Cancer Chemother Pharmacol (2006) 58: 451–459
DOI 10.1007/s00280-005-0183-4

ORIGINAL ARTICLE

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The tumor specific cytotoxicity of dihydronitidine from *Toddalia asiatica Lam*

Received: 14 September 2005 / Accepted: 23 December 2005 / Published online: 8 February 2006
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Abstract Purpose: In recent years, a number of reports have shown the anticancer activity of plant extracts and phytoalkaloid. Methods: We have evaluated the cytotoxicity profiles of 157 extracts prepared from dietary or medical plants growing in the Okinawa island, using 10 different cell lines. In vitro cytotoxicity screening indicated the presence of a highly selective cytotoxic compound in the extract of *Toddalia asiatica Lam*. The known alkaloid (1,3)benzodioxolo(5,6-c)phenanthridine, 12,13-dihydro-2,3-dimethoxy-12-methyl-(dihydronitidine) was identified as an active material from this plant. This alkaloid had highly specific cytotoxicity to human lung adenocarcinoma (A549) cells. Results: The results of the fluorescence activated cell sorter (FACS) analysis and the measurement of caspase-3 activity showed that dihydronitidine induced specific apoptotic cell death in A549 cells. Gene expression analysis in the apoptotic cells found that dihydronitidine variously regulated the cell cycle related genes (CDK2 and CCNE), and up-regulated the cell death related genes specifically in tumor cells. Thus dihydronitidine manifested its characteristics in the tumor selective cytotoxicity, contrasting with the case of a known anticancer agent camptothecin (CPT). Microscopic observation further revealed the specific accumulation of dihydronitidine within the cytosolic organelle, but not in the nuclei of adenocarcinoma. No accumulation was observed with CPT in all cell lines. Conclusion: The data suggested that dihydronitidine toxicity targeted a particular intracellular organelle in the tumor cells.

Keywords Tumor · Alkaloids · Apoptosis · Accumulation · Cytotoxicity · Dihydronitidine

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Introduction

The use of natural products as a Chinese medicine has a long history that began with folk medicine and through the years has been incorporated into traditional medicine. Several drugs currently used in chemotherapy were isolated from plant species or derived from a natural prototype. Most of the current cancer drugs are classified as alkaloids [1]. Alkaloids are a diverse group of low molecular weight, nitrogen-containing molecules found in about 20% of the plant species [2]. Many of the 16,000 alkaloids, for which structures have been described, function in the defense of the plants against herbivores, microbes, viruses, and competing plants. Medical herbal teas contain various bioactive substances represented by flavonoids or alkaloids [3]. The regular consumption of alkaloid is associated with a reduced risk of chronic diseases, including arteriosclerosis, hypertension, diabetes, or cancer [4, 5]. Several alkaloids and analogues have contributed to recent chemotherapeutic treatments [6].

Lung cancer remains the leading cause of cancer related deaths in the world, in both men and women [7].

The poor lung cancer survival rates argue powerfully for more effective approaches to control this disease. In this disease with many unresectable cases, the improvement of the result of chemotherapy is important. Camptothecin (CPT), a plant alkaloid extract from *Camptotheca acuminata*, is a potent inhibitor of the enzyme DNA topoisomerase I [8, 9]. Several CPT derivatives have recently been introduced for cancer therapy, including irinotecan and topotecan [10, 11]. However, the response rates to these reagents remain low, and the overall survival rate has not been improved substantially [12, 13]. With this background, the novel antitumor activity with low side effect has been screened in several studies.

We performed the screening of antitumor activity from food material in the Okinawa islands. Recent result of this screening showed that the traditional medical herbal tea, *Toddalia asiatica Lam.*, in Okinawa had notable tumor selective cytotoxicity. *T. asiatica* Lam. has been used medicinally in Kenya [14]. The fruit has been used as a cough remedy and the roots have been used for the treatment of indigestion and influenza [15, 16]. The leaves also have been used for lung diseases and rheumatism [17]. In Okinawa, the whole stem chips have been used as a remedy for lung diseases. Several bioactive alkaloids have been identified from plants of the genus Toddalia [14, 18].

In this report, we describe the identification and the characterization of the selective cytotoxic compound from *T. asiatica* for the first time. Thus the objective of this investigation was to delineate the lung-tumor selective cytotoxicity of dihydronitidine in vitro, by assessing its effect on apoptosis related events, morphological appearance, and also on gene expression.

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 (100 g) was extracted with 2 l of 50% ethanol (EtOH) at room temperature for 48 h. The extract was concentrated to dryness under a vacuum, and was resuspended in 50% EtOH.

