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NOTE

NC1404, a novel derivative of Bleomycin with modified sugar moiety obtained during the preparation of Boningmycin

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Boningmycin, isolated from the fermentation broth of *Streptomyces verticillus* var. *pingyangensis* n. var,[1] is a member of Bleomycins, which are glycopeptide anticancer antibiotics and widely used in clinical applications for various tumors such as squamous cell carcinomas, germ cell tumors, lymphoma and malignant pleural effusion.[2–4] According to the results of the structure–activity relationship, the molecules of Bleomycins can be divided into three main characterized parts: (1) the metal-binding domain that combines with metal ions and oxygen to form an active complex responsible for DNA cleavage,[5,6] (2) the bithiazole tail that facilitates the Bleomycins binding to nucleic acids and (3) the D-mannosyl-L-gulose moiety that plays a yet unclear role.[7,8] Recently, one research showed that the sugar moiety of the Bleomycins plays a significant role in Bleomycins' tumor cell targeting,[9] while another report suggested that it is not quite essential for the antitumor activity of Bleomycins, but critical for its toxicity.[10] During our research of Boningmycin, a compound NC1404 with modified sugar moiety was obtained and showed lower toxicity and comparable antitumor activity with Boningmycin, which may provide an additional proof of the toxicity of the sugar moiety of Bleomycins. Here we report on the production, isolation, structural elucidation, antitumor activity and primary toxicity of NC1404.

The strain was cultivated on an agar slant containing glucose 1.0%, soluble starch 1.0%, peptone 0.5%, agar 2.0% and NaCl 0.5% (pH 7.2–7.5) at 28 °C for 7 days, then inoculated in 250 ml Erlenmeyer flasks containing 50 ml of the production medium (soluble starch 2.5%, glucose 0.5%, soybean meal 3.5%, KH_2PO_4 0.1%, ZnSO4 0.05% and $CuSO_4$ 0.01% (pH 6.0–6.5)) and incubated at 28 °C on a rotary shaker (220 r.p.m.) for 48 h. Subsequently, 10 ml of obtained preculture was transferred into 500 ml Erlenmeyer flasks containing 100 ml of the production medium. The fermentation was carried out at 28 °C on a rotary shaker at 220 r.p.m. for 7 days. The copper-chelated Boningmycin was isolated from the fermentation broth according to the reported method with minor modifications.[11] Briefly, the fermentation broth (20 l) was adjusted to pH 2 ~ 3 with oxalic acid and filtered. The boningmycin was enriched from the filtrate by macroporous abosorben resin 4006 column (2 l, The Chemical Plant of NanKai University). The obtained crude material was chromatographed on a column of CM-Sephadex C-25 (NH_4^+ form, 200 ml, H&E Co., Ltd, Beijing, China), and eluted with a stepwise gradient of NH_4Cl solution (0.1–0.6 M). As a result, copper-chelated Boningmycin was purified. The solution was combined, desalted, concentrated and lyophilized to yield a blue powder (1155 mg). Then, the obtained blue powder was treated with dithizone(165 mg) in MeOH (37.5 ml). The solution was filtered, and the filtrate was precipitated with four times the volume of Me_2CO. After washed with acetone for three times and filtered, the precipitate was dissolved in distilled water and applied on the column of CM-Sephadex C-25 (NH_4^+ form, 160 ml), subsequently eluted with a stepwise gradient of NH_4Cl solution from 0.1 to 0.4 M. As a result, two compounds Boningmycin (787.5 mg) and NC1404 (15 mg) were obtained.

Compound NC1404 was a white powder with a molecular weight of 1578.44 based on ESI-MS spectrum (Supplementary Figure S1), which gave three quasi-molecular ions at m/z 1579.44 $[M+H]^+$, 790.54 $[M+2H]^{2+}$ and 528.01 $[M+3H]^{3+}$. The molecular weight of NC1404 was 40 units larger than that of Boningmycin. The fragment ions at m/z 688.07 $[M\text{-}Carbamylmannose+H]^{2+}$ indicated that NC1404 has the same carbarmylannose part as Boningmycin. But the fragment ions at m/z 587.00 $[M\text{-}Carbamylmannose\text{-}gulosyl+H]^{2+}$ showed that the gulosyl part of the NC1404 was 40 units larger than that of Boningmycin, which implied that NC1404 is structurally the same as boningmycin except the gulosyl part.

