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Cytotoxicity of Nocobactins NA-a, NA-b and their Ferric Complexes Assessed by Semiempirical Molecular Orbital Method

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Abstract. *Nocobactins NA-a (NBNAa) and NA-b (NBNAb) showed higher cytotoxic activity against human tumor cell lines (HSC-2, HSC-3, HL-60) than against normal human cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast), yielding tumor specificity indices (TS) of 80.0 and 43.9, respectively. We investigated the effect of $FeCl_3$ on these compounds, as judged by changes in their cytotoxicity and absorption spectra. Addition of an equimolar concentration of $FeCl_3$ almost completely abrogated the cytotoxicity and changed the pattern of absorption spectra of NBNAa and NBNAb. Mass spectrometry demonstrated that ferri-nocobactin NA-a (Fe-NBNAa) contains an iron atom, and this chelating complex had two orders lower cytotoxicity than intact NBNAa. A semi-empirical molecular orbital method (CAChe), based on these experimental data, proposed the estimated structure of Fe-NBNAa. The present study suggests that NBNAa and NBNAb are promising compounds for further study of antitumor potential in vivo, although their biological activity is significantly affected by the Fe^{3+} concentration in both intracellular and extracellular milieus.*

Metal chelators such as α,β-unsaturated ketones (1), hydroxyketones (2), β-diketones (3-5), cisplatin-platinum complex (6) and bleomycin-metal complex (7) have been reported to induce apoptosis and antitumor actions against cultured tumor cell lines. We have recently reported that, among three β-diketones, the cytotoxic activity of curcumin, but not that of (-)-3-(trifluoroacetyl)camphor and 3-formylchromone, was inhibited by $FeCl_3$ (8), and that the structure of the Fe(III)-curcumin complex was proposed by a semiempirical molecular orbital method.

Nocobactins NA-a (NBNAa) and NA-b (NBNAb) (Figure 1) are produced by a pathogen *Nocardia farcinica* ATCC 3318, which has been originally identified as *N. asteroids* and then reclassified as the type strain of *N. farcinica* (9-11). NBNAs are mycobactin-like siderophores, which may play a role in the uptake of iron from the proteins of the host by chelation of ferric ion (Fe^{3+}). The genomic sequence of the strain *N. farcinica* IFM 10152, which was isolated from a male Japanese patient, has recently been determined and cell extracts of the strain have shown an iron-dependent cytotoxic activity, suggesting the production of NBNAs (12). In the present study, we first investigated whether these NBNAs show tumor-specific cytotoxicity, using three normal oral human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) and three human tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, promyelocytic leukemia HL-60) as target cells. We next investigated whether the cytotoxic activity of these two compounds against HSC-2 cells is related to their chelating activity, using the following two parameters: change in the cytotoxicity by addition of $FeCl_3$ and that of absorption spectra. Based upon these data, we calculated a molecular model of ferric iron binding NBNAa.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem. Co., St. Louis, MO, USA); $FeCl_3$, dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan).

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Key Words: Nocobactin NAs, chelation, iron, cytotoxicity, tumor-specificity.

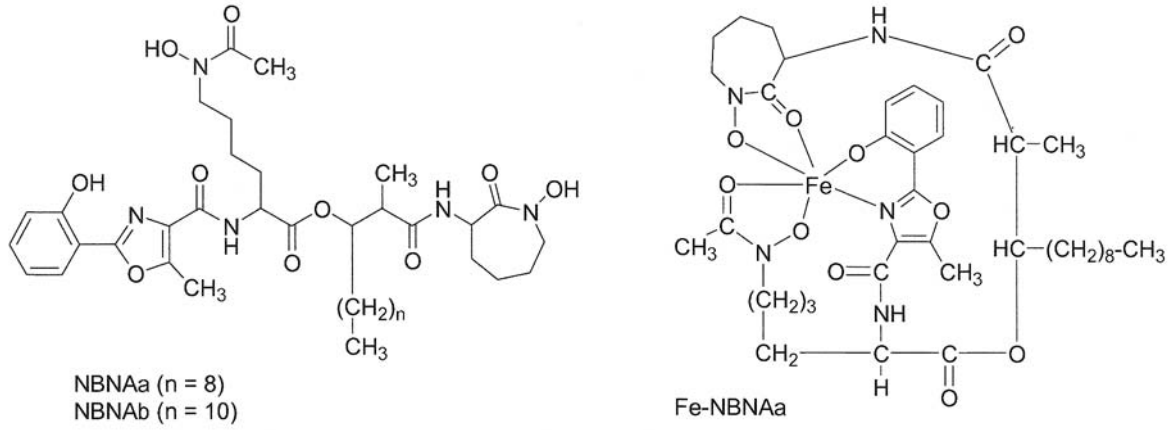

Figure 1. *Structure of nocobactins NA-a (NBNAa), NA-b (NBNAb) and ferri-nocobactin NA-a (Fe-NBNAa).*

