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# Liposome-like nanocapsules of dual drug-tailed betaine for cancer therapy

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ABSTRACT

A novel dual drug-tailed betaine conjugate amphiphile has been firstly synthesized in which the polar headgroup is derived from glycine betaine and the hydrophobic tails are chlorambucil molecules. The newly prepared conjugate undergoes self-assembly to form stable liposome-like nanocapsules as an effective carrier with high drug loading capacity. The nanocapsules showed higher cytotoxic effects to cancer cell lines than those of free chlorambucil in vitro, and inhibited tumor growth effectively *in vivo*. This strategy that utilizes new dual drug-tailed betaine conjugate amphiphile to construct a self-assembled nanoparticle drug delivery system may have great potential in cancer chemotherapy.



## 1. Introduction

Nanosized vehicles are widely used as drug carriers for cancer therapy including micelles, liposomes, nanopartcles (Shchukina and Shchukin, 2011; Porter et al., 2008; Fujii, 1999; Soussan et al., 2009; Gong et al., 2012; Gravel et al., 2012). With the help of these nanocarriers, anticancer drugs can obviously demonstrate many therapeutic advantages over free drugs such as good cytocompatibility, favorable water solubility, high tumor selectivity (Singer et al., 2001; Pankaj et al., 2005; Khandare et al., 2006). However, these drug delivery systems mainly rely on the encapsulation of drug molecules, which often lead to low and unstable drug loading and become an insurmountable obstacle in clinical application (Tong and Cheng, 2008; Yoo et al., 2000; Lee et al., 2005; Caiolfa et al., 2000).

Recently, a new strategy has been developed by conjugating hydrophobic drug molecule with a short hydrophilic group or segment to design drug amphiphiles which can be readily assembled to form nanosized vehicles. The nanovehicles derived from drug amphiphiles have high and stable drug loading capacity. Peptide (Pedersen et al., 2009; Linderoth et al., 2009), poly (ethylene glycol) oligomer (Dahan et al., 2007), phosphorylcholine, dendrimer and drug molecules (Cheetham et al., 2013) are often used as hydrophilic parts to construct drug-carrier conjugates because of their extreme hydrophilicity. As a kind of zwitterion, phosphorylcholine is an effective hydrophilic moiety in the design of drug amphiphiles (Cheetham et al., 2014; Shen et al., 2010; Huang et al., 2014). The drug-phosphorylcholine amphiphiles could form stable liposomal nanovesicles. However, hydrophilic zwitterions applied in the construction of drug amphiphiles are usually limited to phosphorylcholine.

Betaine is another specific type of zwitterionic compound which plays an important role in biological systems (Goursaud et al., 2008; Ueland and Inherit, 2011). Betaine based amphiphiles, alkyl or acyl carboxybetaines, can self assemble to form micelles or capsules because of the nature of zwitterionic heads and alkyl tails (Goursaud et al., 2008; Perttu and Szoka, 2011). However, there has been no report on utilizing zwitterionic betaine to develop drug amphiphiles and nanovesicles. Inspired by the structure of phospholipid which can assemble to form stable liposomes, we first design a novel drug-carboxybetaine conjugate by applying betaine as a hydrophilic head and dual hydrophobic antitumor drug molecules as tails (Fig. 1). Hopefully, the amphiphilic conjugate as a new prodrug could self assemble to form stable nanosized vehicles with extremely high drug loading. In order to confirm the concept, chlorambucil was used as a model as hydrophobic tails in the zwitterionic conjugate in this report.

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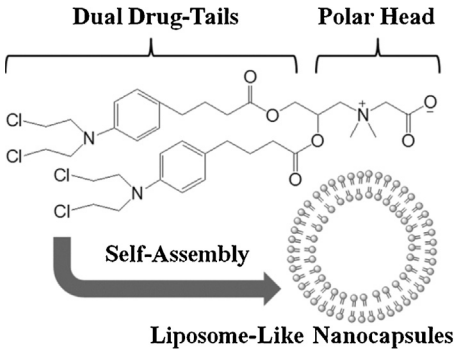


**Fig. 1.** Schematic illustration of dual drug-tailed betaine conjugate.

## 2. Materials and methods

### 2.1. Materials

All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. Chlorambucil (purity > 99%) was purchased from J&K Scientific Co., Ltd. (Shanghai China). 3-(dimethylamino)-1,2-propanediol (purity > 98%) was purchased from Nanjing Wanqing Co., Ltd. (Nanjing China). 1,8-Diazabicyclo [5.4.0] undec-7-ene and carbonyldiimidazole (purities > 99%) were purchased from Aladdin Co., Ltd. (Shanghai China). *Tert*-butyl bromoacetate (purity > 99%) was purchased from Sinopharm Chemical Rengent Co., Ltd. (Shanghai China). Phosphotungstic acid was purchased from Adamas Rengent Co., Ltd. (Shanghai China). MCF-7, HeLa and HepG-2 cells were purchased from the Chinese Academy of Sciences (Shanghai China).

