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Potent Anti-metastatic Activity of Dimeric Sesquiterpene Thioalkaloids from the Rhizome of *Nuphar pumilum*

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Abstract—The methanolic extract and its alkaloid fraction from the rhizomes of *Nuphar pumilum* inhibited invasion of B16 melanoma cells across collagen-coated filters in vitro. Dimeric sesquiterpene thioalkaloids with the 6-hydroxyl group, 6-hydroxythiobinupharidine, 6,6′-dihydroxythiobinupharidine, and 6-hydroxythionuphlutine B, showed potent activity with IC_{50} values of 0.029, 0.087, and 0.36 μM, respectively, but dimeric sesquiterpene thioalkaloids lacking the 6-hydroxyl group (thiobinupharidine, neothiobinupharidine, *syn*-thiobinupharidine sulfoxide, thionuphultine B β-sulfoxide, and neothiobinupharidine β-sulfoxide) and monomeric sesquiterpene alkaloids (nupharidine, deoxynupharidine, 7-epideoxynupharidine, and nupharolutine) showed weak activity. The alkaloid fraction (20 mg/kg/d, po) and the principal dimeric sesquiterpene thioalkaloid 6-hydroxythiobinupharidine (5 mg/kg/d, po) significantly inhibited lung tumor formation by more than 90% 10 days after injection of B16 melanoma cells in mice.

Introduction

Metastatis of cancer, which is the major cause of death in cancer patients, occurs through a complex multistep process consisting of invasion into the circulation from the primary tumor, immigration to distant organs, adhension to endothelial cells, and infiltration into the tissue. Therefore, its blockade has been considered to enhance survival of cancer patients.[1] However, few compounds specifically inhibit the metastatis of cancer such as the matrix metalloprotease (MMP) inhibitor marimastat.[2]

Nupharis Rhizoma, the dried rhizomes of *Nuphar japonicum* DC. and *Nuphar pumilum* (Timm.) DC., has been prescribed for tonic, hemostatic and diuretic purposes in Japanese and Chinese traditional medicines. Chemical studies of this natural medicine have been carried out and a number of sesquiterpene alkaloids such as nupharidine (**9**) and deoxynupharidine (**10**) have been identified from *N. japonicum*.[3] In the course of our studies of natural medicines originating from aquatic plants,[4] we isolated thiohemiaminal type dimeric sesquiterpene thioalkaloids such as 6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), 6-hydroxythionuphlutine B (**4**), and 6′-hydroxythionuphlutine B from the rhizomes of Russian *N. pumilum* and found a new rearrangement reaction of the thiaspiran ring in thiohemiaminal type alkaloids with the 6-hydroxyl group.[5] In addition, new thiaspiran sulfoxide type dimeric sesquiterpene alkaloids designated as nupharpumilamines A–D were characterized.[6] However, in pharmacological studies of nuphar alkaloids, only the central paralysis effect of **10** and immunosuppressive activities of **2**, **3** and **4** have been reported to date.[7]

In the continuing study, the methanolic (MeOH) extract and alkaloid fraction from the rhizomes of *N. pumilum* were found to inhibit invasion of B16 melanoma cells obtained by in vivo selection across collagen-coated filters in vitro. Thus, seven dimeric sesquiterpene thioalkaloids [6-hydroxythiobinupharidine (**2**),[5,8] 6,6′-dihydroxythiobinupharidine (**3**),[5,9] 6-hydroxythionuphlutine B (**4**),[5,9] neothiobinupharidine (**5**),[10] *syn*-thiobinupharidine sulfoxide (**6**),[11] thionuphlutine B β-sulfoxide (**7**),[12] and neothiobinupharidine β-sulfoxide (**8**)[13]] and four monomeric sesquiterpene alkaloids [nupharidine (**9**),[3] deoxynupharidine (**10**),[3] 7-epideoxynupharidine (**11**),[14] and nupharolutine (**12**)[15]] isolated

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from the alkaloid fraction and thiobinupharidine (**1**)[5,16] derived from **2** were examined to clarify the active constituents.

Here, we describe the effects of nuphar alkaloids on invasion of the melanoma cells and their structural requirements for this activity. In addition, the inhibitory effects of the alkaloid fraction and the principal dimeric sesquiterpene thioalkaloid **2** on lung metastasis of melanoma cells in mice are reported.