Despite the spectral complexities, the ^1H NMR spectrum of NC1404 showed characteristic four aromatic proton signals at δ_H 7–9 p.p.m. which belonged to the thiazole ring and imidazole ring of the Bleomycin, indicative of the same kernel structure of NC1404 and Bleomycin as well as Boningmycin.[1,12] The ^{13}C NMR data of NC1404 were tabulated in Table 1 in comparison with those of

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Table 1 ^{13}C NMR data of NC1404 and boningmycin (600 MHz, D_2O)

Assignment		^{13}C shift (p.p.m.)		Assignment		^{13}C shift (p.p.m.)	
		NC1404	Boningmycin			NC1404	Boningmycin
I	CO	174.822	174.785	VI	5	127.932	127.965
	β-CH	69.881	69.868		5′	121.905	121.928
	α-CH	61.965	61.556		β-CH_2	41.905	41.906
	CH_3	21.726	21.718		α-CH_2	34.822	34.829
II	S-CO	178.959	179.042	G	1	101.384	100.325
	R-CO	170.417	170.536		2	73.631	73.034
	2	168.200	168.126		3	69.539	70.625
	4	167.263	167.469		4	72.590	71.939
	6	155.566	155.034		5	71.206	70.012
	5	114.614	115.113		6	64.866	63.155
	α-CH	62.456	62.466		C	102.500	—
	β-CH_2	43.036	42.985		CH_3(e)	20.759	—
	CH_3	13.557	13.679		CH_3(a)	31.042	—
III	CO	180.388	180.33	M	CO	160.879	160.776
	β-CH	77.128	77.178		1	101.727	100.953
	γ-CH	50.476	50.41		3	77.128	77.178
	α-CH	45.417	45.521		5	76.325	76.391
	γ-CH_3	17.723	17.457		2	71.206	71.168
	α-CH_3	14.821	14.872		4	67.738	67.556
					6	63.864	63.758
IV	CO	171.950	171.739				
	2	139.495	139.613	R	(CO)	177.486	177.541
	4	137.694	137.223		a	39.152	39.148
	5	120.685	120.6		b	28.655	28.644
	β-CH	76.712	75.753		c	48.036	48.036
	α-CH	59.643	59.685		d	49.747	49.762
					e	25.655	25.655
V	CO	173.870	173.861		f	25.655	25.655
	α-CH	55.209	55.263		g	49.747	49.731
	β-CH_2	49.747	49.762		h	47.887	47.894
					i	28.438	28.459
VI	2′	173.468	173.568		j	38.869	38.862
	CO	166.310	166.416		CH_3	24.673	24.657
	2	165.566	165.66				
	4	151.608	151.624				
	4′	149.748	149.782				

Boningmycin and analysis of ^1H-^1H COSY, HSQC, DEPT and HMBC spectra.[13] The numbering of the parts in the NC1404 molecule follows the convention used in the previous papers as shown in Figure 1. The signals of the carbons constituting the kernel structure and terminal amine of NC1404 are consistent with those of Boningmycin and the only difference is that NC1404 had three extra carbon signals δ_C 102.500 (G–C), δ_C 20.759 (G–CH_3(e)) and δ_C 31.042 (G–CH_3(a)), which implied that there was an isopropylidene acetal group in the structure of NC1404. In addition, the HMBC correlations from δ_H 1.482 (G–CH_3(e)) and δ_H 1.377 (G–CH_3(a)) to δ_C 102.500 (G–C), δ_H 1.377 (G–CH_3(a)) to δ_c 20.759 confirmed the above hypothesis. Furthermore, the signal at δ_H 1.377 (G–CH_3(a)) showed weak coupling to δ_C 64.866 (G-6) and δ_C 72.590 (G-4), δ_H 3.364 (G-6) to δ_C 102.510 (G–C), which suggested that the isopropylidene acetal group linked to hydroxyl groups of gulosyl moiety (G) at the site of G-6 and G-4 (Figure 2). Comprehensive interpretation of ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, HSQC, DEPT and HMBC spectra of NC1404 further confirmed the expected structure (Figure 1a).

