Synthesis and DNA Cleavage Studies of 2,6-Dimethoxyhydroquinone-3-Mercaptoacetic Acid Conjugates

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Abstract: In an effort to investigate the use of short peptide chains as carriers of new anti-tumor agents, we synthesized four tripeptide-cytotoxic agent conjugates: DMQ-MA-Lys(DMQ-MA)-Phe-Arg-Ome, DMQ-MA-Lys(DMQ-MA)-Ile-Arg-Ome, DMQ-MA-Lys(DMQ-MA)-Val-Arg-Ome, DMQ-MA-Lys(DMQ-MA)-Lys(Cbz)-Arg-Ome. The cytotoxic agent conjugated to the N-terminal and the ξ-amino group of Lysine of the tripeptide is 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA). The tripeptides were synthesized by coupling protected amino acid residues according to Pfp/DCC methods (Pfp: pentafluorophenol, DCC:N,N'-dicyclohexyl-carbodiimide) in solution. Agarose gel electrophoresis showed that these compounds can cleave supercoiled DNA into open-circular form in drug concentration as low as 4-50 μM without H_2O_2 and UV irradiation. Further studies on their cytotoxicity for these conjugates are ongoing.

Keywords: 2,6-Dimethoxyhydroquinone-3-mercaptoacetic acid, tripeptide, cleavage, pBR322 DNA.

In previous paper and work, we reported the synthesis and cytotoxicity studies of a novel series of short chain peptide conjugates of the cytotoxic agent 2, 6-dimethoxyhydro-quinone-3-mercaptoacetic acid[1] (DMQ-MA). The DMQ-MA is a derivative of 2,6-dimethoxybenzoquinone, which is a natural product of fermented wheat germ and found to have a wide spectrum of cytotoxicity against various tumor cell lines under the synergistic activation of L-ascorbic acid (AH_2)[2,3]. Compared with DMQ, DMQ-MA has the moderate water solubility (27°C, DMQ 24mg/100mL, DMQ-MA 500mg/100mL) and low cytotoxicity.

Using high molecular weight compounds as drug carriers to target point is an active research field. But it is limited by the high toxicity of high molecular weight carriers to host cells[4]. Previous studies showed that most of the amino-acid-DMQ-MA conjugates possess higher cytotoxicity index than the parent DMQ-MA[5] and these tripeptide-DMQ-MA conjugates are anticipated to be active in their conjugated form[6].

Experimental

All of the protected amino acids were purchased from Sigma Chemical Co. Medium pressure column chromatography was performed using Merck 230-400 mesh silica gel. CH_2Cl_2 and $CHCl_3$ were purified before use. TLC system was performed on Merck silica gel 60 on aluminum sheets. Low-resolution mass spectra were taken from JEOL

JM-HX 110 instrument and MA SPEC System operating in the FAB mode. Elemental analysis was performed on Perkin-Elmer 240c instrument. Plasmid pBR322 DNA, calf thymus DNA and Tris were purchased from Beijing Sino-American Biotechnology Company. The electrophoresis experiments were carried out on a set of electrophoresis systems by using TAE buffer (pH=7.2, 50 mmol/L Tris acetate, 20 mmol/L sodium acetate and 10 mmol/L sodium chloride) and the gel was stained with ethidium bromide for 20 min after electrophoresis. Images were analyzed on Complete Gel Documenta-tion and analysis system GDS 8000.

DMQ, Boc-Lys(Boc) and DMQ-MA were synthesized in Prof L.Sheh's Lab according to the literature [1], [7] and his US Patent.