Table I. *Tumor-specific cytotoxic activity of nocobactins NA-a (NBNAa) and NA-b (NBNAb).*

	CC_{50} (μM)								
	Normal cells				Tumor cells				
Sample	HGF	HPC	HPLF	(Total)	HSC-2	HSC-3	HL-60	(Total)	TS
NBNAa	34	60	41	(135)	0.37	0.12	1.2	(1.69)	80.0
NBNAb	36	96	38	(170)	0.46	0.31	3.1	(3.87)	43.9
A_{540}	0.449	0.460	0.445		0.673	0.957 (1x10^6/mL)			

$TS = [CC_{50}(HGF) + CC_{50}(HPC) + CC_{50}(HPLF)] / [CC_{50}(HSC\text{-}2) + CC_{50}(HSC\text{-}3) + CC_{50}(HL\text{-}60)]$

Preparation of nocobactin. Nocobactins NA-a and NA-b (9,10) were isolated from clinically isolated *Nocardia farcinica* IFM 10152 (12) with the guide of cytotoxicity against HL-60. The details of production, isolation and characterization of their structures will be reported elsewhere. The complex of NBNAa and Fe (III) was prepared as follows. NBNAa (8.8 mg, 12 μmol) in methanol (5 mL) and 4.9 mg of $FeCl_3 \bullet 6H_2O$ (18 μmol) suspended in a small amount of methanol were mixed (the solution changed immediately to a purple color with the addition of $FeCl_3$), stood overnight at room temperature and evaporated. After partition with $CHCl_3$ and H_2O, the $CHCl_3$ layer was washed three times with H_2O, and then the chloroform was evaporated *in vacuo* to yield 7.9 mg of red residue (Fe-NBNAa).

Ferri-nocobactin NA-a: MALDI-TOF MS: *m/z* 797 $[M + H]^+$, 819 $[M + Na]^+$. UV (MeOH) λ_{max} nm (log ε): 209 (4.79), 260 (sh 4.44), 266 (4.47), 270 (sh 4.44), 276 (sh 4.41), 309 (4.15), 320 (sh 4.13), 340 (inflation 3.87), 467 (3.59). Broad signals were observed on its ^1H NMR spectrum due to a rapid relaxation time accelerated by the iron atom. The ^{13}C NMR spectrum only showed the signals of fatty acid side chain and aromatic carbons due to the iron atom.

Cell culture. Human oral tumor cell lines (HSC-2, HSC-3) and normal human oral cells (HGF, HPC, HPLF) were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere. Normal cells were prepared from the periodontal tissues, according to the guideline of Meikai University Ethics Committee (No.206), after obtaining informed consent from the patients. Since normal human cells have a limited *in vitro* life-span (13), these cells were used at 6-7 population doubling level (PDL). HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

Assay for cytotoxic activity. The cells (other than HL-60 cells) were incubated at 5-6 x 10^3 cells/well in 96-microwell (Becton Dickison Labware, NJ, USA), unless otherwise stated. After 48 hours, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing various concentrations of the test compounds. Near confluent cells were incubated for another 24 hours, and the relative viable cell number was then determined by the MTT method. In brief, the cells were washed once with PBS(-), replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another 4 hours. The cells were lysed with 0.1 mL of DMSO and the absorbance at 540 nm of the cell lysate was determined, using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The absorbance at 540 nm of control cells was usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

