Structural Considerations of NK109, an Antitumor Benzo[*c*]phenanthridine Alkaloid

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Received January 6, 1999

The antitumor activities of a synthetic benzo[*c*]phenanthridine, NK109 (7-hydroxy-2,3-methylenedioxy-5-methyl-8-methoxybenzo[*c*]phenanthridinium hydrogensulfate dihydrate), and of natural benzo[*c*]-phenanthridines were tested in vitro and in vivo. NK109 (**3**) had the highest activity among them. NK109 is similar in structure to fagaronine and fagaridine; however, it has a phenolic-OH at C-7. NK109 exists as a resonance hybrid, the keto-amine and zwitterionic forms in neutral media. The resonance hybrid is cationic and has molecular planarity; these have been considered to be essential for the antitumor activity of the benzo[*c*]phenanthridinium salts. On the other hand, the structurally similar benzo[*c*]phenthridine alkaloids, chelerythrine and sanguinarine, exist as pseudobases under the same conditions. The latter do not exhibit antitumor activity in vivo, probably because they lose both the immonium region and molecular planarity. Thus, **3** may be considered to be novel category of benzo[*c*]phenanthridinium salt from the viewpoint of its structure under biological conditions.

Quaternary benzo[*c*]phenanthridines are commonly isolated from Papaveraceae and Rutaceae plants.[1] These compounds have attracted attention since nitidine (**1**) and fagaronine (**2**) were reported to possess antileukemic activity.[2,3] These two compounds were of interest as anticancer drugs, and preclinical studies at the National Cancer Institute were conducted. The studies indicated that they had low potency and were incompatible with biological fluids, resulting in precipitation in the peritoneal cavity.[4−6] These compounds were not considered to be practical anticancer drugs. Many derivatives of benzo[*c*]-phenanthridine alkaloids have been synthesized and their antitumor activities evaluated,[7−13] but no compound tested had greater activity than **1** and **2**.

In 1989, it was reported that NK109 (**3**, 7-hydroxy-2,3-methylenedioxy-5-methyl-8-methoxybenzo[*c*]phenanthridinium hydrogensulfate dihydrate) displayed a wide antitumor spectrum in vitro.[14] Compound **3** also inhibited DNA topoisomerase II,[15,16] showed antitumor activity against several drug-resistant human tumor cell lines,[17] and had favorable properties for use as an anticancer drug. A clinical study of **3** is now in progress in Japan.

In the present study, we report in vivo antitumor activity of **3** compared with those of other natural benzo[*c*]phenanthridine alkaloids. To explain the greater activity of **3**, we also examined the structure of **3** in a neutral medium as a model for biological conditions.

Results and Discussion

Synthetic alkaloid **3** (X = HSO_4^-·$2H_2O$), naturally occurring benzo[*c*]phenanthridines **1**, **2**, and **4**−**6** (X = Cl^-), and reduced derivative **7** were tested on cultured human cervical tumor cells (HeLa S3) in vitro. The growth-inhibitory curves are shown in Figure 1, and the IC_{50} values are listed in Table 1. The activity of **3** was nearly equal to that of **1** and was greater than that of the other alkaloids (**2**, **4**−**6**). The activities of chelerythrine (**5**), fagaridine (**4**), and 5,6-dihydro-NK109 (**7**) were weaker, suggesting that both the immonium region and the 7-hydroxy group are important for antitumor activity. The activity of these compounds against transplantable murine tumor P-388 in vivo were examined and T/C (%) values are summarized in Table 2.