Purification of the tumor selective cytotoxic material

Partial purification of the extract was carried out by the successive partitioning of the active compound into hexane, diethylether, and n-butanol. The n-butanol fraction was concentrated with the rotary vacuum evaporator. The active fractions were purified by open column chromatography. In brief, a 20×500 mm^2 column was packed with silica gel (wakogel® C-200, Wako Ltd.), and equilibrated with chloroform/methanol (9/1, by vol.). The n-butanol extract was loaded on to the silica gel column, and eluted with the same solvent. The active fraction was further purified by a pre-packed glass ODS column (10×250 mm^2, Yamazen) equilibrated with a mixture of acetonitrile/methanol/acetate (98/1/1, by vol.). All fractions were checked for their selective cytotoxicity.

Identification of chemical structure

The chemical structure of the active component was identified mainly by interpretation of the 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. Mass spectrometric measurements were performed on a GCMS-QP2010 electron ionization mass spectrometer (EI-MS) (Shimadzu, Kyoto, Japan). Conditions for mass spectrometry were as follows: ion source temperature = 200°C, interface temperature = 250°C, detector voltage = 12 kV and scan range m/z = 50–700. Samples were introduced directly into the ionization compartment, and ionized by electron impact at 70 keV. The fluorescence spectrum was measured on a fluorospectrophotometer (RF-5300PC, Shimadzu). Emission spectrum was measured at an excitation wavelength of 397 nm. Excitation spectrum was measured at an emission wavelength of 507 nm.

Cell cultures

The antitumor assay was performed using ten different human normal or cancer cell lines, purchased from the Japan cancer research source bank: WI-38, lung normal diploid fibroblast; OUMS-36, normal human embryo fibroblast cell; OUMS-36T-2F, normal human embryo fibroblast cell (hTRT gene); A549, lung adenocarcinoma cell line; VMRC-LCP, lung squamous cell carcinoma; COLO-201, colon adenocarcinoma; MIA-PaCa2, pancreatic cancer; A431, epidermoid carcinoma; KATOIII, stomach cancer; SKBR-3, breast cancer. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, DMEM or EMEM medium supplemented with 10% fetal bovine serum. The exponentially growing cells were used throughout the experiments.

Cytotoxicity tests

The effects of the EtOH extract or column elute fractions 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 the 96-well plate, and dried aseptically for

30 min. Each cell suspended in the appropriate medium was seeded at 1×10^3 cells (100 µl) per well, and incubated in humidified atmosphere, and 5% CO_2 at 37°C for 48 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 experiments were performed in triplicate, and cell cytotoxicity was expressed as the relative viability of the sample treated cells against untreated controls. D_{50} defines the concentration of the compound that inhibits 50% of the cell growth compared with untreated controls.

Fluorescence activated cell sorter (FACS) analysis

A549 cells (5×10^5 cells in a dish of diameter 90 mm) were incubated for 24 h with or without 480 ng/ml of dihydronitidine. Thereafter, the cells were harvested by trypsin digestion and washed with cold PBS. Cells were collected by centrifugation, and were resuspended in PBS containing 50 ng/ml Annexin V-FITC and 5 µg/ml propidium iodide (PI). After incubation for 30 min on ice in the dark, the samples were subjected to FACS analysis (FACS Calibur, Becton Dickinson Immunocytometry Systems, USA).

Detection of caspase-3 activity

A549 and WI-38 cells in the 96-well microplate (1×10^3 cells/well) were incubated for 24 h with 500, 250, 125, 63 ng/ml of dihydronitidine. Caspase-3 activity in cell lysate was measured by using a caspase-3 fluorometric assay kit (Caspase 3 Assay kit, Sigma Aldrich) according to the manufacturer's instructions.

RNA extraction and gene expression analysis

A549 and WI-38 cells were grown with or without 4.8 µg/ml of dihydronitidine for 4 h. Total RNA was extracted from each cell by the TRIzol Reagent (Invitrogen, Carlsbad, CA), and then was treated with DNase I to remove genomic DNA (Ambion, Austin, TX). Isolated total RNA was labeled as described in the GeneChip® Expression Analysis Technical Manual (Affymetrix®, Santa Clara, CA, USA). Labeled RNA samples were used for the hybridization with the GeneChip® Human Genome U95 microarray (HG-U95A) sets from Affymetrix®. This array represents approximately 12,000 human known genes. To validate the gene expression data, real-time PCR was performed by using SYBER green dye I and Bio-Rad iCycler (Hercules, CA, USA) according to the manufacturer's SYBER green protocol. A549 and WI-38 cells were treated with or without 4.8 µg/ml of dihydronitidine for 8 h. Expression of a given gene (CCNF, CDK2, CDK4, E2F1, PCNA, Mdm2, p19, p53, p38, AKT1, Apaf-1, Bad, Bax, Bcl-2, Bid, CAD, Casp2, Casp3, Casp8, Casp9, CRADD, CYCS, Daxx, FADD, Fas, FasL, FLIP, ICAD, IKK, JNK, NIK, PI3K, RIP, TNF, TNFR1, TRADD, and TRAF2) was determined in triplicate for each cell line and treatment. Expression of the reference gene, β-actin (ACTB), was determined for each cell line and treatment in triplicate. All of the mRNA levels were normalized by the ACTB level, and expressed as an increase in the level compared to the untreated control.