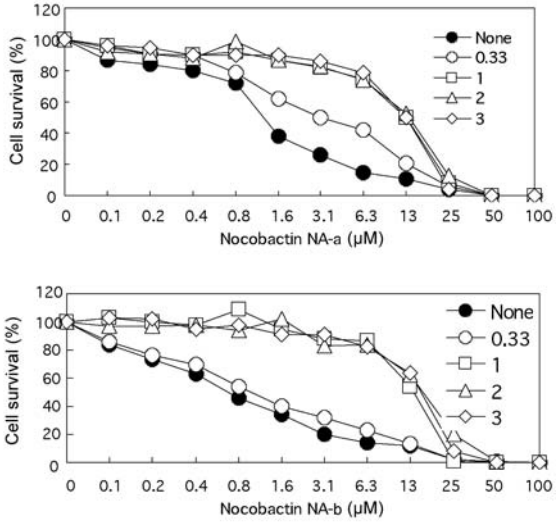

Figure 2. *Effect of $FeCl_3$ on the cytotoxicity of nocobactins NA-a and NA-b against HSC-2 cells. Near confluent HSC-2 cells were incubated for 24 hours with various concentrations of nocobactin NA-a (A) or NA-b (B) mixed with $FeCl_3$ at the following ratios (sample : FeCl3 = 1 : 0 (●), 1 : 0.33 (○), 1 : 1 (□). 1 : 2 (△) or 1 : 3 (◇), and viable cell number (% of control) was determined by the MTT method. Each value represents the mean from 4 determinations. S.D. < 5%.*

Table II. *Effect of $FeCl_3$ on the cytotoxicity of two nocobactins.*

Molar ratio of sample to $FeCl_3$	CC_{50} (μM)
NBNAa: $FeCl_3$	
1 : 0	1.3
1 : 0.33	3.1
1 : 1	12.5
1 : 2	13.4
1 : 3	12.5
NBNAb : $FeCl_3$	
1 : 0	0.7
1 : 0.33	1.0
1 : 1	13.4
1 : 2	16.1
1 : 3	15.6

Near confluent HSC-2 cells were incubated for 24 hours with various concentrations of samples mixed with $FeCl_3$ at the indicated molar ratios, and viable cell number was determined by the MTT method, and the CC_{50} value was determined from the dose-response curve in Figure 2.

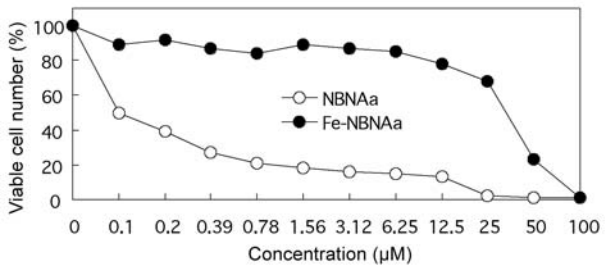

Figure 3. *Comparison of the cytotoxic activity of authentic NBNAa and chelation complex Fe-NBNAa. Near confluent HSC-2 cells were incubated for 24 hours with various concentrations of authentic NBNAa (○) or chelation complex Fe-NBNAa (●), and viable cell number (% of control) was determined by the MTT method. Each value represents the mean from 4 determinations. S.D. < 5%.*

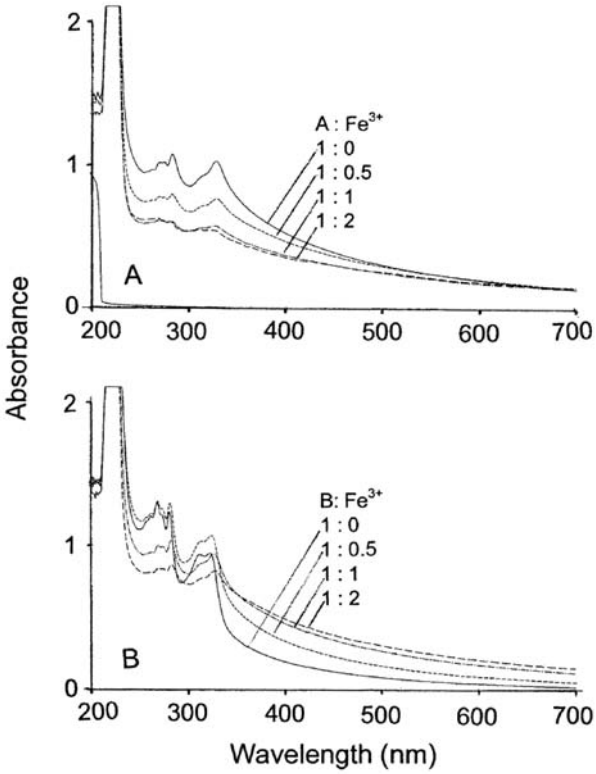

Figure 4. *Absorption spectra of nocobactins NA-a and NA-b in the presence of $FeCl_3$. 10 mM NBNAa (A) or NBNAb (B) (15 μL) and 10 mM $FeCl_3$ (0, 7.5, 15, 30 μL) were mixed in 3 mL of 100 mM Tris-HCl, pH 7.6.*