### 2.2. General

Analytical HPLC was carried out on an Agilent 1100 Infinity Quaternary HPLC System equipped with a VWD UV–vis detector, and a ZORBAX SB-C18 column (Analytical, 4.6 × 150 mm) (Agilent Ltd., USA). Cryo-TEM characterization was performed on a tecnai $G^2$ F20Cryogenic transmission electron microscope using a vitrobot freezing device (FEI Ltd., USA). DLS characterization was performed on a Zetasizer 3000HS dynamic light scattering instrument (Malvern instruments Ltd., UK). MS was performed using a xevo g2 qtof (Waters Co., Ltd., USA). $^1$H and $^{13}$C NMR spectra were obtained using a DPX 500 MHz spectrometer (Bruker Daltonics Inc., USA); the residual solvent protons were used to reference the chemical shift. Coupling constants (*J*) were reported in Hertz (Hz), and splitting patterns were designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad).

### 2.3. Synthesis of dual chlorambucil-tailed betaine conjugate (DCBC)

DCBC was synthesized by three steps showed as follows (Fig. 2).

(i) Chlorambucil conjugation

Chlorambucil (1.818 g, 6 mmol) and CDI (0.972 g, 6 mmol) were dissolved in DCM (20 mL). The solution was stirred for 2 h at 25 °C. 3-(dimethylamino)-1,2-propanediol (0.119 g, 1 mmol) and DBU (0.912 g, 6 mmol) were added to the reaction. The solution was stirred for 24 h at 25 °C and was monitored by TLC (solvent: 40–50% methanol in DCM, visualized by UV light (λ = 254 nm) and phosphomolybdic acid). In order to purify the product (i), the reaction was diluted with 50 mL DCM and the reaction was washed three times with 10 mL 1 M HCl. The DCM solution was dried over $Na_2SO_4$. Solvent was removed by rotary evaporation and then by vacuum. The purity of product was determined by TLC and found to be about 90%. This step had about 85–90% yield. The compound structure was confirmed by MS. MS: $[M+H]^+$ *m*/*z*, 690.4.

(ii) Amine quaternization with *tert*-butyl bromoacetate

The product (i) (about 1 mmol) was solubilized in 30 mL DCM by stirring at room temperature. Next, DIEA (0.516 g, 4 mmol) was added to the reaction. *Tert*-butyl bromoacetate (0.585 g, 3 mmol) was gradually dripped into the reaction in order to avoid excessive heating. The reaction proceeded for 24 h at 40 °C and was monitored by TLC (solvent: 10–20% methanol in DCM, visualized by UV light (λ = 254 nm) and phosphomolybdic acid). The reaction mixture was diluted with 50 mL DCM. The reaction mixture was diluted with 50 mL DCM, washed three times with 10 mL 1 M HCl to remove residual DIEA and *tert*-butyl bromoacetate, and dried over $Na_2SO_4$. Solvent was removed by rotary evaporation and vacuum. The purity of product (ii) was determined by TLC and found to be about 85%. This step had about 80% yield. The compound structure was confirmed by MS. MS: $[M+H]^+$ *m*/*z*, 807.3.

(iii) Deprotection of *tert*-butyl ester

The product (ii) was solubilized in 20 mL DCM and 10 mL TFA. 1 mL of triisopropylsilane was added as a scavenger of carbocations. The reaction was allowed to proceed for 2 h at 25 °C and was monitored by TLC (solvent: 10–20% methanol in DCM, visualized by UV light (λ = 254 nm) and phosphomolybdic acid). The reaction was diluted with 50 mL DCM, washed three times with 10 mL 1 M $NaHCO_3$ to remove residual TFA, and dried over $Na_2SO_4$. Solvent was removed by rotary evaporation and vacuum. The crude product was purified by silica gel column chromatography (DCM: MeOH/65:25) yielding 0.38 g of dual chlorambucil-tailed betaine conjugate (DCBC) (51% overall). The purity of DCBC was determined by HPLC and found to be about 98.2% (Fig. S2). DCBC was confirmed by HPLC, MS, $^1$H NMR and $^{13}$C NMR. MS: $[M+H]^+$ *m*/*z*, 750.2 (Fig. S2). $^1$H NMR (500 MHz, $DMSO_{-d6}$:$CDCl_3$ 1:1): δ 7.04 (4H,

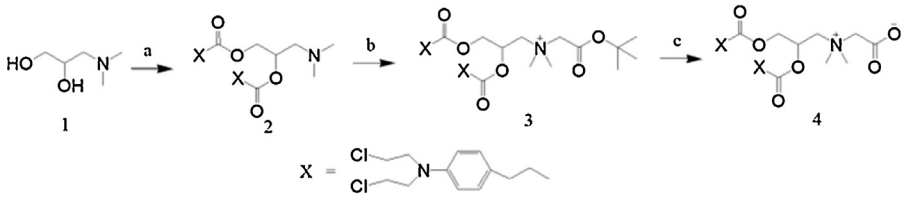


**Fig. 2.** Synthetic procedure followed to obtain dual chlorambucil-tailed betaine conjugate (DCBC). Reagents: (a) (i) CDI, chlorambucil, (ii) DBU; (b) *tert*-butyl bromoacetate, DIEA; (c) TFA, triisopropylsilane.