Morphological observations

The fluorescence of both dihydronitidine and CPT allowed us to examine their incorporation into cells. Cells of A549, WI-38, VMRC-LCP, and OUMS-36T-2F were cultured on glass slides with dihydronitidine or CPT, and were examined by a fluorescence microscope.

Statistical analysis

The data were shown as mean ± standard deviation. Statistical significance was evaluated by the Student's t test. Values of $P<0.05$ were considered as a statistically significant difference.

Results

Selective cytotoxicity of crude extract from *T. asiatica Lam.*

We screened about 150 dietary or medical plants in the Okinawa Island for tumor selective cytotoxicity. The human cell lines, including normal or tumor cells, were used for the estimation of selectivity to various human tissues or tumors. A result from this screening revealed

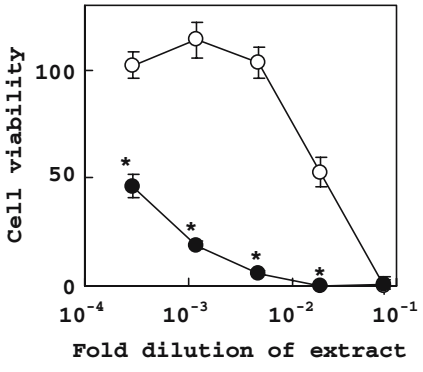

Fig. 1 Selective cytotoxicity of *T. asiatica Lam.* extract. The human fetal lung fibroblast (WI-38) cells (*open circle*) and human lung adenocarcinoma (A549) cells (*filled circle*) were treated with increasing doses of the *T. asiatica* extract. Their viabilities were determined by a cell titer kit, and expressed as the viability ratio of treated to untreated cells. *$P<0.05$ versus the WI-38 cells

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

Atom	^{13}C	^{1}H
1	99.7	7.51 *s*
2	148.7[a]	
3	148.3[a]	
4	104.1	7.48 *s*
4a	125.6	
4b	124.03	
6	53.8	4.08 *s*
6a	123.5	
7	110.7	6.96 *s*
8	147.5	
9	–[d]	
10	106.9	7.28 *s*
10a	124.0	
10b	–[d]	
11	120.2	7.83 *d*, 8.5
12	123.98	7.55 *d*, 8.5
12a	130.3	
N-CH_3	40.7	2.54 *s*
8-OCH_3	55.8[b]	3.87[c] *s*
9-OCH_3	55.6[b]	3.81[c] *s*
O-CH2-O	101.1	6.12 *s*

Analysis conditions were 100 (^{13}C; 30°C) and 400 (^{1}H; 40°C) MHz, in DMSO
[a–c]Assignment may be interchanged
[d]Signal could not be determined

that the crude EtOH extract of *T. asiatica Lam.* showed strong selective cytotoxicity to human lung adenocarcinoma (A549) cells with low toxic effect to human fetal lung fibroblast (WI-38) cells (Fig. 1). The active material of tumor selective cytotoxicity was purified from this crude extract as described in the Materials and methods section.

Identification of chemical structure

The purified active material (5 mg) was dissolved in DMSO-d6 (500 µl) and applied to the NMR spectrometer. ^{1}H and ^{13}C chemical shifts are shown in Table 1. The interpretation of these spectral data identified this active material as dihydronitidine (12,13-dihydro-2,3-dimethoxy-12-methyl-(1,3)Benzodioxolo(5,6-c)phenanthridine). Analysis of the molecular weight by EI-MS gave an $[M]^+$ of 348 as shown in Fig. 2. A comparison of the MS spectrum of this compound with the MS-spectrum library (NIST library) also supported the above identification (Fig. 2).

The fluorescence emission and excitation spectra of dihydronitidine were, respectively, taken with an excitation wavelength at 398 nm and with an emission wavelength at 507 nm. The maximum emission and excitation wavelengths were 507 and 398 nm, respectively (data not shown).

Cytotoxicity of dihydronitidine to various cell lines

The D_{50} of dihydronitidine for various cultured cell lines is listed in Table 2. Cell lines that originated from human normal tissues were less susceptible to dihydronitidine compared to tumor cell lines. Of the cancer cells, only VMRC-LCP cells were rather resistant to dihydronitidine.