The viability of HL-60 cells was determined by trypan blue exclusion. HL-60 cells were incubated at 5 x 10^4/0.1 mL in 96-microwell, and added with various concentrations of the test compounds. After incubation for 24 hours, the viable cell number was determined by counting the cells with a hemocytometer. The density of control cells at harvest was in the range of 8-9 x 10^5/mL. Tumor specificity (TS) was determined by the following equation:

$$TS = [CC_{50}(HGF) + CC_{50}(HPC) + CC_{50}(HPLF)] / [CC_{50}(HSC\text{-}2) + CC_{50}(HSC\text{-}3) + CC_{50}(HL\text{-}60)]$$

Calculation methods. Molecular models of nocobactin NA-a (NBNAa) and its ferric complex (Fe-NBNAa) were minimized with CONFLEX, and then the molecular geometries of these models were calculated by CAChe 6.0. The log P of these models was calculated on these final models, but the Fe atom was un-parameterized.

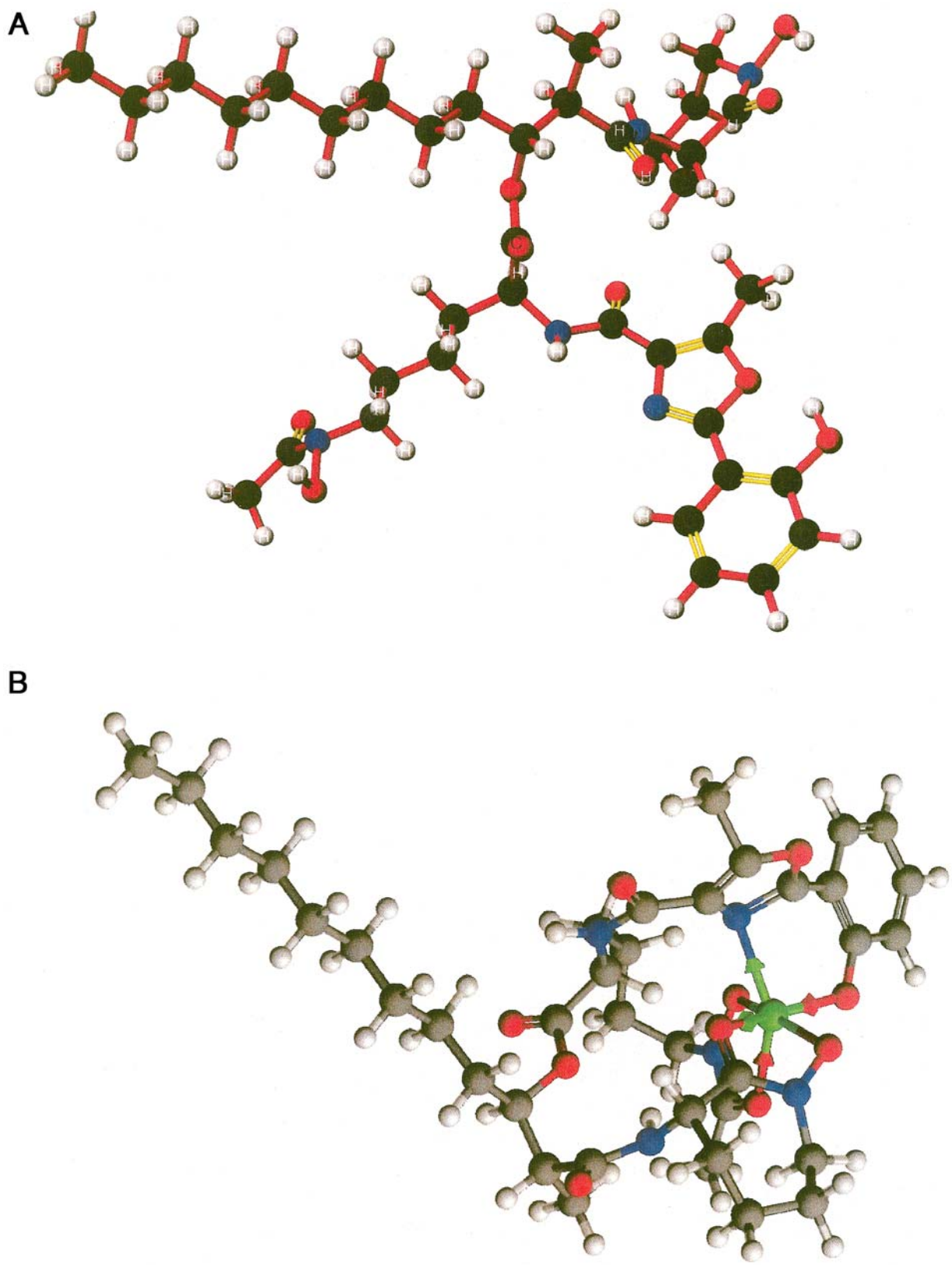

