Cancer Chemother Pharmacol (2010) 65:719–726
DOI 10.1007/s00280-009-1077-7

ORIGINAL ARTICLE

# Tumor-selective cytotoxicity of benzo[c]phenanthridine derivatives from *Toddalia asiatica* Lam.

**Hironori Iwasaki · Takafumi Okabe · Kensaku Takara · Takayoshi Toda · Masayuki Shimatani · Hirosuke Oku**

Received: 15 April 2009 / Accepted: 8 July 2009 / Published online: 23 July 2009


**Abstract**
*Purpose* To develop a novel anti-cancer drug of low side effect against lung adenocarcinoma, the authors screened the bioresources of Okinawa Island, Japan. The medicinal plant *Toddalia asiatica* Lam. contained three benzo[c]phenanthridine derivatives: dihydronitidine (DHN), nitidine (NTD) and demethylnitidine (DMN). Of the three derivatives, DHN had been shown to selectively inhibit the growth of cancer cells in our previous study. Because of similar molecular topology of NTD or DMN to DHN, it can be expected that NTD and DMN also show selective cytotoxicity. The aim of the present study was therefore to examine the selective cytotoxicity of these two compounds in vitro and in vivo.
*Methods* Benzo[c]phenanthridine derivatives were isolated from *T. asiatica* Lam., and their chemical structures were identified by interpretation of NMR and MS spectrum. Of the isolated compounds, NTD and DMN were evaluated for cytotoxicity in vitro or in vivo.
*Results* NTD as well as DHN selectively reduced the growth of murine and human lung adenocarcinoma in vitro with selective intracellular accumulation. NTD has also been proven to be highly effective in vivo to inhibit the growth of both murine and human lung adenocarcinoma in a subcutaneous xenograft model without any deteriorating side effect. In contrast, DMN had no selective cytotoxicity suggesting that 8-methoxy group of NTD is the critical structural feature for the selective cytotoxicity.
*Conclusions* This study thus proves the effectiveness of benzo[c]phenanthridine derivatives as anti-cancer agent in vivo for the first time, and discusses the mechanisms responsible for the selective cytotoxicity.

**Keywords** Nitidine · *Toddalia asiatica* Lam. · Benzo[c]phenanthridinium · Accumulation · Alkaloid · Antitumor

H. Iwasaki (✉) · T. Okabe · M. Shimatani · H. Oku
Center of Molecular Biosciences, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan
e-mail: hiwasaki@comb.u-ryukyu.ac.jp

K. Takara
Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Okinawa 903-0213, Japan

T. Toda
Department of Clinical Laboratory Medicine, School of Medicine, University of the Ryukyus Hospital, Uehara 207, Nishihara, Okinawa 903-0125, Japan

## Introduction

Lung adenocarcinoma is one of the most lethal cancers because of its high incidence and high mortality. Many novel chemotherapeutic agents have been developed against lung cancer, including camptothecin (CPT) derivatives, which are DNA topoisomerase I (TOPO-I) inhibitors. CPT is a potent chemotherapeutic agent, but not effective against lung adenocarcinoma due to tolerance of the cancer cells toward this drug [1, 2]. Several CPT derivatives including irinotecan and topotecan have also been introduced to cancer therapy [2]. However, the response rates to these drugs remain low, and the overall survival rate has not been much improved [3–5]. Furthermore, chemotherapy consisting of CPT derivatives usually encounters serious side effects, which hampers the continuous administration of these drugs to treat the disease. With this background, our intension was to screen a novel anti-cancer agent of low side effect from natural resources.

The medicinal plant *Toddalia asiatica* Lam. growing in Okinawa Islands of Japan has been found to contain several benzo[c]phenanthridinium alkaloids, such as nitidine (NTD), dihydronitidine (DHN) and demethylnitidine (DMN) (Fig. 1). Of the three derivatives, DHN has been shown to have tumor-specific cytotoxicity in our previous study [6]. Because of the similar molecular topology of NTD or DMN to DHN, it can be expected that these derivatives also have tumor-selective cytotoxicity, and therefore we evaluated their cytotoxicity in vitro and in vivo.

The natural benzo[c]phenanthridinium alkaloid has been characterized as topoisomerase poisons. However, because of weak topoisomerase-targeting activity, the benzo[c]phenanthridine derivatives have not been regarded as a useful chemotherapeutic agent. However, it has been an accepted view that the topoisomerase-targeting activity is not a sole factor to explain the cell cytotoxicity of benzophenanthridine derivatives [7]. The present study therefore examined the selective cytotoxicity of these alkaloids in vitro and in vivo for the first time, and discussed its beneficial feature as an anti-tumor agent.

## Materials and methods

### Preparation of crude extracts

Dried stem chips of *T. asiatica* Lam. were purchased from Nakazen Corporation, Okinawa, Japan. Crude extracts were prepared as follows. The dried powder (2,000 g) was extracted with 40 L of 50% ethanol (EtOH) at room temperature for 48 h. The extract was concentrated to dryness under a vacuum and re-suspended in 200 ml of water.