Results and Discussion

Effects of the MeOH extract, alkaloid fraction, and nuphar alkaloids from the rhizomes of *N. pumilum* on invasion of B16 melanoma cells in vitro

The number of B16 melanoma 4A5 cells crossing collagen-coated filters without test sample was 122.8 ± 3.9 cells/well (mean $\pm$ SD, $N = 12$) after incubation for 24 h. The MeOH extract and its alkaloid fraction from the rhizomes of *N. pumilum* inhibited invasion of the collagen matrix by the cells in a concentration-dependent manner with IC_{50} values of 0.05 and 0.023 μg/mL, respectively. Next, effects of the dimeric sesquiterpene thioalkaloids (**1**–**8**) and monomeric alkaloids (**9**–**12**) from the alkaloid fraction (Fig. 1) on the invasion by the cells were examined.

The effects of nuphar alkaloids (**1**–**12**) and reference compounds on the invasive activity of the cells are summarized in Table 1. Reference compounds, curcumin,[17] strongly inhibited invasion with IC_{50} value of 20 μM. The dimeric sesquiterpene thioalkaloids lacking the 6-hydroxyl group [neothiobinupharidine (**5**), *syn*-thiobinupharidine sulfoxide (**6**), thionuphultine B β-sulfoxide (**7**), and neothiobinupharidine β-sulfoxide (**8**)] and monomeric sesquiterpene alkaloids [nupharidine (**9**), deoxynupharidine (**10**), 7-epideoxynupharidine (**11**), and nupharolutine (**12**)] showed weak activity. On the other hand, dimeric sesquiterpene thioalkaloids with the 6-hydroxyl group in the quinolizidine ring [6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), and 6-hydroxythionuphlutine B (**4**)] were found to show strong activity with IC_{50} values of 0.029, 0.087, and 0.36 μM, respectively. However, thiobinupharidine (**1**) derived from **2** also lacked the activity. These findings suggested that the thiohemiaminal structure with the 6-hydroxyl group is essential for strong activity.

Effects of the alkaloid fraction and 6-hydroxythiobinupharidine (2) on lung metastasis of B16 melanoma cells in mice

The effects of the alkaloid fraction and the principal dimeric sesquiterpene thioalkaloid **2** on metastasis of B16 melanoma cells in mice were examined. Ten days after injection of the melanoma cells, ca. 20 colonies of the cells were observed on the surfaces of both lungs in mice. As shown in Table 2, the alkaloid fraction (20 mg/kg/d, po) and **2** (5 mg/kg/d, po) strongly suppressed the lung metastasis of the cells with inhibition of 93 and 94%, respectively.

In conclusion, the alkaloid fraction from the dried rhizomes of *N. pumilum* suppressed invasion of B16 melanoma cells across collagen-coated filters, and three dimeric sesquiterpene thioalkaloids [6 - hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), and 6-hydroxythionuphlutine B (**4**)] were isolated as active principles. The thiohemiaminal structure with the 6-hydroxyl group appeared to be essential for strong activity. In addition, the alkaloid fraction and compound **2** significantly inhibited lung metastasis of the cells in mice. This information may be useful in the design of new lead compounds for development of antimetastatic drugs.

Experimental

Animals

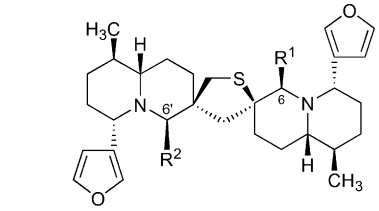
Female ddY mice aged 6 weeks were purchased from Kiwa Laboratory Animals Co., Ltd (Wakayama, Japan) and maintained in an air-conditioned room at 23 ± 2 °C. Standard laboratory chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water were given freely. Test samples were suspended in 5% acacia solution and given orally in a volume of 10 mL/kg.

Cells

Highly lung metastatic cells desired from B16 melanoma 4A5 (RCB0557, Riken Cell Bank, Tsukuba, Japan) were obtained by the in vivo selection method. Briefly, the melanoma cell suspension in phosphate-buffered saline (PBS) (5×10^5 cells/200 μL) was injected intraperitoneally into ddY mice. Two weeks later, metastatic colonies in the mice were isolated and dispersed with pepsin, and the melanoma cells were cultured in culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS [FCS (+)], 100 units/mL penicillin, 100 μg/mL streptomycin. This process was repeated five times, and the highly ddY mouse lung metastatic B16 melanoma cells were obtained and used for the experiments.