In the next analysis, we used CPT as a comparative control of antitumor material. Although CPT has heavy side effects in a clinical trial, this has a potent antitumor activity due to the inhibition of DNA topoisomerase I

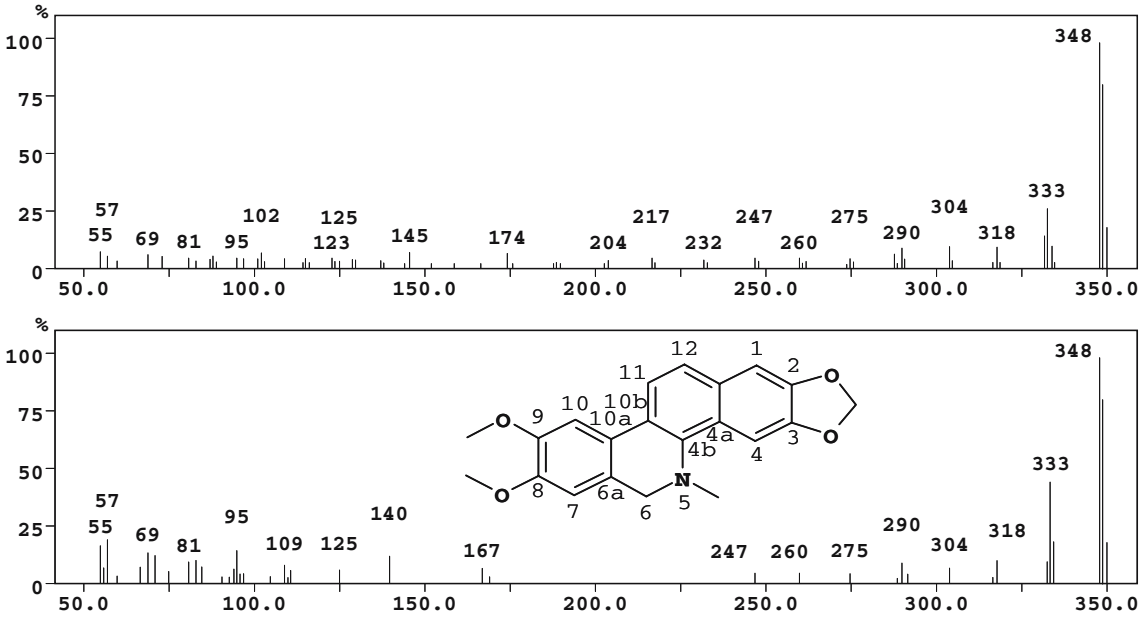

Fig. 2 Mass spectra of the compound extracted from *T. asiatica Lam.* and dihydronitidine. **a** Mass spectrum of the compound isolated from *T. asiatica Lam.* **b** Fitted NIST library mass spectrum. The NIST library matching factors, probability and chemical structure of identification are included

Table 2 Cytotoxicity of dihydronitidine to various cell lines

Origin	Cell line	D_{50}(μg/ml)
Normal	WI-38 (lung normal diploid fibroblast)	55.11
	OUMS-36 (normal human embryo fibroblast cell)	41.13
	OUMS-36T-2F (normal human embryo fibroblast cell (hTRT gene))	56.15
Tumor	A549 (lung adenocarcinoma cell line)	0.19
	VMRC-LCP (lung squamous cell carcinoma)	38.52
	COLO-201 (colon adenocarcinoma)	3.21
	MIA-PaCa2 (pancreatic cancer)	2.38
	A431 (epidermoid carcinoma)	1.95
	KATOIII (stomach cancer)	4.60
	SKBR-3 (breast cancer)	3.31

activity. It was reported that nitidine, the analogue of dihydronitidine, also has the same activity. To gain more insight into the action manner of dihydronitidine, cancerous, and normal lung cells were treated with dihydronitidine (Fig. 3a, c) or CPT (Fig. 3b, d) for 24 and 48 h, respectively. These results showed that the CPT was more toxic to lung squamous cell carcinoma (VMRC-LCP) in short incubation time (24 h) with a relatively lower cytotoxicity to normal (WI-38) cells (Fig. 3b). However, treatment with CPT for 48 h significantly damaged both the normal (WI-38) and adenocarcinoma (A549) cells (Fig. 3d), compatible with the serious side effect of this compound. In contrast to CPT, dihydronitidine has little toxic effect on WI-38 cells even after a treatment of 48 h (Fig. 3b). These data also showed a different feature in the selective cytotoxicity of each agent. Furthermore, CPT was more toxic to VMRC-LCP cells than to A549 cells in the 24 h treatment, while dihydronitidine only affected A549 cells in both treatment times (Fig. 3a, b). This result implied that the mechanism of action of dihydronitidine differed from that of CPT.