NC1404 may be an artifact deriving from Boningmycin with acetone during the process of decoppering. To prove this, Boningmycin was dissolved in methanol, four-fold volume of acetone was added into the solvent and a small amount of hydrochloric acid was added dropwise under stirring to form a large amount of precipitate. The precipitate was analyzed by HPLC (performed on an SHIMADZU 10A instrument using an ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 μm) on a binary LC system (solvent A: 80 mM acetic

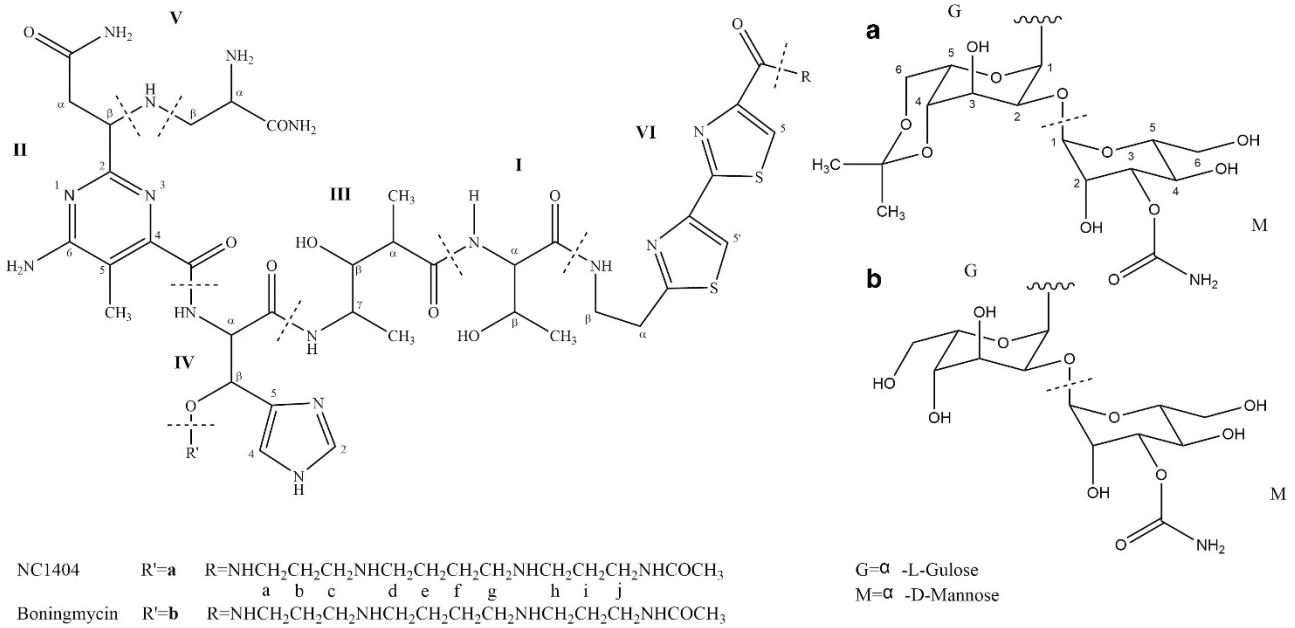

Figure 1 The Structures of NC1404 and Boningmycin.