Materials

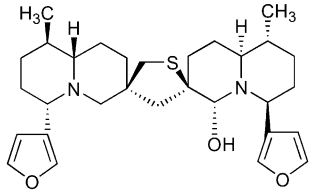
The extraction, fractionation, and isolation of the alkaloids (**2**–**12**) from Russian Nupharis Rhizoma were described previously.[5] Briefly, the MeOH extract from the dried rhizome of *N. pumilum* was partitioned into $CHCl_3$–1 M aqueous HCl (1:1). The 1 M aqueous HCl phase was made to a pH ca. 10 with concd NH_4OH and then extracted with EtOAc. The EtOAc-soluble fraction (the alkaloid fraction) was subjected to ordinary silica gel ($CHCl_3$–MeOH–Et_2NH) and NH–Chromatorex (*n*-hexane–CH_2Cl_2–EtOAc→$CHCl_3$–MeOH) and finally HPLC (Develosil ODS-HG-5, MeOH–H_2O–Et_2NH) to furnish **2** (0.019% from the natural medicine), **3** (0.020%), **4** (0.003%), **5** (0.001%), **6** (0.001%), **7** (0.0005%), **8** (0.0003%), **9** (0.0002%), **10** (0.002%), **11**



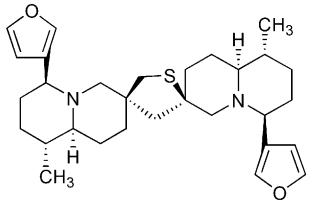
thiobinupharidine (**1**): $R^1 = R^2 = H$

6-hydroxythiobinupharidine (**2**): $R^1 = OH$, $R^2 = H$

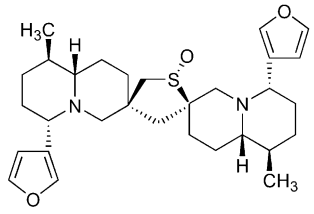
6,6'-dihydroxythiobinupharidine (**3**): $R^1 = R^2 = OH$



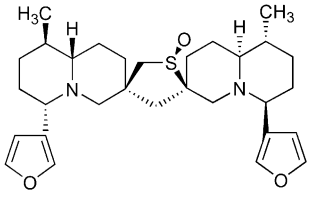
6-hydroxythionuphlutine B (**4**)



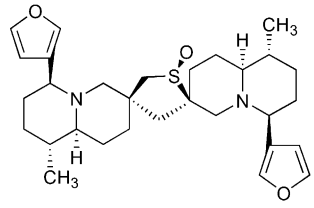
neothiobinupharidine (**5**)



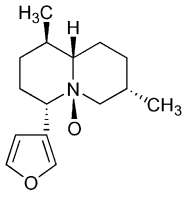
syn-thiobinupharidine sulfoxide (**6**)



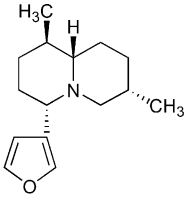
thionuphlutine B β-sulfoxide (**7**)



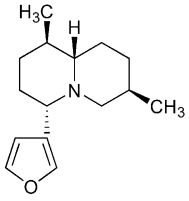
neothiobinupharidine β-sulfoxide (**8**)



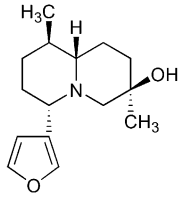
nupharidine (**9**)



deoxynupharidine (**10**)



7-epideoxynupharidine (**11**)



nupharolutine (**12**)

Figure 1. Nuphar alkaloids isolated from the rhizome of *Nuphar pumilum*.

Table 1. Inhibitory effects of alkaloid constituents from *Nuphar pumilum* on invasion of B16 melanoma cells

	Inhibition (%)				
	Concn 0.01	0.1	1	10	100 (μM)
Thiobinupharidine (**1**)	—	5.2±17	9.7±8.3	15.6±5.3	22.7±8.8[a]
6-Hydroxythiobinupharidine (**2**)	50.9±5.5**	57.5±3.1**	66.5±1.8**	84.8±0.3**,[a]	86.8±2.4**,[a]
6,6′-Dihydroxythiobinupharidine (**3**)	40.3±4.1**	43.1±6.9**	68.5±3.1**	83.2±2.3**,[a]	86.6±1.5**,[a]
6-Hydroxythionuphlutine B (**4**)	5.9±14.6	31.6±11.9	62.7±2.2**	61.4±3.6**,[a]	47.1±4.6**,[a]
Neothiobinupharidine (**5**)	—	11.9±6.8	18.9±4.1	33.2±4.1	28.3±2.1[a]
syn-Thiobinupharidine sulfoxide (**6**)	—	16.9±11	38.3±14.1	42.2±5.1	32.5±5.3[a]
Thionuphlutine B β-sulfoxide (**7**)	—	−8.1±4.5	10.2±1.5	9.2±10.5	0.0±4.5
Neothiobinupharidin β-sulfoxide (**8**)	—	10.6±5.0	18.5±2.2	12.8±3.5	15.7±4.6
Nupharidine (**9**)	—	19.6±3.1	34.7±2.7**	41.4±2.5**	47.2±3.8**
Deoxynupharidine (**10**)	—	−5.1±4.9	14.0±4.7	28.5±5.2	19.1±2.6
7-Epideoxynupharidine (**11**)	—	5.1±3.7	25.3±3.7	20.9±1.3	23.0±4.1
Nupharolutine (**12**)	—	25.5±9.6	1.7±4.8	16.8±11.5	−0.6±10.3