### Purification of the tumor-selective cytotoxic material

Partial purification of the extract was performed by liquid–liquid extraction using hexane, diethylether and *n*-butanol. The *n*-butanol fraction was concentrated with a rotary vacuum evaporator. After being resolved in methanol (MeOH), it was purified through a silica gel column (20 mm × 500 mm, wakogel® C-200, Wako Ltd.) using MeOH/chloroform (1:2–1:0). The active fractions obtained were concentrated and purified with pre-packed glass ODS column (10 mm × 250 mm, Yamazen) mixture of acetonitrile/methanol/acetate (98/1/1, by vol.). The active fractions were purified again under the same condition. All fractions were checked for their cytotoxicity.

### Identification of chemical structure

The chemical structures of components were identified by interpretation of NMR and MS spectra. The $^{13}C$ and $^{1}H$ NMR spectra were recorded on a JEOL α-500 spectrometer. Dimethylsulfoxide (DMSO) was used as the internal standard for $^{13}C$ and $^{1}H$ NMR spectroscopy.

### Cell cultures

The anti-tumor assay was performed using ten different normal and cancer cell lines: WI-38, human lung normal diploid fibroblast cell line; OUMS-36T-2F, normal human embryo fibroblast cell line (hTRT gene); A549, human lung

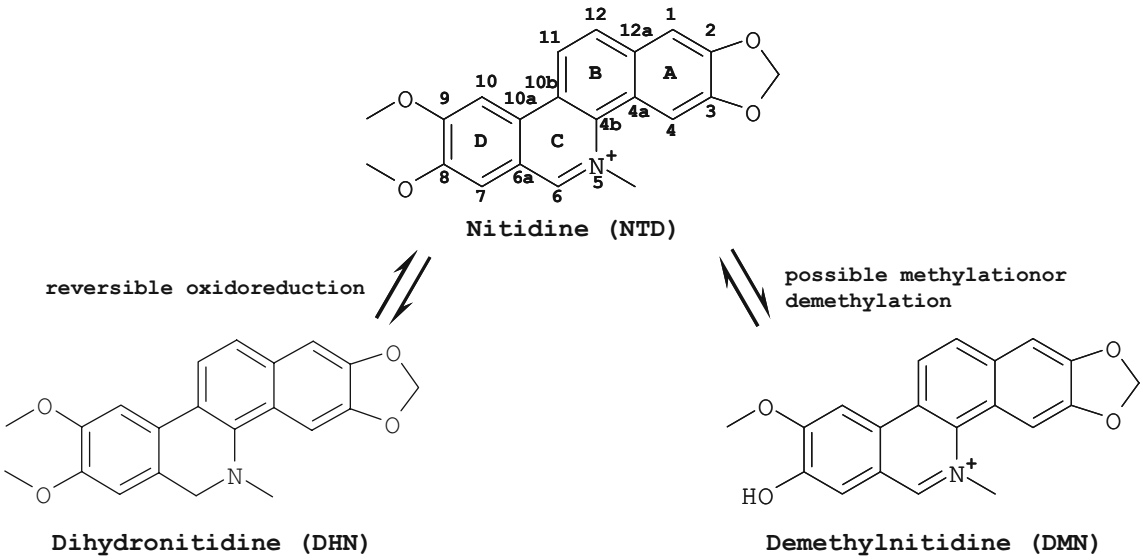


**Fig. 1** Chemical structure of NTD, DHN and DMN. The tautomeric reduction form (DHN) and the possible demethylation form (DMN) of NTD in plant cells

adenocarcinoma cell line; VMRC-LCP, human lung squamous carcinoma cell line; WI-38 VA13, SV40 virus transformed human lung fibroblast cell line; ACC-MESO-4, human malignant pleural mesothelioma cell line; PC-14, human lung undifferentiated adenocarcinoma cell line; RERF-LC-KJ, Japanese lung adenocarcinoma cell line; BLKCL.4, murine embryonic fibroblast cell line; and LLC, Lewis murine lung cancer cell line. These cell lines were obtained from Human Science Research Resources Bank (HSRRB) (human cell lines) and Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University) (murine cell lines). Cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$ in RPMI 1640 medium, DMEM and EMEM medium supplemented with 10% fetal bovine serum. Exponentially growing cells were used throughout the experiments.

### Cytotoxicity tests

The effects of the samples on the growth of various cancer cells were determined by measuring the cell viability using a commercial cell titer kit (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega). Briefly, the sample or vehicle was added to a 96-well plate and dried aseptically for 30 min. The cells were suspended in an appropriate medium and seeded at $1 \times 10^3$ cells (100 μl) per well and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C for 24 h. The cell number after the treatment was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay kit. All the measurements were performed in triplicates, and the cell cytotoxicity was expressed as the relative viability of sample treated cells against untreated controls. The 50% lethal dose ($D_{50}$) is defined as the concentration of the compound that inhibits 50% of cell growth, compared with untreated controls.

### Gene expression analysis

Gene expression analysis was performed in the mRNA from A549 and WI-38 cells treated with or without NTD. This analysis was essentially followed as described in the Affymetrix Expression Analysis Technical Manual [8]. The prepared cRNA was hybridized to an Affymetrix HG-U95Av2 chip. Data analysis was performed using GeneChip software. The entire experiment was performed three times.