FACS analysis

To determine whether apoptosis or necrosis was involved in the cytotoxicity of dihydronitidine, A549 and WI-38 cells were stained with Annexin V and PI after 24 and 48 h of dihydronitidine treatment, and analyzed by flow cytometry. Treatment with dihydronitidine for 24 h increased the early apoptotic cell population (annexin V+/PI−, 34.4%) (Fig. 4a) compared with the negative control (6.2%) (Fig. 4c). The late apoptotic cells were markedly increased by 48 h of treatment (annexin V+/PI+, 30.6%) (Fig. 4b). The total percentage of apoptotic cells was 54.0% for 48 h of treatment. Thus, dihydronitidine induced the apoptotic cell death of A549 cells in a time-dependent manner. Dihydronitidine treatment, however, caused no increase in the apoptotic population of normal WI-38 cells. Extension of treatment time to 48 h also had no effect on the population of early apoptotic cells (Fig. 4d–f).

Caspase-3 activity

To further gain an insight into the apoptosis inducing mechanism of dihydronitidine, we next monitored the caspase-3 activities in the lysate of A549 and WI-38 cells.

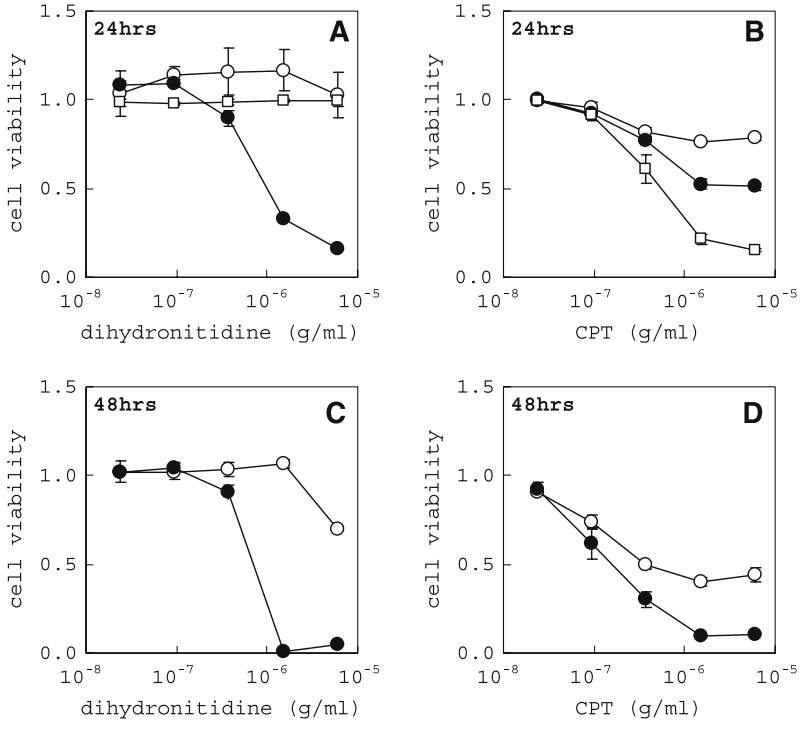

Fig. 3 Cytotoxicity of dihydronitidine and CPT. WI-38 (*open circle*), A549 (*filled circle*), and VMRC-LCP (*open square*) cells were treated with dihydronitidine (**a**, **c**) or CPT (**b**, **d**) for 24 (**a**, **b**) or 48 h (**c**, **d**). Their viabilities were determined by the cell titer kit, and expressed as the viability ratio of treated to untreated cells