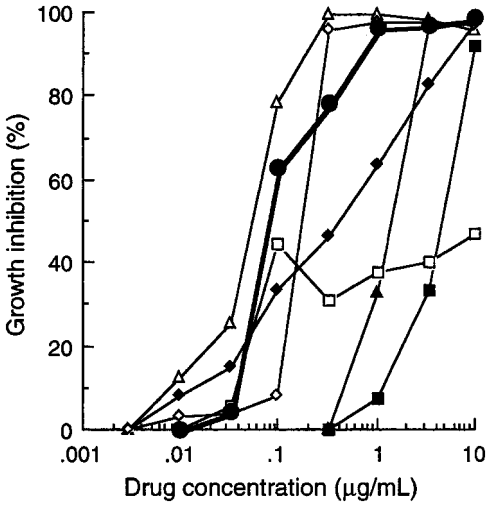

Figure 1. Growth inhibitory effect of benzo[*c*]phenanthridine alkaloids against HeLa S3 cells: NK109 (**3** as HSO_4^-; ●), nitidine (**1** as Cl^-; △), fagaronine (**2** as Cl^-; ◆), chelerythrine (**5** as Cl^-; ▲), sanguinarine (**6** as Cl^-; ◊), fagaridine (**4** as Cl^-; ■), and dihydro-NK109 (**7**; □).

Table 1. Growth Inhibitory Effects of NK109 (**3**) and Naturally Occurring Benzo[*c*]phenanthridine Alkaloids (**1**, **2**, **4**−**7**) Against HeLa S3 Cells

compound	IC_{50} (μg/mL)
NK109 (**3**)	0.08
chelerythrine (**5**)	1.24
sanguinarine (**6**)	0.16
nitidine (**1**)	0.05
fagaronine (**2**)	0.39
fagaridine (**4**)	3.99
5,6-dihydro-NK109 (**7**)	16.2

Compounds **5** and **6** were highly toxic. They both induced toxic death at a dosage of 25 mg/kg. Compounds **1** and **2** showed toxicities at doses of 50 and 75 mg/kg, respectively. The T/C values of all the natural benzo[*c*]phenanthridines tested were near 100% at the optimal dose (just below the toxic dose). None showed any life-prolonging properties. On

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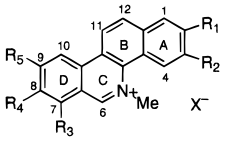
10.1021/np990005d CCC: $18.00
Published on Web 05/06/1999

Table 2. Antitumor Activities of NK109 (3) and Naturally Occurring Benzo[*c*]phenanthridiniums (**1**, **2**, **5**, **6**) Against P-388 Leukemia[a]

compound	dose (mg/kg)	T/C (%)	median survival time (days)
control		100	9.3
NK109 (**3**)	150.0	261[b]	23.5
	75.0	159[b]	14.3
	50.0	147[b]	13.2
chelerythrine (**5**)	25.0	12	1.1
	12.5	111	10.0
	6.25	111	10.0
sanguinarine (**6**)	50.0	12	1.1
	25.0	25	2.3
	12.5	102	9.5
fagaronine (**2**)	75.0	14	1.3
	50.0	102	9.5
	25.0	102	9.5
nitidine (**1**)	75.0	46	4.3
	50.0	102	9.5
	25.0	108	10.0

[a] Evaluated for 30 days. [b] Statistically significant at $p < 0.01$ compared with control based on Log−Rand and Wilcoxon tests.

the other hand, **3** exhibited an increase in the T/C value according to the dose escalation and showed statistically significant activity even at a dosage of 50 mg/kg. At the maximum dosage (150 mg/kg), the T/C value reached 261%, and two mice among five survived during the observational period (30 days). A diminution of body weight was observed at this dosage, caused by an adverse effect on the digestive system; however, it was recoverable (Figure 2). Although all of the natural benzo[*c*]phenanthridines possessed cytotoxicities in vitro, they also showed toxicity in vivo.