### Fluorescence microscopic observation

The A549, WI-38 VA13, OUMS-36T-2F and VMRC-LCP cells of different susceptibility to NTD were treated for 0, 1, 2 or 4 h with a medium containing 3 μM of NTD. Cells treated with vehicle buffer were the control. Control and NTD-treated cells after the required incubation period were washed three times with PBS. With the group of 4 h treatment, cells were subsequently incubated with NTD-free medium for 1 or 4 h to observe the decay of fluorescence due to cellular turnover of NTD. NTD fluorescence was visualized by OLYMPUS BX-41 fluorescence microscope under the following conditions: 400–440 nm Ex filer, 455 nm dichroic mirror and 475 nm barrier filter.

### Animal studies

In human lung adenocarcinoma (A549) xenograft model, 4-week-old BALB/cAJcl-nu/nu male mice (22–26 g body weight) were purchased from CLEA Japan Inc. (Tokyo, Japan). A549 cells ($1 \times 10^6$) were inoculated intradermally on day −15. After 15 days, these mice were divided into two groups (six mice per group), by tumor volumes, and treated with PBS or NTD (day 0). NTD suspended in PBS(–) at concentration of 1.0 mg/ml was administrated by intraperitoneal injection with 0.1 mg/day per body during this study period. The tumor volume and body weight were measured on day 0, 6 and 14. The measurement of tumor size was terminated on day 14, because tumor necrosis was observed.

In murine lung adenocarcinoma (LLC) xenograft model, 4-week-old C57BL/6JJcl male mice (22–26 g body weight) were purchased from CLEA Japan Inc. (Tokyo, Japan). LLC cells ($2 \times 10^4$) were inoculated intradermally on day −7. After 7 days, these mice were divided into two groups (six mice per group) by tumor volumes, and treated with PBS or NTD (day 0). The schedule of NTD treatment has been described above. The tumor volume and body weight were measured on day 0, 4 and 7. Since the tumor xenograft sloughed off probably due to necrosis in some cases on and after day 7, no further measurements were made.

The whole blood of each individual murine LLC xenografts mouse was collected, allowed to clot and centrifuged at 1,500*g* for 20 min to separate the serum. To assess the hepatic parenchymal injury, plasma GPT activity was measured by Transaminase C-II-test wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

All animal experiments were approved by the Animal Care and Use Committee of the University of the Ryukyus.

### Statistical analysis

The data were expressed as mean ± standard deviation. The statistical significance was evaluated by Student's *t* test. Values of $p < 0.05$ were considered to be statistically significant.

## Results

### Preparation and purification of NTD and DMN

We obtained three alkaloids including dihydronitidine (DHN) during the course of purification of tumor-specific cytotoxicity from *T. asiatica* Lam. Purification and identification of DHN has been described in the previous study [6]. About 5 mg of each purified material was dissolved in methanol-d4 (500 µl) and applied to the NMR spectrometer. The interpretation of spectral data identified these materials as nitidine (NTD) and demethylnitidine (DMN) (Table 1, Fig. 1). The occurrence ratio of DHN, NTD and DMN in the extract was 1:100:5.

### Cytotoxicity of NTD and DMN in vitro

The tumor-selective cytotoxicity of NTD and DMN were evaluated. In the case of human lung cell lines, WI-38 as normal cells and A549 as lung adenocarcinoma, NTD showed strong tumor selectivity as was the case for DHN in our previous study [6] (Fig. 2a). In contrast, DMN had no cytotoxicity to each cell line (Fig. 3). DMN, the demethyl derivatives of NTD at the 8-methoxy group, therefore showed no selective cytotoxicity (Fig. 3).

The cytotoxicity of NTD to other human cell lines was surveyed. The $D_{50}$ of NTD for various cultured cell lines is listed in Table 2. Cell lines originating from human normal tissues were less susceptible to NTD, compared to tumor cell lines, except for human lung squamous cell carcinoma (VMRC-LCP) and human malignant pleural mesothelioma (ACC-MESO4).

To determine whether this selective cytotoxicity is reproducible with the lung adenocarcinoma of other species, we tested the cytotoxicity of NTD to murine lung adenocarcinoma cells. The $D_{50}$ of NTD to murine embryonic fibroblast cells (BLKCL.4) and lung adenocarcinoma cells (LLC) was >30 µM and 14.0 ± 1.0 µM, respectively (Fig. 2b). Thus, the tumor-selective cytotoxicity of NTD was also observed with murine cultured cell lines as was the case for human cell lines.

### Gene expression analysis in A549 and WI-38 cell lines

The expression levels of apoptosis relation genes (p53, BAX, p21) are shown in Fig. 4. In the A549 cell line, these apoptosis promoting genes were up-regulated significantly. In WI-38 cell line, no variation was observed.