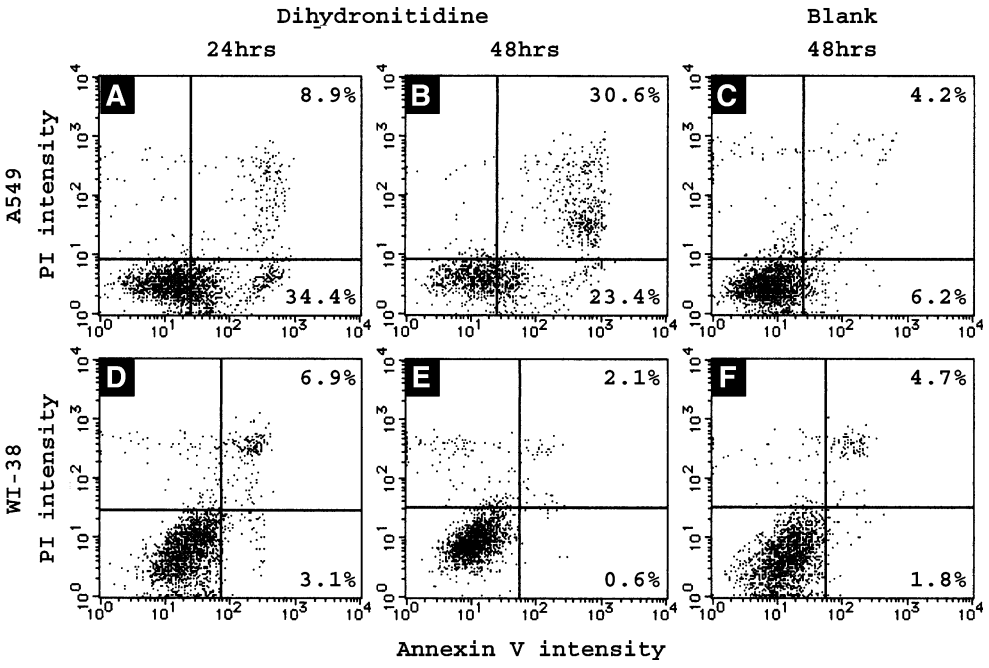

Fig. 4 Flow cytometric analysis of apoptosis in A549 and WI-38 cells after treatment of PBS and dihydronitidine. A549 cells (**a**–**c**) or WI-38 cells (**d**–**f**) (1×10^5/ml) were treated with either 480 ng/ml of dihydronitidine or PBS. **a**, **d** With 480 ng/ml of dihydronitidine for 24 h. **b**, **e** With 480 ng/ml of dihydronitidine for 48 h. **c**, **f**, With PBS as a negative control for 48 h. Cells were subsequently stained with Annexin V-FITC (*x*-axis) and PI (*y*-axis). Fifty thousand cells were then analyzed using a FACS scan to determine the percentage of Annexin V/PI = positive/negative (early apoptosis) and Annexin V/PI = positive/positive cells (late apoptosis)

Addition of dihydronitidine to the culture medium specifically lowered the viability of cancer cells (Fig. 5a), and simultaneously increased the caspase-3 activity only in the lysate of cancer cells (Fig. 5b), in a concentration-dependent manner. The caspase activity of A549 cells at 500 ng/ml of dihydronitidine was about threefold that of untreated cells. Dihydronitidine caused no significant change in both viability and caspase-3 activity of WI-38 cells (Fig. 5a, b).

Gene expression analysis

To obtain a clue to the mechanisms for tumor selective cell death, we performed the real-time PCR of 37 genes involved in the signal transduction pathway leading to cell proliferation or cell death: apoptosis and necrosis. With respect to A549 cells, 6 of the 37 genes listed in Fig. 6 showed the statistically significant changes in response to dihydronitidine. However, dihydronitidine induced no changes in the expression of these genes in normal WI-38 cells (data not shown). As shown in Fig. 6, dihydronitidine decreased the expression of CDK2, and increased the mRNA levels of proliferating cell nuclear antigen (PCNA), Fas, Bax, p53, and Mdm2.

Morphological observations

As revealed in the foregoing section, dihydronitidine is a fluorescent material and emits blue fluorescence at 507 nm. We observed dihydronitidine or CPT treated

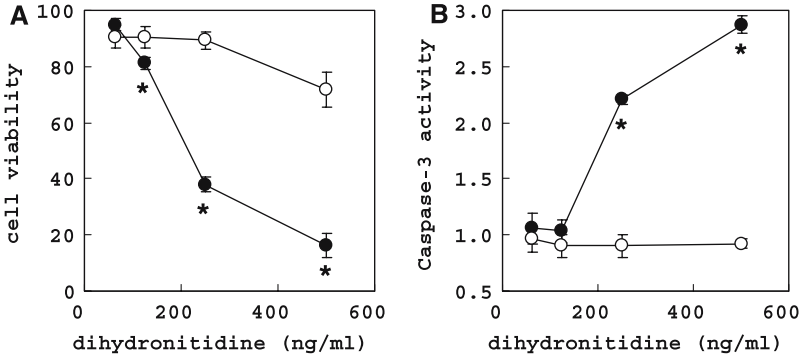

Fig. 5 Effect of dihydronitidine on caspase-3 activities in A549 and WI-38 cells. Cells (1×10^4/ml) were treated with 500, 250, 125, 63 ng/ml of dihydronitidine for 24 h. After incubation, cell viabilities (**a**) and caspase-3 activities were assayed (**b**). Each value was expressed as the ratio of treated to untreated negative-control cells. *Y-axis* indicates -fold increase over vehicle control. $^*P<0.05$ versus the WI-38 cells