Boc-Lys(Cbz)-Arg-Ome **1a**: N_α-Boc-N_ξ-Cbz-Lysine hydrochloride (0.5 g, 1.315 mmol) in 10 mL of CH_2Cl_2 was stirred with Pfp (0.363 g, 1.97 mmol) in ice bath for 15 min and then DCC (0.27 g, 1.315 mmol) was added, stirred further at 0°C for 15 min, and then stirred at room temperature for 2 hours, filtered and the solvent was removed in *vacuo*. Arg-Ome (0.344 g, 1.35 mmol) in 2mL of DMF 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 with CH_2Cl_2, 1%, 2-10%, 11% CH_3OH/CH_2Cl_2 100 mL respectively. The solvents were evaporated to get a white solid. Rf=0.54 (CH_3OH : CH_2Cl_2=15 : 85).

Boc-Phe-Arg-Ome 2a, Boc-Ile-Arg-Ome 3a, and Boc-Val-Arg-Ome 4a were synthesized in a similar method as for 1a.

Table 1 Data of elemental analysis, MS and yields for compounds 1a-4a

Compounds	Elemental Analysis C %	H %	N %	[MH$^+$]	Yields %
1a	56.73 (56.92)	7.64 (7.50)	15.27 (15.18)	551	76
2a	57.93 (58.15)	7.58 (7.66)	16.09 (16.46)	436	71
3a	53.86 (53.97)	8.73 (8.92)	17.46 (17.11)	402	75
4a	52.71 (52.85)	8.53 (8.12)	18.09 (18.43)	388	68

( ) represents the Found data

Boc-Lys(Boc)-Lys(Cbz)-Arg-Ome **1b**: **1a** (0.217 g, 0.4 mmol) in 3 mL of CH_2Cl_2 was stirred with 2 mL of trifluoroacetic acid (TFA) for 1 hour. The solvents were removed in *vacuo*, and the residue was the TFA salt of **1a**. Boc-Lys(Boc) (0.14 g, 0.4 mmol) in 5 mL of CH_2Cl_2 was treated with Pfp (0.11 g, 0.6 mmol) in ice bath for 15 min, DCC (0.09 g, 0.4 mmol) was added, continued to react at 0°C for 15 min and then at room temperature for 90 min. The reaction mixture was added to the TFA salt of **1a**, and DIEA was added to adjust the pH to 7.0. After 120 min, the reaction mixture was filtered, and the filtrate was diluted with CH_2Cl_2 and washed with citric acid (10%, 30 mL), saturated $NaHCO_3$ (30 mL), and water (20 mL) successively, dried over $MgSO_4$ and evaporated in *vacuo* to give the crude solid. It was purified with silica gel chromatography and eluted stepwise with CH_2Cl_2, 1%, 2-12%, 15% CH_3OH/CH_2Cl_2 to afford the tripeptide as a white solid. Rf=0.57 ($CH_3OH:CH_2Cl_2$=20:80).

Boc-Lys(Boc)-Phe-Arg-Ome **2b**, Boc-Lys(Boc)-Ile-Arg-Ome **3b**, and Boc-Lys(Boc)

-Val-Arg-Ome **4b** were synthesized in a similar way as for **1b**.

Table 2 Data of elemental analysis, MS and yields for compounds **1b-4b**

Compounds	Elemental Analysis			$[MH^+]$	yields%
	C%	H%	N%		
1b	57.07 (57.32)	7.97 (7.62)	14.40 (14.59)	779	71
2b	57.92 (58.31)	7.99 (8.21)	14.78 (14.53)	664	66
3b	55.32 (55.71)	8.74 (8.33)	15.58 (15.66)	630	67
4b	54.63 (54.32)	8.62 (8.21)	15.93 (16.27)	616	63