Nitidine (**1**) $R^1 + R^2 = OCH_2O$; $R^3 = H$; $R^4 = R^5 = OCH_3$; $X^- = Cl^-$

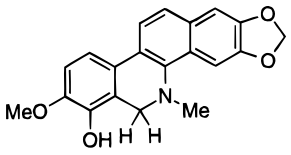
Fagaronine (**2**) $R^1 = OH$; $R^2 = OCH_3$; $R^3 = H$; $R^4 = R^5 = OCH_3$; $X^- = Cl^-$

NK109 (**3**) $R^1 + R^2 = OCH_2O$; $R^3 = OH$; $R^4 = OCH_3$; $R^5 = H$; $X^- = HSO_4^-{\bullet}2H_2O$

Fagaridine (**4**) $R^1 + R^2 = OCH_2O$; $R^3 = OCH_3$; $R^4 = OH$; $R^5 = H$; $X^- = Cl^-$

Chelerythrine (**5**) $R^1 + R^2 = OCH_2O$; $R^3 = R^4 = OCH_3$; $R^5 = H$; $X^- = Cl^-$

Sanguinarine (**6**) $R^1 + R^2 = R^3 + R^4 = OCH_2O$; $R^5 = H$; $X^- = Cl^-$



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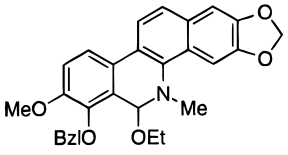

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Structurally, **3** is a phenolic benzo[*c*]phenanthridine alkaloid similar to **2** and **4**. Nevertheless, the phenolic hydroxide at C-7 in **3** is unique and allows for an acid−base equilibrium. The p*Ka* value of **3** calculated by the absorbance method[18] was found to be 5.3. Subsequently, we successfully isolated both forms in equilibrium and determined their structures (**3** and **9**) by ^1H and ^{13}C NMR analyses. During determination of the keto-amine structure (**9**), we noticed that the color of **9** differed in some solvents. For example, in a basic aqueous medium, it was purple, and in CH_2Cl_2, it was violet. The shift in the absorption maxima observed according to the polarity of the solvents suggested a fluctuation in the structure of **9**.[18] Thus, the base form of NK109 is not a fixed structure (**9**) in a strict

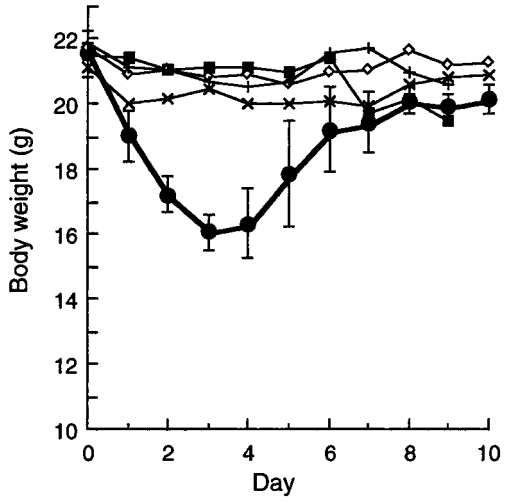


Figure 2. Change in body weight of NK109-treated mice and control mice: NK109 (**3**): 150 mg/kg (●), 75 mg/kg (×), 50 mg/kg (◊), 25 mg/kg (+), and control (■). Values are expressed as mean; vertical lines of data of 150 mg/kg indicate s.e.mean.

sense, but is a resonance hybrid. Its limiting structures are **9** and the zwitterion form **10** (Scheme 1).

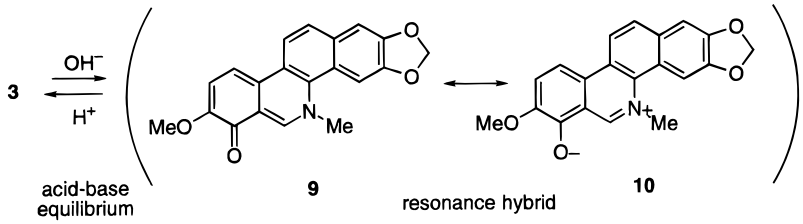
NMR experiments supported these assignments. NMR spectra of **3**, **7**−**9** were measured in DMSO-d_6. The *N*-methyl protons of the quaternary form **3** are deshielded by the nitrogen cation; therefore, its peak shifted downfield to δ 4.92. The normal tertiary amines, **7** and 7-benzyloxy-6-ethoxy-5,6-dihydro-NK109 (**8**) gave signals at δ 2.48 and 2.61, respectively. The *N*-methyl proton of the keto-amine form **9** exhibited a signal at δ 4.11. This value is shifted downfield compared with **7** and **8**, and resembled that of **3**, indicating that the lone pair of the nitrogen delocalizes to the adjacent unsaturated bond. Therefore, there is a strong contribution of the zwitterion form **10**. This effect and contribution increases in polar solvents. For example, the *N*-methyl protons of **9** gave a signal at δ 4.56 in CD_3-OD.

