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Novel peptide derivatives of bleomycin A_5: Synthesis, antitumor activity and interaction with DNA

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Abstract—A series of novel amino acid and peptide derivatives of bleomycin (BLM) A_5 were synthesized. All the compounds possessed significant antitumor activities in vitro against HL-60, BGC-823, PC-3MIE8, and MDA-MB-435 cell lines. Their antitumor activities against MDA-MB-435 were 10-fold higher than BLM A_5. The DNA cleavage studies indicated that the hydrophobic amino acid or peptide derivatives of BLM A_5 could induce higher cleavage ratio of double to single strand DNA than BLM A_5. From the DNA binding studies, we found that the derivatives containing either D-conformation amino acid or basic amino acid could facilitate DNA binding of BLM.

1. Introduction

Bleomycins (BLMs) are a group of structurally related antibiotic compounds that were originally isolated from the culture broth of *Streptomyces verticillis* as copper chelates by Umezawa et al. in the early 1960s.[1,2] This family of compounds only differs at the C-terminus, which contains several dozen natural congeners.[3,4] The BLMs have been used clinically for treatment of cancers, including Hodgkin's lymphomas, carcinomas of skin, testicular tumors, and quamous cell carcinomas of the cervix, head, and neck.[5,6] The therapeutic efficacy of BLM is believed to derive from its ability to bind to and oxidatively degrade cellular DNA, and the process is metal-ion and oxygen dependent.[7–10] However, like many other antitumor drugs, BLMs also manifest several clinical limits, at high doses BLMs can induce pulmonary fibrosis and lead to fatal hypoxemia.[11–13] Furthermore, BLMs are also severely limited by tumor resistance and lack of cell selectivities.[14,15]

The structure of BLMs is generally divided into four functional domains, as listed in Figure 1. The C-terminus domain was believed to relate to their renal and lung toxicity and antitumor activity.[16,17] Our previous work, which focused on alteration at the C-terminus of BLMs with different alkyl groups showed that the hydrophobicity of the C-terminus played an important role in their DNA binding property and antitumor activity.[18,19] It has been reported that many tumors contain elevated levels of plasminogen activator and thus produce elevated levels of protease plasmin in the milieu of tumors, and there are many differences in the enzyme system between tumor and normal cells.[20] Based on these differences, some plasmin-activated prodrugs for cancer chemotherapy had been designed and synthesized by Charkravarty.[21,22] These prodrugs contained Val-Leu-Lys tripeptide, which exhibited about a 7-fold improved selective cytotoxicity for tested tumor cells. In order to increase the cell selectivity and bioactivity of BLMs, in this paper, we introduced the tripeptide (Val-Leu-Lys), related dipeptide and amino acid to the C-terminus of BLM A_5. We also studied the DNA binding and cleavage properties of these novel BLM analogs.

2. Chemistry

The BOC protected amino acid and peptide were synthesized and purified by the general method according to the literature.[23] The method used to prepare peptide and amino acid derivatives of BLM A_5 (**1**) is illustrated in Scheme 1, the primary amino group in β-aminoalanine moiety was protected by forming Cu(II) coordinate complex **2** according to our previous reports.[18,19] Com-

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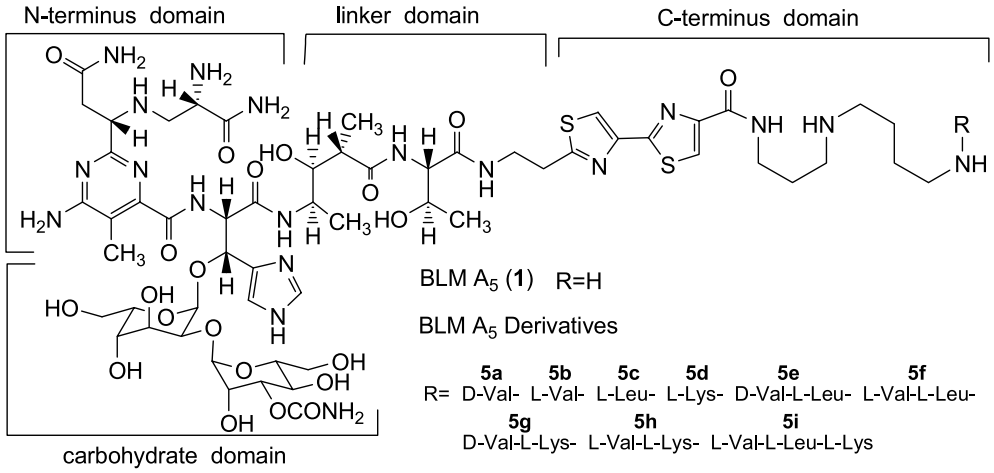
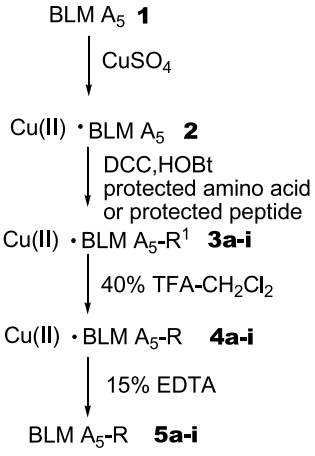


