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Synthesis of 2,6-Dimethoxyhydroquinone-3-mercaptoacetyl -peptide-chlorambucil Conjugates

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Abstract: This paper reports a continous study of the use of short chain peptides as carriers of a potential antitumor agents: 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA). In an effort to carry out anti-cancer drug design, we synthesized another two new DMQ-MA-peptide-chlorambucil (CRB) derivatives: DMQ-MA-Lys(CRB)-Arg-OMe, DMQ-MA-Lys(DMQ-MA)-Lys(CRB)-Arg-OMe. These peptide-chlorambucil conjugates were synthesized by coupling protected amino acids in solution and the next conjugation was achieved by reacting with pentafluorophenyl ester of DMQ-MA in DMF. The CRB in side chain was coupled by deblocking the lysyl-carbobenzyloxy protecting group Z and then reacting with the pentafluorophenyl ester of chlorambucil (CRB). Further study on cytotoxicity, DNA binding, and sequence specificity of DNA alkylation of these two new conjugates are investigating.

Keywords: 2,6-Dimethoxyhydroquinone-3-mercaptoacetic acid, chlorambucil, peptide.

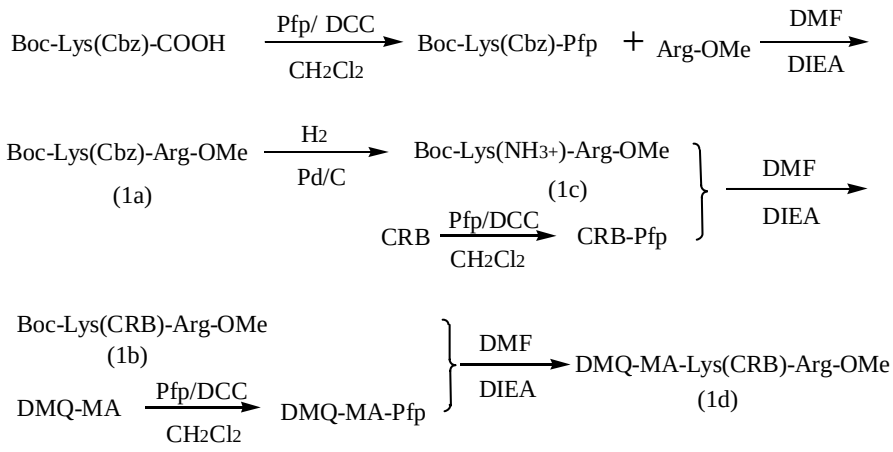
In previous paper, we had reported the synthesis of three conjugates of the cytotoxicity agents: 2,6-Dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA)[1], which is a derivative of 2,6-dimethoxy-*p*-benzoquinone (DMQ). DMQ is a naturally fermented product of wheat germ and was found to have a wide spectrum of cytotoxicity against various tumor cell lines under the synergistic activation of L-ascorbic acid[2-5] . Owing to the very low aqueous solubility of DMQ, we prepared derivatives of the DMQ-MA. Most of the DMQ-MA-peptide conjugates were synthesized by liquid-phase synthetic methods according to **Scheme 1** (Pfp:pentafluorophenol, DCC:N,N'-Dicyclohexyl-carbodiimide), and many of them displayed enhanced *in vitro* antitumor activities under the activation of the L-ascorbic acid (AH_2) as compared to the parent compound DMQ-MA. In this paper, we used short chain peptides as carriers of DMQ-MA, and compared with previous compounds, the most significant characteristics in these two conjugates are that chlorambucil was coupled in the side chain of lysine.

The main purpose of this drug design is based on the following reason:

Alkylating agents such as chlorambucil (CRB) are useful against chronic lymphocytic leukaemia[6] . These agents are known to alkylate DNA preferentially at guanine N7 positions[7], causing intra- or inter- strand cross-linking of DNA and interfering with DNA replication. Though CRB has been used in clinical diagnosis for more than 35 years, the mechanism of its' action are not fully understood. Targeting of ADJ/PC6 plasmacytoma cells and DNA by a CRB-spermidine conjugate was reported to

be more cytotoxic and also produced more DNA interstrand cross-linking than CRB[8]. It appears that the approach of drug targeting depends on the ionic interaction of positively charged spermidine (carrier) with the negatively charged phosphoryl groups of DNA[9]. Some reports indicated that CRB enters and exits chronic lymphocytic leukaemia lymphocytes by simple diffusion[10,11]. All these study on the CRB shows that it possesses some specific characteristics, which can be used in anti-cancer drug design. In this paper, we reported two novel DMQ-MA-peptide-CRB conjugates, and envisage that the involvement of DMQ-MA and CRB will enhance the cytotoxity significantly while remain the relatively appropriate solubility with the use of Lys and Arg.

Scheme 1.



Material and methods

All of the protected amino acid derivatives were purchased from Sigma Chemical Co. Medium pressure column chromatography was performed using Merck 230-400 mesh silica gel. TLC system was performed on Merck silica gel 60 on aluminum sheets. Low resolution mass spectra was taken from JEOL JMX-HX 110 instrument operating in the FAB mode at Tunghai University, Taiwan. Elemental analysis were performed at Cheng-Kung University, Taiwan.