### Observation of NTD accumulation by fluorescence microscopy

Tumor-specific accumulation of DHN was observed in the previous study [6]. Because of the similar molecular topology of NTD to DHN, the same mechanism might be involved in the anti-tumor activity of NTD. The accumulation and excretion of NTD were therefore observed under various treatment conditions. The chosen four cell lines (A549, WI-38 VA13, OUMS-36T-2F and VMRC-LCP) were different in susceptibility to NTD (Fig. 5). Higher accumulations were observed in cells more sensitive to NTD after only 1 h of treatment (Fig. 5a-ii). In addition, the

**Table 1** $^{13}C$ and $^{1}H$ NMR spectral data of isolated active material

| Position | Nitidine | Demethylnitidine |
|---|---|---|
| $^{13}C$ NMR spectral data | | |
| 1 | 105.4 | 105.3 |
| 2 | 152.0[a] | 152.6[a] |
| 3 | 151.0[a] | 150.8[a] |
| 4 | 107.1 | 107.1 |
| 4a | 121.6[a] | 121.7 |
| 4b | 134.5[a] | n.d. |
| 6 | 150.5 | 69.3 |
| 6a | 125.9 | n.d. |
| 7 | 109.5 | 112.0 |
| 8 | 153.9 | 152.6 |
| 9 | 160.7 | 142.5 |
| 10 | 103.8 | 103.7 |
| 10a | 121.6 | 133.1 |
| 10b | 134.3[a] | n.d. |
| 11 | 119.8 | 119.8 |
| 12 | 131.7 | 131.6 |
| 12a | 121.4[a] | 121.0 |
| N-$CH_3$ | 52.1 | 52.0 |
| 8-$OCH_3$ | 57.1 | – |
| 9-$OCH_3$ | 57.8 | 57.7 |
| O–$CH_2$–O | 104.3 | 104.2 |
| $^{1}H$ NMR spectral data (J Hz) | | |
| 1 | 7.54[s] | 7.56[s] |
| 4 | 8.21[s] | 8.24[s] |
| 6 | 9.61[s] | 9.56[s] |
| 7 | 7.79[s] | 7.65[s] |
| 10 | 8.19[s] | 8.19[s] |
| 11 | 8.67[d(9.0)] | 8.69[d(9.0)] |
| 12 | 8.17[d(9.0)] | 8.18[d(9.0)] |
| N-$CH_3$ | 4.92[b] | 4.86[b] |
| 8-$OCH_3$ | 6.26[s] | 6.26[s] |
| 9-$OCH_3$ | 4.10[s] | – |
| O–$CH_2$–O | 4.25[s] | 4.27[s] |

Analysis conditions were 100 ($^{13}C$; 25°C) and 400 ($^{1}H$; 25°C) MHz in $CD_3OD$

[a] Assignment may be interchanged

[b] The signal was overlapped by the signal of the solvent

n.d., Clear signal could not be determined

–, None

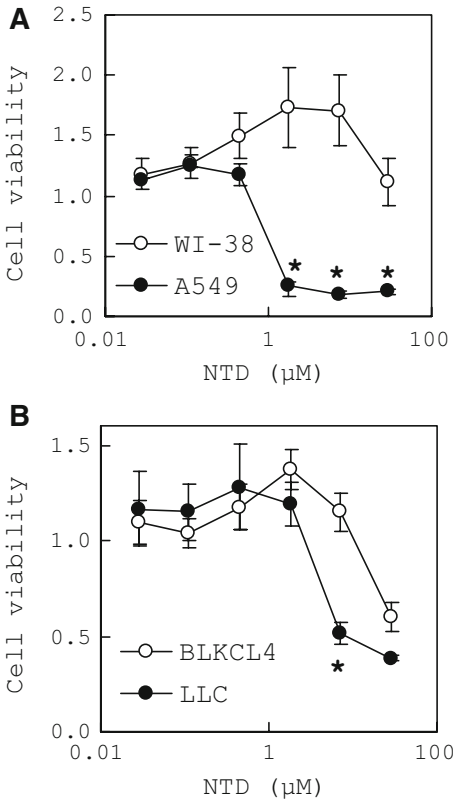


**Fig. 2** Tumor-selective cytotoxicity of NTD to human and murine cells. Human normal lung cells (WI-38, *open circle*) and human lung adenocarcinoma cells (A549, *filled circle*) were treated with NTD for 24 h. (**a**) The embryonic fibroblast cells established from C57BL/6 mice (BLKCL.4, *open circle*) and murine Lewis lung cancer cells (LLC, *filled circle*) were similarly treated with NTD for 24 h. (**b**) Cell viabilities were expressed as the viability ratio of treated to untreated cells. * $P < 0.01$ versus the WI-38 or BLKCL.4 cells

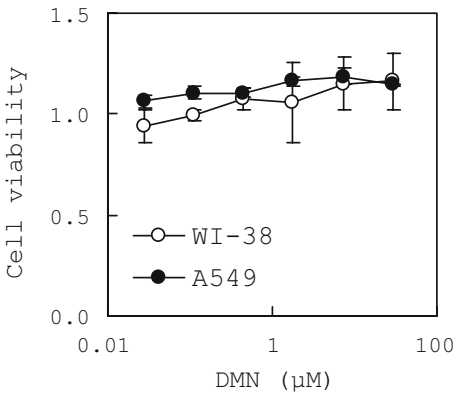


**Fig. 3** Effect of DMN on the viabilities of human cells. Human normal lung cells (WI-38, *open circle*) and human lung adenocarcinoma cells (A549, *filled circle*) were treated with demethylnitidine for 24 h. Cell viabilities were expressed as the viability ratio of treated to untreated cells

accumulated NTD was not removed from the cell by simply changing the medium to the NTD-free one. It was also noted that the cells resistant to NTD (OUMS-36T-2F and VMRC-LCP) always showed lower fluorescence intensity, reflecting the lower loading of this compound to these cells. The low fluorescence intensity in these resistant cells was consistently observed in several treatment conditions.