Quaternary benzo[*c*]phenanthridines possessing oxygenated subsitituents at the 7,8-position, such as **5** and **6**, are known to exist as pseudobases in alkaline environments (Scheme 2).[19] We also detected the pseudobase form of **3** under limited conditions. A dilute solution of **3** in aqueous NaOH (1 N) and EtOH (1:1) gradually lost its characteristic purple color. When **3** becomes colorless, it exists as the pseudobase **11** (Scheme 3).[20] However, **3** was precipitated as the keto-amine form (**9**) from alkaline aqueous solution; therefore, the pseudobase form of NK109 (**11**) could not be isolated. Consequently, its pseudobase was detected only in strong alkaline and diluted solution, for example, for UV measurement. Furthermore, in a pH 9 solution, **9** does not lose its purple color. We considered that the contribution of the pseudobase form of **3** could be normally neglected.

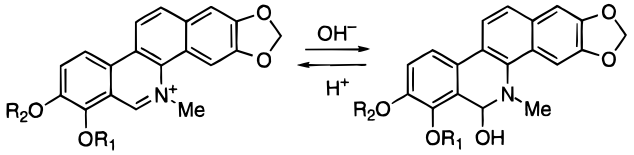
We investigated the structural properties of NK109 in detail and conclude that NK109 can exist as a quaternary cation (**3**) or as a resonance hybrid of the keto-amine (**9**) and zwitterion (**10**). Natural benzo[*c*]phenanthridines, such as **5** and **6**, exist as pseudobases in alkaline medium. The lone pair of the nitrogen atom in a resonance hybrid is delocalized to the conjugated double bond. This suggests that the immonium region and the D ring are on the same plane. The most characteristic property of NK109 is that it can exist as a planar molecule under both acid and base conditions.

Many researchers have studied the benzo[*c*]phenanthridinium alkaloids, and some have concluded that the

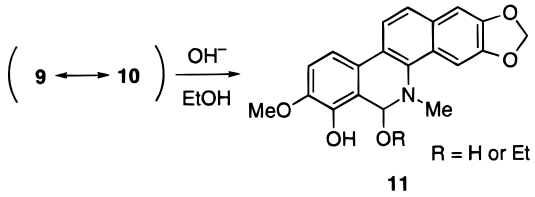
Scheme 1. NK109, a Resonance Hybrid, Limited by the Keto-Amine Structure (**9**) and the Zwitterion (**10**)



Scheme 2. Pseudobase Forms of 7,8-Oxygenated Derivatives



Scheme 3. Pseudobase Form of NK109



compounds possessing strong antitumor activity were 8,9-oxygenated; the most promising compounds among them are **1** and **2**.[21,22] They also concluded that the 7,8-oxygenated benzo[*c*]phenathridinium alkaloids had no antitumor activity in vivo.[21] In the present study, we found that **3** possessed significant antitumor activity in vivo.