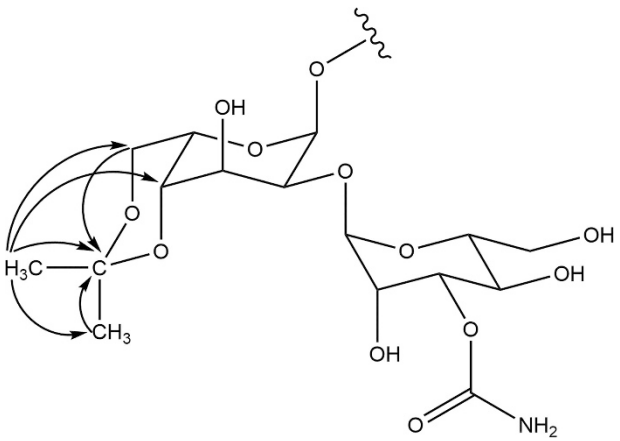

Figure 2 Selective HMBC correlations of the sugar moiety of NC1404.

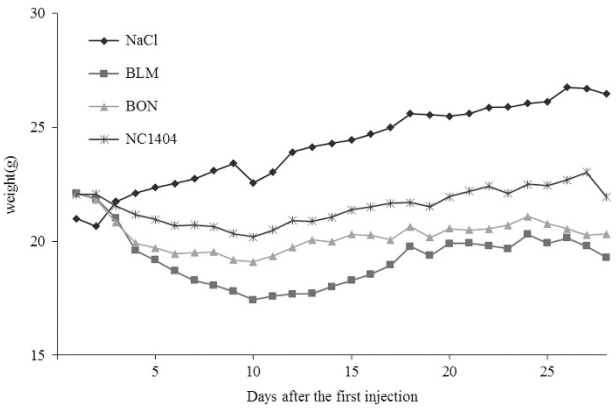

Figure 3 C57BL/6J mice's weight after injection of Bleomycin, Boningmycin, NC1404, and NaCl. The abbreviations are as follows: BLM = Bleomycin, and BON = Boningmycin A full color version of this figure is available at the *Journal of Antibiotics* online.

Table 2 Activities (IC_{50}, μM) of NC1404 in inhibiting the growth of cultured tumor cells

Cell line	*NC1404*	*Boningmycin*	*Bleomycin*
HepG2	1.34±0.10	1.41±0.06	2.96±0.34
HeLa	3.06±0.32	2.76±0.28	4.22±0.29
HaCaT	3.52±0.16	4.18±0.08	5.11±0.27
MCF7	1.70±0.47	1.64±0.18	2.25±0.45
HCT116($p53^{-/-}$)	0.97±0.09	1.03±0.13	1.93±0.30
HCT116	0.77±0.05	1.05±0.16	1.55±0.07

acid and 40 mM sodium 1-hexanesulfonate aqueous solution, adjusted with ammonia to a pH of 3.8, solvent B: methanol: acetonitrile (70:30 v/v); flow rate 1 ml min^{-1}; 60% A, 40% B; UV detection at 254 nm and oven temperature at 30 °C)). As a result, the formation of NC1404 was detected as shown in Supplementary Figure S7, which confirmed the speculation that NC1404 is an artifact during the process of decoppering.

To assess whether the modification in sugar moiety of NC1404 has an effect on the antitumor activity, an MTT assay was carried out using the reported method.[14] The IC_{50} values of NC1404, Boningmycin and Bleomycin against HepG2, HeLa, HaCaT, MCF-7, HCT116 (p53 − / −), HCT116 cells were listed in the Table 2, which showed that NC1404 had comparable antitumor activity with Boningmycin, but higher potency than Bleomycin.

Then, we evaluated the primary toxicity of NC1404 by comparing the effect of NC1404, Bleomycin and Boningmycin on the body weights of C57BL/6J mice. The experiment had carried out that 12 mg kg^{-1} per day of the test compounds were i.p. daily for seven days and then observed the loss of the body weights for 28 days. In contrast to the control group, all the compound-injected groups

showed the weight loss. However, the weights of NC1404 group mice reduced significantly less than that of the other groups (Figure 3).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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