**Table 2** Cytotoxicity of nitidine to various cell lines

| Origin | Cell name | $D_{50}$ (μM) |
|---|---|---|
| Normal | OUMS-36T-2F (normal human embryo fibroblast cell (hTRT gene)) | >30.0 |
| | WI-38 (lung normal diploid fibroblast) | >30.0 |
| Tumor | A549 (lung adenocarcinoma cell line) | 0.9 |
| | VMRC-LCP (lung squamous cell carcinoma) | >30.0 |
| | ACC-MESO-4 (malignant pleural mesothelioma cell line) | >30.0 |
| | PC-14 (lung adenocarcinoma cell line) | 15.5 |
| | RERF-LC-KJ (lung adenocarcinoma cell line) | 5.2 |

## Effect of NTD on the growth of lung adenocarcinoma implant

To assess the therapeutic potency of NTD, we conducted daily consecutive treatments of human lung adenocarcinoma cells (A549) inoculated into the dorsal skin of BALB/cAJcl-nu mice. The implanted tumors grew to 94.0 ± 29.3 ml 15 days after inoculation (day 0 of Fig. 6a). These 12 mice were then divided into two groups of comparable tumor volumes, and the treatment with NTD and PBS(–) was started (day 0 of Fig. 6a).

Intraperitoneal administration of NTD (0.1 mg/body per day i.p.) for 15 days significantly reduced the tumor volume of A549 (day 0–14 of Fig. 6a).

A similar experiment was conducted with the murine lung adenocarcinoma cells (LLC) inoculated into the dorsal skin of C57BL/6JJcl mice. The implanted tumors grew to 25.2 ± 11.5 ml, 7 days after inoculation (day 0 of Fig. 6b). These 12 mice were then divided into two groups of comparable tumor volumes and treated with NTD or PBS(–) (day 0 of Fig. 6b).

Intraperitoneal administration of NTD (0.1 mg/body per day i.p.) for 8 days was highly effective in reducing tumor volume of LLC xenograft (day 0–7 of Fig. 6b).

Behavior disorders, commonly manifested as lethargy, poor grooming and anorexia, were not observed throughout the treatment period in both animal experiments. In addition, no anatomical abnormalities were observed in all groups. There was no significant difference in body weight between two groups in all tests (data not shown).

Some chemotherapeutic agents if used alone may be associated with severe hepatopathy. Before dissection, we collected blood samples from LLC xenograft C57BL/6JJcl mice and measured the serum level of glutamic pyruvic

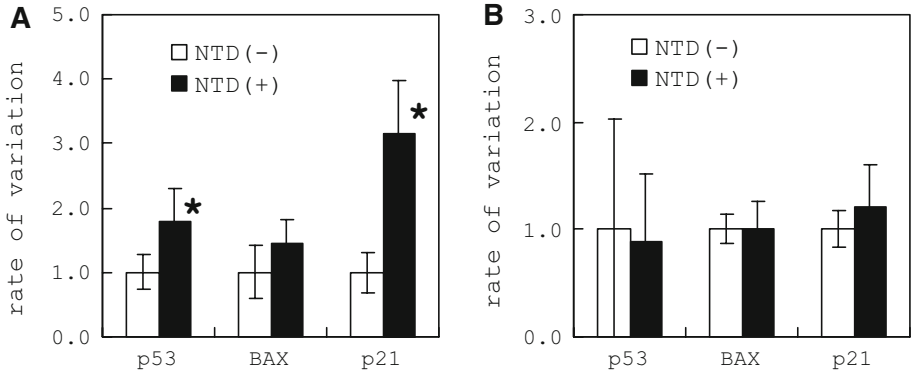


**Fig. 4** Effect of NTD on the expression levels of various genes. Values represent the relative changes in gene expression against the untreated control cells (**a** A549, **b** WI-38). Values are mean ± SD of triplicate analysis. * $P < 0.05$ versus the untreated group