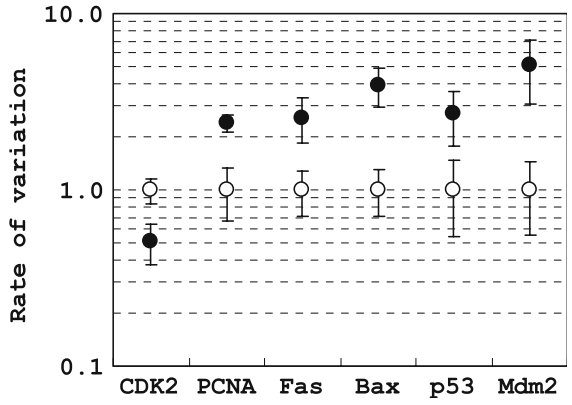

Fig. 6 Effect of dihydronitidine on the expression levels of various genes. Values represent the relative changes in gene expression against the untreated control in A549 cells. The data were indicated as the rate of variation. *Graph* shows averages of the triplicate analyses with a 95% confidence interval of untreated cells (*open circle*) and treated cells (*closed circle*)

A549, VMRC-LCP, WI-38, and OUMS-36T-2F cells with a fluorescence microscope (Fig. 7a). As seen in the figures, a strong fluorescence due to dihydronitidine was only seen in the A549 cells, but not in the other cells. This fluorescence was not observed with all CPT treated cells, even with the CPT susceptible cells of VMRC-LCP. The magnified image of A549 cells accumulating dihydronitidine gave additional information on the intracellular distribution of dihydronitidine (Fig. 7b). The fluorescence in A549 cells was specifically localized within a small particle organelle of cells. This result showed that dihydronitidine accumulated in the extra-nuclear compartment.

Discussion

Our current study conducted the screening of a cancer specific chemotherapeutic agent from subtropical bioresources such as food materials and plant medicines. This view is very important to develop a chemotherapeutic agent of no side effect. This work found that the *T. asiatica Lam.*, commonly used as a herbal tea in the Okinawa islands, contained a compound of effective tumor selective cytotoxicity against lung disease. This study identified the effective component as alkaloid dihydronitidine classified into benzophenanthridine.

Plant-derived alkaloids currently in clinical use include the antitumor agents CPT, taxol, vincristine, and vinblastine [19]. These alkaloids exert antitumor activity through the inhibition of DNA topoisomerase I (CPT), microtubule stabilization (taxol), microtubule interference (vincristine), or depolymerization of microtubule (vinblastine) [20–23]. Although no report on the biological activity of dihydronitidine has been available, it has been reported that nitidine- or dihydronitidine-analogues classified into benzophenanthridine also exert their antitumor activity through the inhibition of topoisomerase I [24–26]. For this reason, we considered CPT to be the most appropriate reference antitumor agent in this study. CPT is a naturally occurring alkaloid isolated from plant extracts [27, 28]. Although these are widely used for the therapy of various cancers, strong side effects have also been reported [29, 30]. Dihydronitidine showed selective cytotoxicity to lung adenocarcinoma (A549) cells but not to normal lung (WI-38) cells (Fig. 3). This selectivity was not seen with the CPT