Figure 5. *Predicted conformation of nocobactin NA-a (A) and nocobactin NA-a and Fe^{3+} complex (1 : 1 molar ratio) (B). (A): Gray color, H; black color, C; red color, O; blue color, N. (B): White color, H; gray color, C; red color, O; blue color, N; green color (center), Fe.*

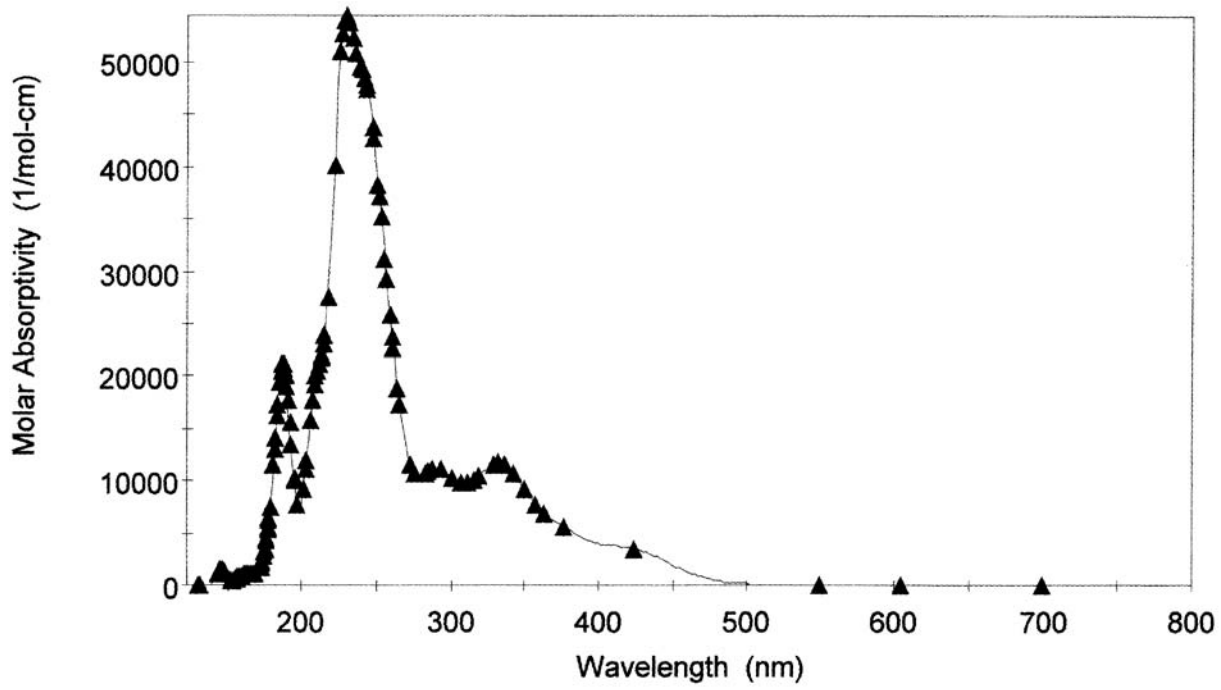

Figure 6. *Predicted absorption spectra of Fe-nocobactin NA-a.*

Results and Discussion

Tumor-specific cytotoxicity. Nocobactin NA-a showed higher cytotoxic activity against human tumor cell lines (CC_{50} of NBNAa against HSC-2, HSC-3 and HL-60 cells were 0.37, 0.12 and 1.2 μM, respectively) than against human normal cells (CC_{50} against HGF, HPC and HPLF cells were 34, 60 and 41 μM, respectively), yielding the tumor specificity index (TS) of 80.0.

Similarly, nocobactin NA-b also showed higher cytotoxicity against tumor cell lines (CC_{50}=0.46, 0.31 and 3.1 μM, respectively) than normal cells (CC_{50}=36, 96 and 38 μM, respectively), yielding the TS value of 43.9 (Table I).

It may be noteworthy that the TS value of NBNAa (TS=235) on squamous cell carcinoma (HSC-2 and HSC-3) was comparable to that of doxorubicin (TS=255) (14).

