or ig once in two days with **3i-HCl** for 3 weeks. At the end of the experiment, animals were euthanized and the weights of tumors were measured.

Table 4. Anti-tumor efficacy of bufalin, sunitinib and **3i-HCl** against MV-4-11 xenografts in nude mice.

Compound	Dosage (mg/kg)	Administration route	Mice (n) Initial/end	Tumor weight (g) X ± SD	Inhibition rate (%)
Solvent	/	iv, QOD × 21d	16/16	1.9508 ± 0.1202	-
Sunitinib	40	ig, QD × 21d	8/8	0.0000 ± 0.0000*	100.00%
bufalin	1	ip, QOD × 21d	8/8	2.4146 ± 0.2589	-23.77%
3i-HCl	1	iv, QOD × 21d	8/8	2.2448 ± 0.4022	-15.07%
3i-HCl	2	iv, QOD × 21d	8/8	0.0000 ± 0.0000*	100.00%
3i-HCl	2	ig, QOD × 21d	8/8	1.5118 ± 0.2714	39.04%
3i-HCl	4	ig, QOD × 21d	8/8	0.0000 ± 0.0000*	100.00%

[a] The *in vivo* experiment was carried out in nude mice bearing MV-4-11 xenografts.

As shown in Table 4, sunitinib at a dose of 40 mg/kg/d by ig significantly inhibited tumor growth. According to a previous report, the LD_{50} value of bufalin (ip.) in mice was about 2.2 mg/kg [22]. The result indicated that both bufalin and **3i-HCl** showed no inhibitive effects on tumor growth *in vivo* at the dose of 1 mg/kg/d, while **3i-HCl** inhibited tumor growth by 100% at the dose 2 mg/kg by iv ($P < 0.01$). **3i-HCl** also showed strong inhibitive effects on tumor growth at a dose of 4 mg/kg/d by ig and the tumor inhibition rate of **3i-HCl** was also 100% ($P < 0.01$).

In conclusion, we have synthesized a series of new bufalin-3-yl nitrogen-containing-carbamate derivatives from bufalin by a two-step reaction route, and evaluated their cytotoxicity against Hela cells. Among them, **3i-HCl** had a strong cytotoxic effect on various tumor cells with IC_{50} = 0.30–10.78 nM. Furthermore, **3i-HCl** significantly inhibited tumor growth by 100% at a dose of 2 mg/kg by iv, or 4 mg/kg by ig. These bufalin-3-yl nitrogen-containing-carbamate derivatives will provide better insight into the effect of the C3 site on cytotoxic activity for designing potential cardiac glycoside antitumor agents.

Acknowledgments

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Razafitsalama J, Adriantsiferana R, Rasamison VE, Kingston DGI. Cytotoxic Cardenolide Glycosides of Roupellina (Strophanthus) boivinii from the Madagascar Rainforest (1). J Nat Prod 2007;70:1766–1770.

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Graphical abstract

Synthesis and biological evaluation of bufalin-3-yl nitrogen-containing-carbamate derivatives as anticancer agents

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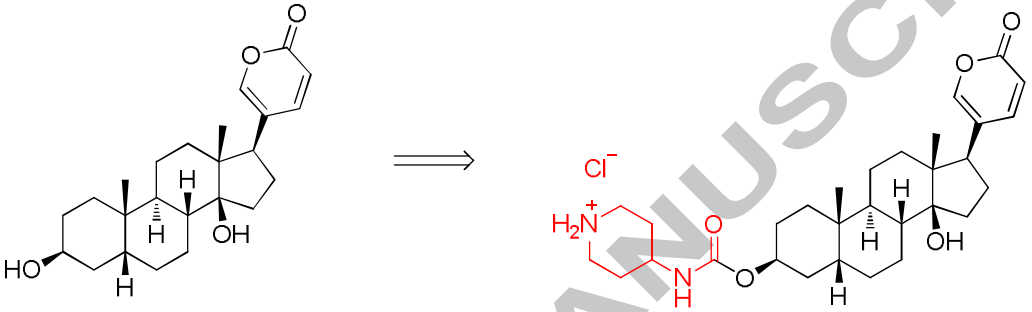

Highlights

► Highly efficient method to synthesize bufalin-3-yl nitrogen-containing-carbamate derivatives. ► Bufalin-3-yl nitrogen-containing-carbamate derivatives show significant cytotoxicity. ► **3i-HCl** significantly inhibits tumor growth *in vivo* by 100% **at the dose** 2 mg/kg by iv, or 4 mg/kg by ig.