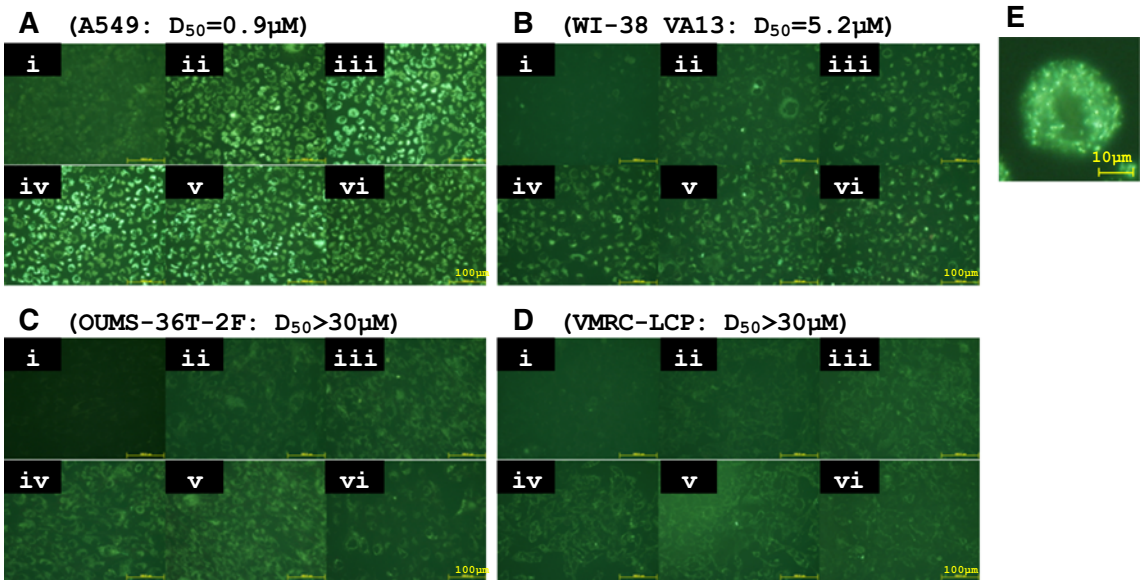


**Fig. 5** Fluorescence microscopic images of various cells treated with NTD. Cell lines of different susceptibility to NTD were treated with 3 μM of NTD for various times. Each plate was observed under UV illumination. *i–iv* for each cell (**a**–**d**) show the fluorescence images of the cells treated with 3 μM NTD for 0, 1, 2, and 4 h, respectively. *i* and *iv* show the images of the cells treated with 3 μM for 4 h and subsequently incubated with NTD-free medium for 1 and 4 h, respectively. Enlarged image of A549 cells treated with 3 μM for 2 h (**e**)

transaminase (GPT), markers of liver damages. This test showed no sign of liver damage by the daily consecutive NTD treatment (Fig. 7).

## Discussion

The present study described the tumor-specific cytotoxicity of benzo[c]phenanthridine derivatives extracted from the medicinal plant *T. asiatica* Lam. There were three benzophenanthridine derivatives in the extract of *T. asiatica* Lam.: nitidine (NTD), dihydronitidine (DHN) and demethylnitidine (DMN) (Fig. 1). Of the three derivatives, NTD has been proven to reduce the growth of both human and murine lung adenocarcinoma in a subcutaneous xenograft model for the first time. No side effect was noted for NTD on the basis of the serum levels of liver injury marker GPT (Fig. 7). The pharmacological significance of this study therefore lies in that this work provides the first in vivo evidence of natural benzo[c]phenanthridine derivatives as effective anti-cancer agent of low side effect.

As expected from the molecular topology, NTD was found to inhibit the growth of tumor cells selectively, as was the case for DHN (Fig. 2, Table 2). The gene expression analysis also supported that the pathway of apoptosis induction were similar to DHN (Fig. 4). NTD has been characterized as dual poison, in inhibiting both topoisomerase I and II [7, 9–11]. However, NTD has not been proven to be highly effective as anti-tumor agent in vivo, probably due to its weak activity to inhibit topoisomerase or due to its limited solubility to formulate [7, 9]. In general, there is a poor correlation between cytotoxicity and topoisomerase-targeting activity among the benzophenanthridine derivatives [11]. These observations suggest that in the absence of

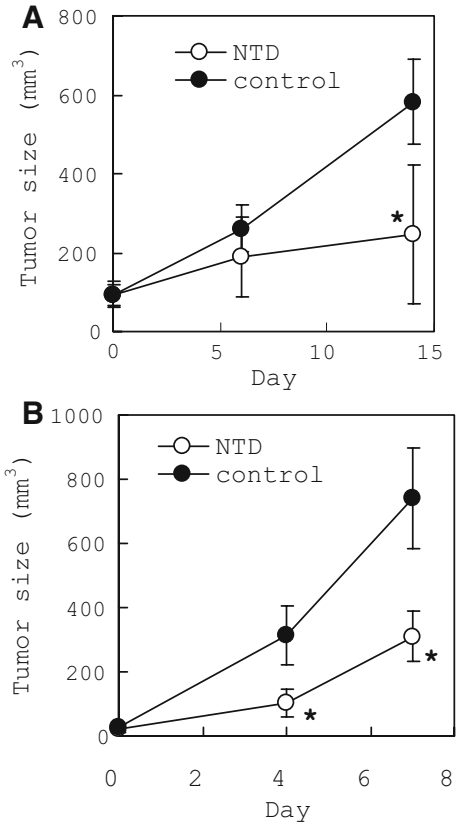


**Fig. 6** Suppression of human (**a**) or murine (**b**) lung adenocarcinoma xenograft growth by NTD. BALB/cAJcl-nu/nu mice bearing human lung adenocarcinoma (A549) xenografts (**a**) and C57BL/6JJcl mice bearing murine lung cancer (LLC) (**b**) xenografts were separated into two treatment groups: PBS(–) as controls (*filled circle*) or NTD 0.1 mg/day/body i.p. (*open circle*) on day 0. Tumor size was measured with calipers. Values are mean ± SD of six animals per group. * $P < 0.01$ versus the control group