Chelating activity. The cytotoxic activity of NBNAa against HSC-2 cells gradually declined on the addition of increasing concentrations of $FeCl_3$ (Figure 2A). Addition of an equimolar concentration of $FeCl_3$ (NBNAa: Fe^{3+}=1:1) reduced the cytotoxicity of NBNAa about 10-fold (CC_{50} was increased from 1.3 to 12.5 μM) (Table II). The addition of a higher concentration of $FeCl_3$ did not further reduce the cytotoxicity of NBNAa (only up to 12.5-13.4 μM) (Table II).

Similarly, the addition of an equimolar concentration of $FeCl_3$ (NBNAb : Fe^{3+} = 1:1) reduced the cytotoxic activity of NBNAb about 20-fold (CC_{50} was increased from 0.7 to 13.4 μM) (Figure 2B). Further addition of $FeCl_3$ only slightly reduced the cytotoxic activity of NBNAb (up to 15.6-16.1 μM) (Figure 2B, Table II).

These data suggest that NBNAa and NBNAb form a chelating complex with Fe^{3+} at a 1:1 molar ratio as well as Fe-mycobactins (15-17). To confirm this, we made the complex of NBNAa and $FeCl_3$, and then applied it to mass spectrometry. The protonated molecular ion of Fe-NBNAa was observed at *m/z* 797, which corresponded well with the total of the molecular weight of NBNAa (Mw 743) and Fe^{3+} (Aw 56) minus 3 H^+ (3 binding sites) plus 1 H^+ (protonation from the matrix; α-cyano-4-hydroxycinnamic acid), thus confirming that nocobactin NA-a formed a 1:1 complex with Fe^{3+}. The complex thus prepared showed about two orders lower cytotoxic activity as compared with authentic NBNAa (CC_{50}= 35 μM (complex) *vs.* 0.1 μM (authentic), respectively) (Figure 3).

Absorption spectra. NBNAa showed several peaks at 270-276, 282, 314 (shoulder) and 328 nm (Figure 4A). Addition of $FeCl_3$ at the molar ratio of 0.5 to NBNAa slightly reduced the absorption intensity (maybe due to low solubility of Fe-NBNAa), without changing the relative intensity of these peaks. The addition of a higher molar ratio of $FeCl_3$ (NBNAa : $FeCl_3$ = 1 : 1 or 1 : 2) further reduced the absorption intensity, almost reaching the baseline (Figure 4A).

NBNAb showed several peaks at 268, 281, 312 and 323 nm (Figure 4B). Addition of $FeCl_3$ at the molar ratio of 0.5 to NBNAb did not apparently change the relative intensity of these peaks. The addition of a higher molar ratio of $FeCl_3$ ([NBNAb] : $FeCl_3$ = 1 : 1 or 1 : 2) considerably reduced the absorption intensity of these peaks and caused a shift of wavelength from 323 to 327 or 328 nm, but an increase near 460 nm which indicated the absorption spectrum of Fe-nocobactin (9) (Figure 4B). The absorption spectral data further supported that both NBNAa and NBNAb interact with iron, as reported by Ratledge and Snow (9). Assuming that these compounds and Fe^{3+} make a chelation complex at a 1 : 1 molar ratio, the structure of this complex was predicted with CAChe 6.0 (Figure 5). The structure for the complex can be obtained when Fe^{3+} is bound to five oxygen atoms (red color) and one nitrogen atom (blue color) (Figure 5B). The predicted absorption spectrum of the chelating complex shows peaks at 280, 325 and 425 nm (Figure 6), corresponding with the experimental data (Materials and Methods). On the calculated molecular model, three hydroxyl groups are blocked with a ferric iron, but the water solubility of Fe-NBNAa estimated by 1-octanol/water partition coefficient (log p=5.302), which was calculated on the basis of the molecular model, is similar to that of NBNAa (log p=5.503); the ratio of partition coefficients of these compounds is 1: 1.59 (Fe-NBNAa : NBNAa).

The present study suggests that the biological activity of NBNAa and NBNAb may be significantly affected by the Fe^{3+} concentration in both intracellular and extracellular milieus. We have recently found that apoptosis-inducing agents do not always show tumor-specific cytotoxicity, and that tumor-specific compounds do not always induce apoptosis (1-3). This suggests that tumor specificity is a more important factor than the apoptosis-inducing activity for evaluation of antitumor potential. In this sense, nocobactins NA-a and NA-b are promising antitumor compounds.

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