Figure 1. Structures of BLM A_5 (**1**) and BLM A_5 derivatives.



	R^1 (**3**)	R (**4**, **5**)
a	D-(Boc)Val-	D-Val-
b	L-(Boc)Val-	L-Val-
c	L-(Boc)Leu-	L-Leu-
d	L-$(Boc)_2$Lys-	L-Lys-
e	D-(Boc)Val-L-Leu-	D-Val-L-Leu-
f	L-(Boc)Val-L-Leu-	L-Val-L-Leu-
g	D-(Boc)Val-L-(Boc)Lys-	D-Val-L-Lys-
h	L-(Boc)Val-L-(Boc)Lys-	L-Val-L-Lys-
i	L-(Boc)Val-L-Leu-L-(Boc)Lys-	L-Val-L-Leu-L-Lys-

Scheme 1.

pound **2** was coupled with large excess corresponding protected amino acid or peptide at −5 to 1 °C for 12–24 h in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) in MeOH, providing **3a**–**i** in 60–95% yields. Amino group that protected copper complexes **3a**–**i** were treated with trifluoroacetic acid (TFA) in dichloromethane to remove the BOC to afford the copper complexes **4a**–**i**. Cu(II) was removed from **4a**–**i** by using 15% EDTA, and the products were purified with HP-20 column to afford the desired compounds **5a**–**i** in 40–70% yields based on BLM A_5.[24]

The structures of all novel compounds were characterized by FAB-MS, TOF-MS, ^{1}H NMR, and ^{13}C NMR. The conjugated position of amino acid or peptide to **1** was confirmed by comparing the ^{1}H and ^{13}C spectrum between **1** and compounds **5a**–**i**. Compared with **1**, the proton signals of the terminal methylene within the C-terminal spermidine substituent of compounds **5a**–**i** shifted downfield (from 2.85 to 3.14–3.23 ppm, respectively) in ^{1}H NMR, while the carbon signals shifted upfield (from 40.58 to 39.19–39.52 ppm, respectively) in ^{13}C NMR, indicating that the position to which the amino acid or peptide conjugated was the primary amino group within the C-terminus spermidine substituent, which is in accordance with our previous reports.[18,19]

3. Biological activity

The antitumor activities of novel BLM derivatives **5a**–**i** together with **1** against HL-60, BGC-823, PC-3MIE8, and MDA-MB-435 cell lines in vitro were tested by using the tetrazolium salt (MTT) assay.[25] The 50% inhibition concentrations (IC_{50}) of the test compounds are reported in Table 1. Compared with **1**, the antitumor activities of the novel BLM A_5 derivatives **5a**–**i** were dramatically increased against MDA-MB-435 cell line in vitro, but for other test cell lines (HL-60, BGC-823, and PC-3MIE8), these compounds exhibited similar antitumor activities. The results showed that the amino acid or peptide derivatives of BLM A_5 exhibited some selective cytotoxicities. From the data in Table 1, it was found that the tripeptide derivative of BLM A_5 **5i** was the most effective compound among the test compounds, which may have originated from its higher permeability to cell membrane or uptake by cells.