Synthesis

DMQ and DMQ-MA was synthesized in Prof Sheh's laboratory.

Boc-Lys(Cbz)-Arg-OMe **1a:** N_α-Boc-N_t-Cbz-Lysine hydrochloride (1.61mmol) in CH_2Cl_2 was stirred with Pfp (2.41mmol) in ice bath for 10 min and then DCC (1.61mmol) was added, stirred further for 15 min with ice bath, and then stirred at room temperature for 4 hours, filtered and the solvent was removed *in vacuo*. Arg-OMe (1.61mmol) in DMF then was added, adjusted pH=7.0 with DIEA (N,N-Diisopropylethylamine), reacted for 3.5 hours at room temperature, distilled under reduced pressure and

evaporated to give a white solid, which was purified by column chromatography and eluted with CH_2Cl_2, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% CH_3OH in CH_2Cl_2 (100ml) respectively, evaporated to get a white solid and TLC system (CH_3OH: CH_2Cl_2=15:85) Rf=0.52.Yields: 71%.

Boc-Lys(CRB)-Arg-OMe **1b:** The protected peptide **1a** (0.75mmol) was dissolved with methanol and added 0.1ml concentrated hydrochloride acid. The mixture was hydrogenated over palladium on charcoal for 2 hours, filtered over celite and the solvents was distilled under reduced pressure, then get a white solid **1c** Boc-Lys(NH_3^+)-Arg-OMe. CRB in DMF (1.116mmol) was esterified with Pfp (1.614mmol) and DCC (1.116mmol) at room temperature. After sirring for 3.5 hours, the reaction mixture was added to **1c**. DIEA was used to adjust the pH to 7.2. The reation was allowed to proceed for 3 hours. The solvents were removed *in vacuo* and the crude product was purified by silica gel column chromatography using stepwise elution (CH_2Cl_2/MeOH: CH_2Cl_2, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% MeOH in CH_2Cl_2) to give a white solid **1b**.Yield: 70%.

DMQ-MA-Lys(CRB)-Arg-OMe **1d:** Boc-Lys(CRB)-Arg-OMe **1b** (0.43mmol) in CH_2Cl_2 was stirred with trifluoroacetic acid (TFA) for 50 min and the solvents removed *in vacuo*. DMQ-MA (0.54mmol) in CH_2Cl_2 was stirred with Pfp (0.75mmol) in ice bath for 10 min and then DCC (0.54mmol) was added, stirred further for 15 min with ice bath, and then continued stirring at room temperature for 3.5 hours, filtered and dried under vaccum for half an hour. Then the TFA salt in CH_2Cl_2 was added. DIEA was added to adjust the pH to neutral. After 1.5 hour, the reaction mixture was filtered, diluted with CH_2Cl_2 and washed successively with citric acid, saturated $NaHCO_3$ and saturated NaCl, after which it was dried and evaporated to give a crude solid. Silica gel chromatography using stepwise elution: CH_2Cl_2, 1%, 2%, 3%, 4%, 5%,6%, 7%, 8%, 9%, 10% CH_3OH in CH_2Cl_2, afforded the pink product **1d.** Yields: 68%. TLC system (CH_3OH:CH_2Cl_2=15:85) Rf=0.55. Elemental analysis, Analysis calcd for $C_{37}H_{55}N_7O_9SCl_2$: C:52.61, H:6.52, N:11.61, Found : C:52.47, H:6.39, N:11.70. LRMass(FAB): [MH^+]:845.

2a,Boc-Lys(Boc)-COOH was sythesized according to literature 12. **2b**,Boc-Lys(Boc)-Lys(CRB)-Arg-OMe,
2c,DMQ-MA-Lys(DMQ-MA)-Lys(CRB)-Arg-OMe, was prepared following a procedure similar to that for **1b** and **1d**. The results are as follows, **2c:**Yields: 70%. TLC system (CH_3OH:CH_2Cl_2=20:80) Rf=0.51. Elemental analysis, Analysis calcd for $C_{53}H_{77}N_9O_{15}S_2Cl_2$: C:52.39, H:6.34, N:10.38, Found : C:52.50, H:6.28, N:10.42. LRMass(FAB): [MH^+]:1215.

Results and discussion

In this experiment, there were some tricks and difficulties. The hydrogen process need to be dealt with very carefully and must be determined with TLC system in order to react completely while not over-reaction. We still need to pay much attention to the purification and protect against decomposition of DMQ-MA by the Michael retrograde reaction *in situ*. Based on many previous works, we are trying our best in carrying out the anti-cancer drug design. In this paper, Arg and Lys were used as carriers to bring the cytotoxic agent DMQ-MA and CRB to the target tumor molecular and by involvement

two DMQ-MA or CRB, which possess significant antitumor activity, we design these two compounds in order to enhance their cytotoxicity to inhibit human pulmonary adenocarcinoma cell line (PC-9 cells) and oral epidermoid carcinoma cell line (KB). We hope to clarify their reaction mechanism to DNA or protein in the next step.

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