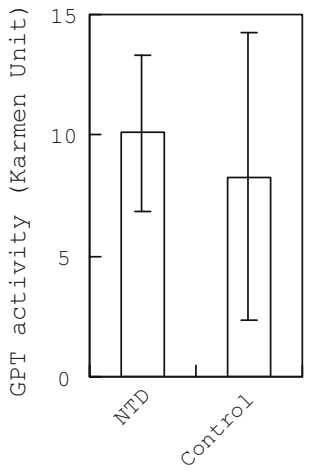


**Fig. 7** Effect of NTD on serum GPT activity of mice bearing murine LLC cells. Whole blood was collected from the heart of mice bearing LLC cells and analyzed for GPT activity. Values are mean ± SD of six animals per group

potent intrinsic topoisomerase-targeting activity, other mechanism may substantially contribute to the cytotoxicity. This could be the present case of NTD, as shown in both in vitro and in vivo. The $D_{50}$ of NTD for A549 cancer cells (0.9 μM) was much lower than that for a well-known topoisomerase inhibitor, CPT (>3 μM). Thus, the cytotoxicity of NTD against A549 cells was higher than that of CPT, despite one-tenth of its topoisomerase-targeting activity [6]. Furthermore, the dose response profile for the cytotoxicity of CPT differed from that for NTD with 48 h of exposure: NTD showed selective cytotoxicity up to 48 h of exposure, while CPT inhibited the growth of both normal and cancer cells almost to the same extent (data not shown). This observation in the present study was largely comparable to that for DHN described in our previous study [6]. This observation therefore supports the view that other mechanisms rather than the inhibition of intrinsic topoisomerase activity is involved in the selective cytotoxicity of NTD. In this context, Sanders et al. reported a topoisomerase-directed selective cytotoxicity of protoberberines against glioblastoma cells [12]. They proposed an accelerated uptake of the drug by the glioblastoma cells to explain the selective cytotoxicity of protoberberine, and suggested the involvement of ligand translocation process associated with the specialized membrane invaginations known as caveola [12]. The surfaces of most cells are studded by tiny, flask-shaped membrane invaginations called caveola [12]. Caveola has been proposed to pinch off and form vesicles that sequester tracer molecules of low molecular weight in the extracellular fluid that flows into the cells [12]. The protoberberine loaded cells, thus, gave off bright fluorescence associated with cytoplasmic vesicles [12].

The present results also demonstrated the accumulation of NTD in the cancer cell specific-manner, which appeared to largely agree with that for protoberberine. Viewed under fluorescence microscope, cancer cells susceptible to NTD had blight fluorescence associated with the cytoplasmic vesicles (Fig. 5). Thus, the cancer cell-specific accumulation of NTD also may be explained by active transport of NTD via the membrane invagination. The structural requirement for the selective transport of protoberberine has been identified as the presence of iminium nitrogen, which might be necessary for the receptor-mediated incorporation of positively charged drugs [12].

In contrast, the current study identified the most important structure for the cancer cell-specific cytotoxicity of NTD as 8-methoxy group (Figs. 1, 3). Demethylation of NTD at the 8-position completely abolished the selective cytotoxicity. This finding obviously ruled out the possibility of a selective uptake of NTD via the interaction of iminium cation with the cell surface components responsible for the selective drug uptake. Given that iminium cation in NTD molecule is the critical factor for the selective

cytotoxicity, loss of the positive charge by the saturation of 5, 6 double bond should abolish the cytotoxicity. However, DHN bearing no positive charge at nitrogen heteroatom had been shown to retain the selective cytotoxicity in our previous study, with roughly comparable activity to NTD. The $D_{50}$ of DHN cytotoxicity for A549 cells in our previous study was 0.5 μM [6], and close to 0.9 μM observed for NTD in this study (Table 2). Furthermore, DHN as well as NTD accumulated specifically in the cancer cells [6]. Thus, our present and previous studies suggest that the selective transport of NTD may be mediated via membrane invagination, but with different ligand–receptor systems suggested for the incorporation of protoberberines [12].

Several lines of studies have been conducted to characterize the structural features of NTD associated with its topoisomerase-targeting activity [13]. The previous structure–activity data with NTD suggested that the replacement of the methylenedioxy moiety within A-ring with 2,3-dimethoxy substituents results in a substantial loss of topoisomerase-targeting activity [11, 13]. It has also been suggested that demethylation of the 8-methoxy group influences the topoisomerase-targeting activity, but not the critical factor [10, 13]. Steric hindrance associated with the substituents at the 8-position appeared to affect the stabilization of cleavable complex between topoisomerase and DNA, as the 8-hydroxy derivative was more active than the methoxy derivative [10]. Therefore, it can be speculated that the topoisomerase-targeting activity of DMN was more potent than that of NTD. Nevertheless, DMN, demethyl derivative of NTD, showed no selective cytotoxicity (Fig. 3), which in turn suggests that the difference in the incorporation of NTD into cell is primarily responsible for the expression of the cancer-selective cytotoxicity.