Table 1. In vitro antitumor activities of **5a**–**5i** and **1** against human tumor cell lines

Compound	Cell line IC_{50} (μM)			
	HL-60	BGC-823	PC-3MIE8	MDA-MB-435
1	0.25	1.25	1.48	1.64
5a	0.66	1.39	1.83	0.13
5b	0.31	0.80	0.80	0.13
5c	0.37	0.73	1.27	0.17
5d	0.51	0.92	1.08	0.10
5e	0.63	0.87	1.32	0.32
5f	0.27	0.93	1.18	0.09
5g	0.98	1.01	3.32	0.22
5h	0.74	1.17	1.91	0.10
5i	0.07	0.60	1.14	0.07

4. DNA cleavage

The therapeutic efficacy of BLM is believed to derive from its ability to bind to and oxidatively degrade cellular DNA, and double-strand DNA cleavage has been proposed to be important for therapy.[26] The DNA cleavage properties of **5a**–**i** together with **1** to pSP64 plasmid DNA were tested, and the results are shown in Figure 2. According to the results, all tested BLM derivatives **5a**–**i** exhibited similar or a little stronger DNA cleavage efficiency to **1**, but interestingly, compounds **5a**–**c**, **e**, **f**, and **i** induced extraordinarily high double to single strand DNA cleavage ratio, and there was nearly no Form II band (single DNA cleavage product) found in the picture for compounds **5a**–**c**, **e**, and **f**. It seemed that the higher double to single strand cleavage ratio was beneficial to the hydrophobic amino acid or peptide conjugated to BLM A_5 C-terminus.

5. DNA binding

DNA binding properties between BLM molecules and DNA backbone determined by the C-terminus of BLM are the basis for the DNA cleavage activity. In order to compare the effects on DNA binding affinity resulting from the alteration of C-terminus, we determined the DNA binding properties including apparent binding constant (K_b) (Fig. 3) and thermal denaturatation alteration (ΔT_m) (Fig. 4) by using calf thymus DNA (CT-DNA) with the methods reported.[27,28] According to the results in Figures 3 and 4, BLM A_5 derivatives with L-lysine or L-lysine as N-terminal peptide conjugated to BLM A_5 C-terminus (**5d, g, h**, and **i**) exhibited much stronger DNA binding affinity than hydrophobic amino acid or peptide conjugated BLM A_5 derivatives (**5a**–**c**, **e**, and **f**). These results were consistent with those of DNA cleavage properties (Fig. 2). For this kind of BLM derivatives, it seemed that lower DNA binding affinity could enhance the double to single DNA cleavage ratio. In addition, compounds **5a, e**, and **g** exhibited a little stronger DNA binding affinity than compounds **5b, f**, and **h**, respectively, which resulted from the stereochemistry of the valine or valine at the C-terminal peptide conjugated to the C-terminus of BLM A_5. The structure of D-conformation derivatives seemed to facilitate the adoption of a compact bound conformation of BLM for DNA binding.

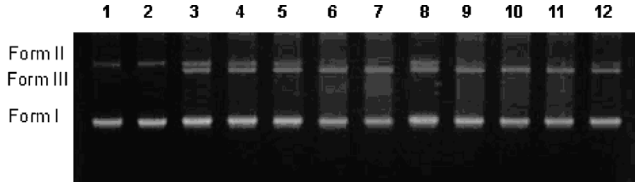


Fig. 2. Agarose gel illustrating the cleavage reaction of supercoiled pSP64 by Fe(II)-compound. The reaction was performed in 20 mM Tris–HCl, pH 8 buffer, each reaction mixture contained 200 ng pSP64 plasmid DNA in a total reaction volume of 15 μl and was incubated at 25 °C for 60 min. Lane 1, DNA alone; lane 2, 4.0 μM Fe(II); lane 3, 2.0 μM **1**, 4.0 μM Fe(II); lane 4, 2.0 μM **5h**, 4.0 μM Fe(II); lane 5, 2.0 μM **5g**, 4.0 μM Fe(II); lane 6, 2.0 μM **5f**, 4.0 μM Fe(II); lane 7, 2.0 μM **5e**, 4.0 μM Fe(II); lane 8, 2.0 μM **5d**, 4.0 μM Fe(II); lane 9, 2.0 μM **5c**, 4.0 μM Fe(II); lane 10, 2.0 μM **5b**, 4.0 μM Fe(II); lane 11, 2.0 μM **5a**, 4.0 μM Fe(II); lane 12, 2.0 μM **5i**, 4.0 μM Fe(II).