Each value represents the mean±SEM ($N = 4$). Significantly different from the control, $^{**}p < 0.01$.
[a]Cytotoxic effect was observed.

Table 2. Inhibitory effects of the alkaloid fraction and 6-hydroxythiobinupharidine (**2**) on lung metastasis of B16 melanoma cells in mice

	Dose (mg/kg, po)	*N*	Numbers of colonies	Inhibition (%)
Control	—	6	19.5±5.3	—
Alkaloid fraction	20	5	1.4±0.5**	93
6-Hydroxythiobinupharidine (**2**)	5	6	1.2±0.7**	94
Curcumin	20	6	2.5±0.6**	87

Each test compound was given orally once a day. Each value represents the mean±SEM. Significantly different from the control, $^{**}p<0.01$.

(0.0008%), and **12** (0.004%). The dehydroxyl derivative **1** was synthesized by $NaBH_4$ reduction of **2**.[5]

DMEM was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). FCS, penicillin, and streptomycin were purchased from Gibco BRL, Life Technologies, Inc. (Rockville, MD, USA). MTT was purchased from Dojindo Co. Ltd (Kumamoto, Japan). Cell Culture Insert™ (0.3 cm^2/well, pour size 3 μm) were purchased from Becton Dickinson & Company (Franklin Lakes, NJ, USA). Twenty-four- and 98-well microplates and 25 cm^2 culture flask were purchased from Sumitomo Bakelite Co., Ltd (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries Co. Ltd (Osaka, Japan).

Collagen matrix invasion assay

The invasion assay of B16 melanoma cells was performed using Cell Culture Insert™ and 24-well microplates. The upper side of each filter of Cell Culture Insert™ was pre-coated with collagen (type I). Briefly, 100 μL of 3 mg/mL collagen solution (type I, from porcine tendon, Wako) was added onto the each filter and incubated at room temperature for 2 h. The excess collagen solution was carefully aspirated and the filter was washed with PBS and DMEM without FCS [FCS (−)]. Cell Culture Insert™ with collagen-coated filters were inserted into the 24-well microplates with 700 μL/well of DMEM [FCS (+)] and pre-incubated for 20 min. A mixture of B16 melanoma cells (5×10^5 cells) suspended in 150 μL DMEM [FCS (−)] and test sample solution in 150 μL DMEM [FCS (−)] were then added onto the filters and incubated for 24 h. After incubation, the cells crossing the filters were fixed with 25% glutaraldehyde solution and stained with crystal violet, and the numbers of cells were counted under a microscope. The test sample was dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO in the medium was 0.1%. Curcumin was used as a reference compound. All experiments were performed in quadruplicate, and results are expressed as percentage inhibition of invasion.

Cytotoxicity was determined by MTT colorimetric assay. Briefly, after 20 h incubation of the melanoma cells (5×10^5 cells/200 μL/well) and test compounds in DMEM [FCS(+)] in 96-well microplates, MTT (10 μL/well, 5 mg/mL in PBS) solution were added to the wells. After a further 4 h in culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced by the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm).

Assay for experimental lung metastasis of the melanoma cells

B16 melanoma cells were resuspended to a concentration of 2.5×10^6 cells/mL in PBS. Mice were given an intravenous injection of the melanoma cells (5×10^5 cells/200 μL). Ten days later, the mice were killed and the lungs were excised and tumor colonies were counted. Curcumin was used as a reference compound.

Statistics

Values are expressed as means±SD or SEM. For statistical analysis, one-way analysis of variance followed by Dunnett's test was used. Probability (*p*) values less than 0.05 were considered significant.

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