Caolo and Stermitz explained the antitumor activities of benzo[*c*]phenanthridinium alkaloids by the concentration of the quaternary cation in 50% EtOH−buffer (pH 7).[22] Thus, the 50% EtOH−buffer reflected biological conditions in which 7,8-oxygenated derivatives preferred the pseudobase form and the 8,9-oxygenated ones preferred the quaternary form. They concluded that the cationic form is needed for the antitumor activity. Several 6-alkoxy derivatives of **5** and **6** were synthesized by Stermitz and co-workers.[21] All compounds were tested for their antileukemic activity, but none was active. Because **5** and **6** converted into a pseudobase, they presumably lost antitumor activity. Walterová and co-workers determined the pK_{R+} values in H_2O−EtOH (1:1).[23] Those of **6**, **5**, and **1** were 5.75, 6.67, and 9.76, respectively. Evidently **1** exists in the quaternary form in a neutral medium. Thus, the preferred structure of NK109 under biological conditions is the resonance hybrid. It sufficiently retains both the quaternary cationic property and molecular planarity. NK109 is the only known 7,8-oxygenated benzo[*c*]phenanthridine compound possessing significant antitumor activity in vivo.

In conclusion, the benzo[*c*]phenanthridine alkaloids require both the immonium region and molecular planarity in order to exhibit antitumor activity.[22,24] Compounds **1** and **2** exist as intact cationic forms under biological conditions and showed some antitumor activity. Compounds **5** and **6** have potential activity; however, they lose the cationic structure to form the pseudobase. Compound **3** is converted to a resonance hybrid that has sufficient properties required for antitumor activity. This is due to the hydroxyl group at the 7-position. Thus, NK109 (**3**) is a compound belonging to a novel category of benzo[*c*]phenanthridine alkaloids based on its structure under biological conditions.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi 535 melting point apparatus. NMR spectra were obtained using Varian Gemini-200 and JEOL JNM-GX400 spectrometers. All chemical shifts were reported in parts per million relative to the internal standard of tetramethylsilane (TMS, δ 0.00). UV spectra were recorded on a Shimadzu UV-2200 and a Hitachi 220A spectrometer. MS were recorded on a Micromass Limited Auto Spec-Q spectrometer. Elemental analyses were performed on a Yanako CHN Corder.

Tumors. The HeLa S3 tumor cell was purchased from Dainippon Pharmaceutical Co., Ltd., and cloned in our laboratory. The P-388 leukemia cell was obtained from the Cancer Chemotherapy Center, Tokyo, Japan, and was maintained by intrapertoneally transplantation (1×10^4 cells/body) to DBA/2 mouse.

Materials. Chelerythrine (**5**) and sanguinarine (**6**) were perchased from Sigma Chemical Company. The quaternary cation form of NK109 (**3**) and 7-benzyl-6-ethoxy-5,6-dihydro-NK109 (**8**) were synthesized in our laboratory.[25] Nitidine (**1**), fagaronine (**2**),[20] and fagaridine (**4**)[26] were also synthesized in our laboratory. Other chemicals were obtained from commercial suppliers and were used without further purification.

Animals. CDF_1 mice (female) were purchased from Charles River, Japan, and used in experiments at the age of six weeks. Each treatment group was composed of five animals.

Calculation of pK_a. Solutions of **3** (5.125 μg/mL) were prepared using the following media: phospholic acid−citric acid buffers (pH 4.5, 5.0, 5.5, 6.0), 0.1N HCl, and 0.1N NaOH. The UV−vis spectra of these solutions were obtained, and then the pK_a value was calculated by the absorbance at 270 nm for each solution using following equation: $pK_a = pH + \log [(\epsilon - \epsilon_{A^-})/(\epsilon_{HA} - \epsilon)]$.