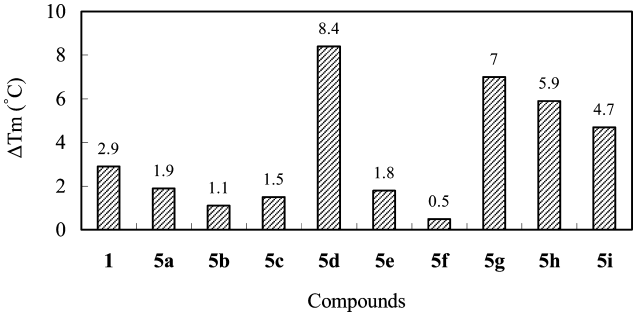


Figure 3. The alteration of the melting temperature ΔT_m (°C) for CT-DNA bind to compounds **5a**–**i** and **1**.

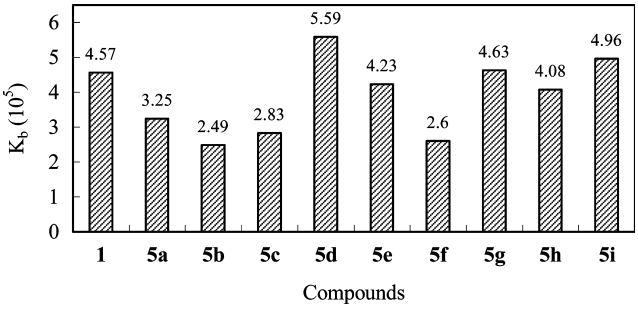


Figure 4. The apparent binding constants (K_b) of compound **5a**–**i** and **1** to CT-DNA.

6. Conclusion

In the aggregate, amino acid and peptide derivatives of BLM A_5 can be easily prepared by DCC condensation in methanol. Compared with BLM A_5, the C-terminus of BLM A_5 modified with amino acid and peptide could exhibit similar antitumor activities in vitro against HL-60, BGC-823, and PC-3MIE8 cell lines, and interestingly, this series of compounds showed a little strong selectivity of cytotoxicity against MDA-MB-435 cell line. From the primary studies on the SAR, we conclude that hydrophobic amino acid or peptide derivatives of BLM A_5 can induce higher double to single strand

DNA cleavage ratio than BLM A_5, and BLM derivatives with D-conformation amino acid and basic amino acid are easier to adopt for a compact and bound conformation of BLM for DNA binding than others.

Acknowledgments

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Supplementary data

The melting point, FAB-MS, TOF-MS, ^{1}H NMR, and ^{13}C NMR data of all BLM derivatives (**5a**–**i**) and BLM A_5 are given in the Supplementary Material.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.06.021.

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24. General procedure: Cu(II)·BLM A_5(**2**). An aqueous solution containing 90 mg (63 mmol) of **1** was treated with 112 mg (70 mmol) $CuSO_4$, the combined solution was maintained at 0–4 °C for 30 min. The resulting solution was lyophilized to obtain **2** as a blue powder (95 mg, 95%). Cu(II)·BLM A_5-R^1(**3a**–**i**) DCC (290 mmol) was added to 15 mL methanol solution containing protected amino acid or peptide (290 mmol), and then HOBt (290 mmol) were added. The mixture was stirred at 0 °C for 30 min. A solution of **2** (58 mmol) in 3 mL methanol was then added to the reaction mixture, and the reaction mixture was stirred at −5 °C for 12 h. After removing the precipitated DCU by filtration, the filtrate was concentrated under diminished pressure, and the crude product was purified by column chromatography (silica gel G) using MeOH/10% NH_4AC/10% NH_3 (100:10:1) as eluent. The product mixture was evaporated to remove the solvent at reduced pressure, and lyophilized to give **3a**–**i** resulting in 60–95% yield as a blue powder. Cu(II)·BLM A_5R(**4a**–**i**) 5 mL 40% TFA in dichloromethane solution was added to dried **3a**–**i**. The mixture was stirred at room temperature for 30 min, then the solvent and excess TFA were removed at reduced pressure, and lyophilized to afford the copper complex **4a**–**i**. BLM A_5R(**5a**–**i**). Demetallation of **4a**–**i** was accomplished by stirring with 15% EDTA(10 mL) at 30 °C for 1 h, then the mixture was passed through an ion exchange resin (HP-20) column slowly, washed with water successively, and then eluted with acidic methanol MeOH/2 mM HCl (4:1), the eluate was evaporated to remove the solvent and lyophilized, this procedure was repeated two times to afford **5a**–**i** in 90–95% yield as a white powder.
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