( ) represents the Found data

DMQ-MA-Lys(DMQ-MA)-Lys(Cbz)-Arg-Ome **1c**: **1b** (0.44 g, 0.565 mmol) in 2 mL of CH_2Cl_2 was stirred with 2 mL of TFA for 1 hour and the solvents were removed in *vacuo*. DMQ-MA (0.44 g, 1.68 mmol) in 10 mL of $CHCl_3$ was stirred with Pfp (0.467 g, 2.4 mmol) in ice bath for 15 min, then DCC (0.35 g, 1.69 mmol) was added, stirred at 0°C for another 20 min and at room temperature for 2 hours, filtered and the filtrate were dried under vacuum for 30 min. Then the TFA salt of **1b** in 3 mL of DMF was added, and adjusted the pH with DIEA to 7.0. The reaction mixture was filtered after 2 hours, then diluted with $CHCl_3$ and washed with citric acid, saturated $NaHCO_3$ and water successively, dried over $MgSO_4$ and evaporated to give a solid. It was purified with silica gel chromatography and eluted stepwise with CH_2Cl_2, 1%, 2-12%, 15% $CH_3OH/CHCl_3$ to afford a purple solid. Rf=0.53 ($CH_3OH:CH_2Cl_2$=20:80).

DMQ-MA-Lys(DMQ-MA)-Phe-Arg-Ome **2c**, DMQ-MA-Lys(DMQ-MA)-Ile-Arg -Ome **3c**, DMQ-MA-Lys(DMQ-MA)-Val-Arg-Ome **4c** were synthesized in a similar way as for **1c**.

Table 3 Data of elemental analysis, MS and yields for compounds **1c**-**4c**

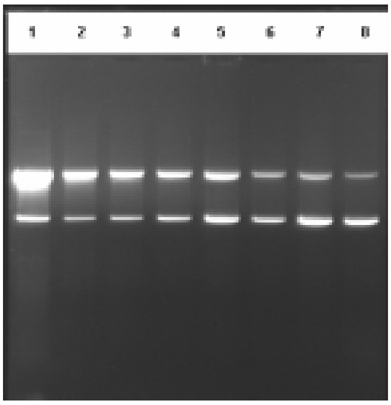
Compounds	Elemental Analysis			$[MH^+]$	Yields %
	C %	H %	N %		
1c	53.11 (52.87)	6.21 (6.02)	10.55 (10.31)	1063	67
2c	53.22 (53.49)	6.02 (6.32)	10.35 (10.17)	948	65
3c	51.26 (51.52)	6.46 (6.23)	10.73 (11.06)	914	63
4c	50.72(51.03)	6.34 (6.21)	10.90 (11.17)	900	67

( ) represents the Found data

DNA strand cleavage experiments

To study the action of the above tripeptide-DMQ-MA conjugates on the cleavage of nucleic acids, we performed electrophoretic analysis on the nicking of supercoiled DNA by tripeptide-DMQ-MA conjugates in the presence of AH_2 (ascorbic acid). The intensity of the bands was quantitated by the GDS 8000 computer software Gelworks 1D. Supercoiled Plasmid DNA values were corrected by a factor of 1.47 as a result of its lower staining capacity by ethidium bromide[8]. The experiment results show that these compounds start to cleave supercoiled DNA into open circular form in drug concentrations as low as 4-50 μM in the presence of AH_2, and some of them can even cleave the CCC form to OCC and linear form. The cleavage of pBR322 DNA was markedly inhibited by the addition of glycerol (>700 μM), sodium benzoate (>700 μM), mannitol (>400 μM) and DMSO (>700 μM). These studies suggest that hydroxyl radical were involved in the cleavage. Strand cleavage reaction is dependent on the peptide-DMQ-MA′s concentration, which means that the degree of DNA cleavage is drug-dose-dependent. At high concentrations, the open circular DNA is further cleaved into the linear form.

Figure 1 DNA cleavage patterns of **1c**.



Incubation in PBS at 37°C for 10 min and at 65°C for 10min. Band (a) represents the open-circular form (OCC form), band (b) represents the linear form and band (c) is the supercoiled form (CCC form). Lane 8: DNA alone; Lane 7: DNA+AH_2 (5μM); Lane 6-1: 5, 10, 20, 50, 100, 150 μM **1c** and AH_2 (5μM).

The DNA cleavage patterns of **2c**, **3c**, and **4c** are similar with that of **1c**. In the present work, no H_2O_2 or UV irradiation is required. Studies on the cytotoxicity of these conjugates are in progressing.

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