Preparation of Keto-amine Form of NK109 (9). A solution of the quaternary form of NK109 (**3**)[25] (2.05 g, 5.35 mmol) in 500 mL of H_2O was neutralized with 6N NaOH. The solution's color changed from orange to purple, and then the purple solid precipitated. The reaction mixture was extracted with MeOH (75 mL) and CH_2Cl_2 (675 mL). The organic layer was washed with H_2O (500 mL), dried over Na_2SO_4, and evaporated in vacuo to give the keto-amine **9** (2.02 g) as a violet solid: mp 234 °C; ^{1}H NMR (DMSO-d_6) δ 3.76 (s, 3H), 4.41 (s, 3H), 6.24 (s, 2H), 7.03 (d, J = 8.0 Hz, 2H), 7.19, (d, J = 8.0 Hz, 1H), 7.55 (s, 1H), 7.89 (d, J = 9.0 Hz, 1H), 7.97 (s, 1H), 8.26 (d, J = 9.0 Hz, 1H), 9.02 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 48.0, 55.3, 98.5, 101.9, 102.7, 105.1, 118.0, 118.2, 118.8, 120.3, 125.0, 126.8, 127.4, 131.2, 131.4, 147.3, 147.5, 150.1, 151.0, 169.0; FABMS *m/z* 333 [$M^{\bullet+}$] and 334 [$M^+ + H$]; *anal.* C 67.67%, H 4.79%, N 3.60%, calcd for $C_{20}H_{15}NO_4 \cdot 0.25CH_2Cl_2 \cdot 0.1H_2O$, C 67.94%, H 4.87%, N 3.91%.

5,6-Dihydro-NK109 (7). To a solution of the quaternary form of **3**[25] (500 mg, 1.07 mmol) in H_2O (25 mL) and MeOH (25 mL) was added sodium cyanoborohydride (87 mg, 1.39

mmol) in 2 mL of MeOH at room temperature. After stirring for 2 h, the resulting precipitate was filtered and washed with H_2O. The residue was dried in vacuo to give **7** as a light purple solid: mp 252−253 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.48 (s, 3H), 3.86 (s, 3H), 4.16 (s, 2H), 6.14 (br s, 2H), 6.97, (d, J = 8.5 Hz, 1H), 7.30 (s, 1H), 7.32 (d, J = 8.5 Hz, 1H), 7.52 (s, 1H), 7.53 (d, J = 8.5 Hz, 1H), 7.76 (d, J = 8.5 Hz, 1H), 8.80 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 41.1, 48.0, 55.7, 99.6, 101.0, 104.1, 110.3, 113.6, 118.7, 120.1, 123.5, 124.2, 125.1, 125.6, 130.2, 141.9, 142.8, 147.0, 147.4, 147.7; FABMS m/z 335 [$M^{\bullet+}$] and 336 [$M^+ + H$]; *anal.* C 70.68%, H 5.11%, N 3.97%, calcd for $C_{20}H_{17}NO_4 \cdot 0.08H_2O$, C 71.05%, H 5.49%, N 4.14%.

Growth Inhibition Assay. HeLa S3 cells were cultured in Eagle's Minimum Essential Medium (Flow Labs., McLean, VA) supplemented with 10% calf serum and 60 μg/mL of kanamycin, and was incubated at 37 °C in 5% CO_2−95% air. The resulting cell suspension was seeded in a 96-well plate (0.2 mL to each well) and incubated for 24 h. After dissolution with 5% glucose solution or DMSO, the test compounds were diluted with 5% glucose solution at concentrations from 0.01 to 10 μg/mL. Each solution (10 μL) was added to each well individually and incubated for an another 72 h. After the nuclei were dyed by methylene blue, the number of cells in each well was determined by measurement of the absorbance at 660 nm. The growth-inhibitory effect was assessed by comparing the growth rate of the drug-treated cells and that of control, and then the IC_{50} values (%) were determined.

Antitumor Experiments in Vivo. Female CDF_1 mice (6 weeks old) were intravenously inoculated with P-388 leukemia cells (1×10^5 cells/body) on day 0. Each compound was dissolved with 5% glucose solution or EtOH solution containing Cremophore (1:1) and was diluted with 5% glucose solution to the appropriate concentration. The prepared drug solutions (0.1 mL per 10 g of mouse) were injected into the tail vein as a single injection on day 1. The antitumor activity was evaluated by the T/C (%) values and change in body weight. The T/C (%) values were obtained by comparing the median survival time of the animals in the drug-treated group (T) and that of the control (C).

Acknowledgment. We greatly appreciate Prof. Hanaoka, Kanazawa University, Japan, for providing some of the benzo[*c*]phenanthridine alkaloids.

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NP990005D