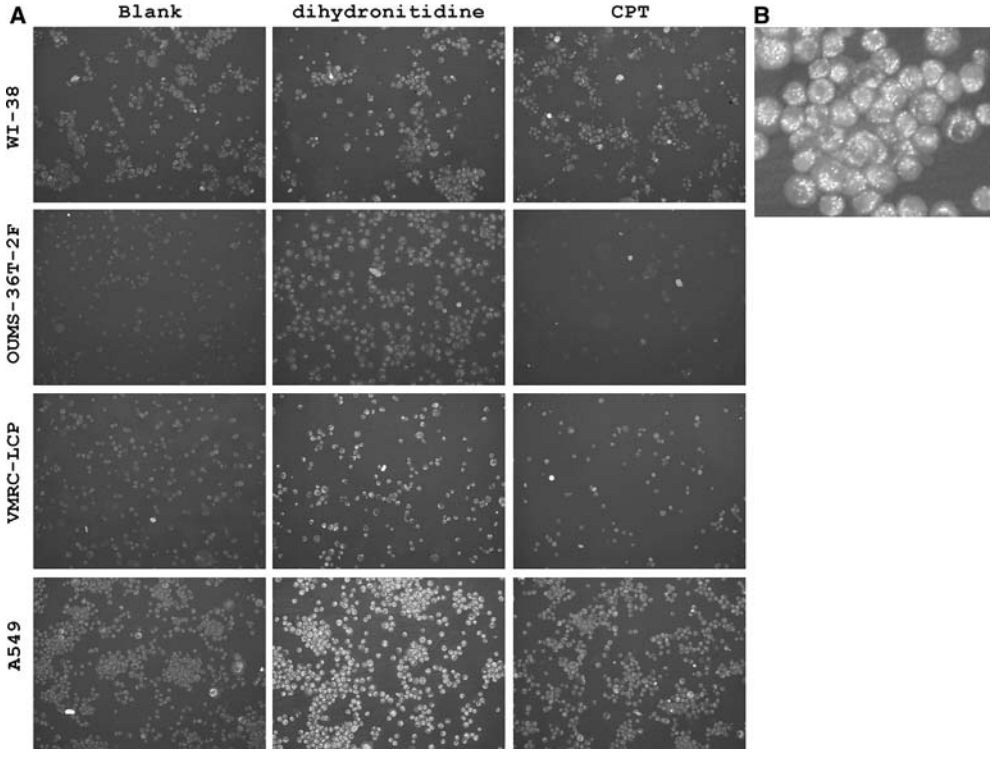

Fig. 7 Fluorescence microscopic images of dihydronitidine or CPT treated cells. Cells were treated with 250 ng/ml of dihydronitidine, or CPT for 24 h. Each plate was observed under UV illumination. **a** Fluorescence image of each observation (×100). **b** Enlarged image of A549 cells treated with dihydronitidine

treatment. CPT treatment for 24 h shows strong cytotoxicity to the VMRC-LCP cells rather than against the A549 cells.

Treatment with CPT for 48 h inhibited the growth of A549 and WI-38 cells similarly. This may explain the side effect of CPT chemotherapy in clinical cure. If the mechanism of action of dihydronitidine is similar to that of CPT, the responses of the cells to this compound should be comparable to those observed with CPT. However, Fig. 3 showed the difference between these two compounds, and suggested that the mechanism of actions of dihydronitidine differed from that of CPT.

This A549 cell specific cytotoxicity was shown to be the caspase-3-dependent apoptosis (Figs. 4, 5). Several signal transduction systems and genes have been shown to be involved in the apoptotic death pathway. We verified whether the expression of the gene related with cell proliferation or the death pathway was modulated by dihydronitidine (Fig. 6). Of the genes involved in the regulation of cell cycling, the expression of CDK2 was decreased by dihydronitidine treatment. CDK2 activation requires the association with cyclin E (CCNE). The CCNE expression level also showed a decreasing tendency (data not shown). These genes were closely related to the G1/S transition of the mitotic cell cycle [30]. Decrease in the expression of these genes suggested that the cell cycle progression was inhibited in A549 cells. However, PCNA was increased inversely. PCNA acts as a processivity factor for DNA polymerase, which is involved directly in DNA synthesis, and the PCNA level is correlated with the proliferative state of cells [31]. These controversial changes in the mRNA expression remain unexplained.

Marked increases in Fas, Bax, and p53 gene expression were noted only in A549 cells. The Fas/FasL system is a key signaling transduction pathway of apoptosis [28, 29]. Up-regulation of the Fas expression has been demonstrated to induce apoptosis in hydrogen peroxide treated A549 cells [30]. Therefore, the apoptotic cell death by dihydronitidine (Figs. 4, 5) may be explained in part by the specific up-regulation of the Fas gene in A549 cells. In addition, because Bax is a primary target of the p53 gene and is involved in a p53-regulated pathway for the induction of apoptosis [31, 32], the increase of the Bax gene expression reasonably explained the apoptotic cell death of A549 cells. Mdm2 is a p53 targeting gene that acts in a negative feedback loop to inhibit p53 by blocking the access of transcription cofactors to the p53 transactivation domain and by inducing p53 degradation [33–35]. Another tumor suppressor protein, p19, also showed an increasing tendency (data not shown). This gene binds and renders Mdm2 inactive in the regulation of the p53 protein by translocating Mdm2 into the nucleolus [33,36] and also by inhibition of the ubiquitin ligase activity of Mdm2 [37]. More definitive experiments such as a proteomic analysis may be needed before the exact pathway in cell death signaling is known.

An important finding on the mechanism of action of dihydronitidine was obtained by fluorescence microscopy. Dihydronitidine specifically accumulated in the cytosolic compartment of only A549 cells (Fig. 7). Thus, the difference in the mechanism of action between dihydronitidine and CPT may lie in their intracellular concentrations. This observation suggested that dihydronitidine exerts its cytotoxicity by accumulation in a particular cytosolic organelle, suggesting that the primary target of dihydronitidine differed from that of CPT. The intracellular concentration of dihydronitidine increased to the saturation level after 2 h of treatment. These results suggested that the increase in the intracellular dihydronitidine concentration precedes the up-regulation of the death receptor or p53 signaling. Therefore, we postulated that cell death was elicited by accumulation of the dihydronitidine into the intracellular organelle. Although the mechanisms for the cancer cell specific accumulation of dihydronitidine has not been clarified, this feature may be beneficial for the application to chemotherapeutic agents of low side effect. Although there still remain many questions to be answered, the selectivity of dihydronitidine may become a potential to be exploited for various applications.

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