There was a positive correlation between intracellular accumulation of NTD and susceptibility to this drug in the cancer cell lines (Fig. 5). The susceptible cancer cell line accumulated NTD more than the normal or rather resistant cells lines. Taking the weak topoisomerase-targeting activity of NTD into consideration, it could be speculated that the lower potency would be offset by increased cellular absorption with the consequences of reduced cell proliferation. Thus, the susceptibility of cancer cell appeared to simply reflect the extent to which the topoisomerase activity was inhibited by the accumulated NTD. However, topoisomerase protein is primarily located in the nucleus [14], and no accumulation of NTD in the nucleus was noted in the present study (Fig. 5e). NTD accumulates in the extra-nuclear compartment, as was the case for DHN, in the form of small vesicles. It is therefore unlikely that the direct effecter of NTD in the cancer cells is the inhibition of DNA replication, catalyzed by topoisomerase. DHN accumulated in A549 cells has been shown to induce apoptosis via the modulation of Fas, Bax and p53 gene expressions in our previous study [6]. In the present study, NTD also exerts its anti-cancer activity by a similar mechanism, as DHN does, although identification of the direct cellular target is yet to be achieved (Fig. 4).

NTD has been characterized as a topoisomerase poison and found to show a different spectrum of cytotoxicity against various cancer cells, although its potency was almost only one-tenth of CPT. The variable responses to these natural alkaloids, between cancer cell lines or between normal and cancer cells, could be due to difference in their capability to take up the drugs from the extracellular environment. Identification of transport system responsible for the selective accumulation of NTD or natural alkaloids is significant since the selective cytotoxicity could form the basis of therapies directed toward cancer cells without any side effect. Clarification of the underlying mechanism for this selectivity, therefore, may open up another possibility for cancer chemotherapy.

## References


1. Kojima A, Hackett NR, Ohwada A, Crystal RG (1998) In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. J Clin Invest 101:1789–1796
2. Takimoto CH, Wright J, Arbuck SG (1998) Clinical applications of the camptothecins. Biochim Biophys Acta 1400:107–119
3. Kraus-Berthier L, Jan M, Guilbaud N, Naze M, Pierre A, Atassi G (2000) Histology and sensitivity to anticancer drugs of two human non-small cell lung carcinomas implanted in the pleural cavity of nude mice. Clin Cancer Res 6:297–304
4. Flescher E (2007) Jasmonates in cancer therapy. Cancer Lett 245:1–10
5. Ancuceanu RV, Istudor V (2004) Pharmacologically active natural compounds for lung cancer. Altern Med Rev 9:402–419
6. Iwasaki H, Oku H, Takara R, Miyahira H, Hanashiro K, Yoshida Y, Kamada Y, Toyokawa T, Takara K, Inafuku M (2006) The tumor-specific cytotoxicity of dihydronitidine from *Toddalia asiatica* Lam. Cancer Chemother Pharmacol 58:451–459
7. Li D, Zhao B, Sim SP, Li TK, Liu A, Liu LF, LaVoie EJ (2003) 8, 9-Methylenedioxybenzo[i]phenanthridines: topoisomerase I-targeting activity and cytotoxicity. Bioorg Med Chem 11:3795–3805
8. Affymetrix I (2002) GeneChip expression analysis technical manual. Affymetrix, Inc., Santa Clara, CA
9. Wang HK, Morris-Natschke SL, Lee KH (1997) Recent advances in the discovery and development of topoisomerase inhibitors as antitumor agents. Med Res Rev 17:367–425
10. Li D, Zhao B, Sim SP, Li TK, Liu A, Liu LF, LaVoie EJ (2003) 2, 3-Dimethoxybenzo[i]phenanthridines: topoisomerase I-targeting anticancer agents. Bioorg Med Chem 11:521–528
11. Makhey D, Li D, Zhao B, Sim SP, Li TK, Liu A, Liu LF, LaVoie EJ (2003) Substituted benzo[i]phenanthridines as mammalian topoisomerase-targeting agents. Bioorg Med Chem 11:1809–1820
12. Sanders MM, Liu AA, Li TK, Wu HY, Desai SD, Mao Y, Rubin EH, LaVoie EJ, Makhey D, Liu LF (1998) Selective cytotoxicity of topoisomerase-directed protoberberines against glioblastoma cells. Biochem Pharmacol 56:1157–1166
13. Makhey D, Gatto B, Yu C, Liu A, Liu LF, LaVoie EJ (1996) Coralyne and related compounds as mammalian topoisomerase I and topoisomerase II poisons. Bioorg Med Chem 4:781–791
14. Rothenberg ML (1997) Topoisomerase I inhibitors: review and update. Ann Oncol 8:837–855