

Inotodiol, a Lanostane Triterpenoid, from *Inonotus obliquus* Inhibits Cell Proliferation through Caspase-3-dependent Apoptosis

MASAAKI NOMURA¹, TATSUO TAKAHASHI¹, AIMI UESUGI¹, REIKO TANAKA² and SHINJIRO KOBAYASHI^{1,3}

¹Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences and

³Organization for Frontier Research in Preventive Pharmaceutical Sciences, Hokuriku University, Kanazawa, Ishikawa 920-1181;

²Department of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences, Takatsuki, Osaka 569-1094, Japan

Abstract. Background: To investigate the antitumor effect of *Inonotus obliquus* Pilat, the antiproliferative effect of lanostane triterpenoids from a chloroform extract of *I. obliquus* sclerotia against mouse leukemia P388 cells was assessed. Materials and Methods: Cell viability was measured by MTT assay. Caspase-3/7 activity and DNA fragmentation were evaluated to analyze apoptosis induction. The *in vivo* antitumor effect was evaluated by the number of survival days of mouse leukemia P388-bearing female CDF₁ mice. Results: The chloroform extract of *I. obliquus* sclerotia inhibited proliferation of the P388 cells. Among the triterpenoids examined, only inotodiol inhibited P388 cell proliferation. DNA fragmentation and caspase-3/7 activation were observed in the P388 cells treated with inotodiol (30 μ M). A caspase-3 inhibitor, DEVD-CHO (*N*-acetyl-Asp-Glu-Val-Asp-al, 100 μ M) partially inhibited the DNA fragmentation and growth-inhibition induced by inotodiol. The intraperitoneal administration of 10 mg/kg inotodiol prolonged the number of survival days of the P388-bearing

mice. Conclusion: Inotodiol inhibits cell proliferation through apoptosis induction by activating caspase-3.

Inonotus obliquus (Pers.: Fr.) Pilat [*Fuscoporia obliqua* (Pers.: Fr.) Aoshima] (*I. obliquus*) is a white-rot fungus belonging to the family *Hymenochaetaceae* Donk, and is widely distributed over Europe, Asia, and North America. In Japan, it is distributed in the *Betula platyphylla* var. *japonica* forests of Hokkaido. Since the 16th century, the sclerotia of this fungus, called 'Chaga', has been used as a folk medicine for cancer, digestive system diseases and tuberculosis in Russia and western Siberia (1, 2). Mizuno *et al.* (3) reported that polysaccharides isolated from sclerotia of *I. obliquus* have an anticancer effect. Polysaccharides, such as krestin and lentinan, have been used in clinical cancer therapies (4), and it is likely that the anticancer effect of Chaga involves an immunomodulating effect. On the other hand, extracts of *I. obliquus* have been identified to have various biological effects, including antioxidant (5, 6), antiviral (7), antifungal (8), anti-inflammatory (9), anticarcinogenic (10) and anticancer effects (3, 11-14). Extracts of *I. obliquus* directly inhibited *in vitro* proliferation of cancer cells (11-13), and triterpenoids from the extracts showed significant anti-proliferative effects (12). The triterpenoids also have anti-fungal and antitumor promotion effects (8, 10). These findings suggested that extracts of *I. obliquus*, including the triterpenoids, also show anticancer effects through direct growthinhibition of cancer cells. However, the mechanisms of the antiproliferative effect of *I. obliquus* extracts have not been well documented.

In this study, the antiproliferative effect of a chloroform extract of *I. obliquus* sclerotia against mouse leukemia P388 cells was assessed. The antiproliferative effect and mechanism of action of the lanostane triterpenoids, inotodiol, tramentenolic acid, lanosterol and 3 β -hydroxylanosta-8,24-dien-21-al against P388 cells were also investigated.

Abbreviations: DEVE-CHO, *N*-acetyl-Asp-Glu-Val-Asp-al; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IC₅₀, 50% growth-inhibitory concentration; *I. obliquus*, *Inonotus obliquus* (Pers.: Fr.) Pilat [*Fuscoporia obliqua* (Pers.: Fr.) Aoshima]; inotodiol, lanosta-8,24-diene-3 β ,22R-diol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; % ILS, percentage increase in life-span; tramentenolic acid, 3 β -hydroxylanosta-8,24-dien-21-oic acid.

Correspondence to: Shinjiro Kobayashi, Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Ishikawa 920-1181, Japan. Tel: +81 76 2291165, Fax: +81 762292781, e-mail: s-kobayashi@hokuriku-u.ac.jp

Key Words: Inotodiol, lanostane triterpenoids, cell proliferation, apoptosis, caspase-3.

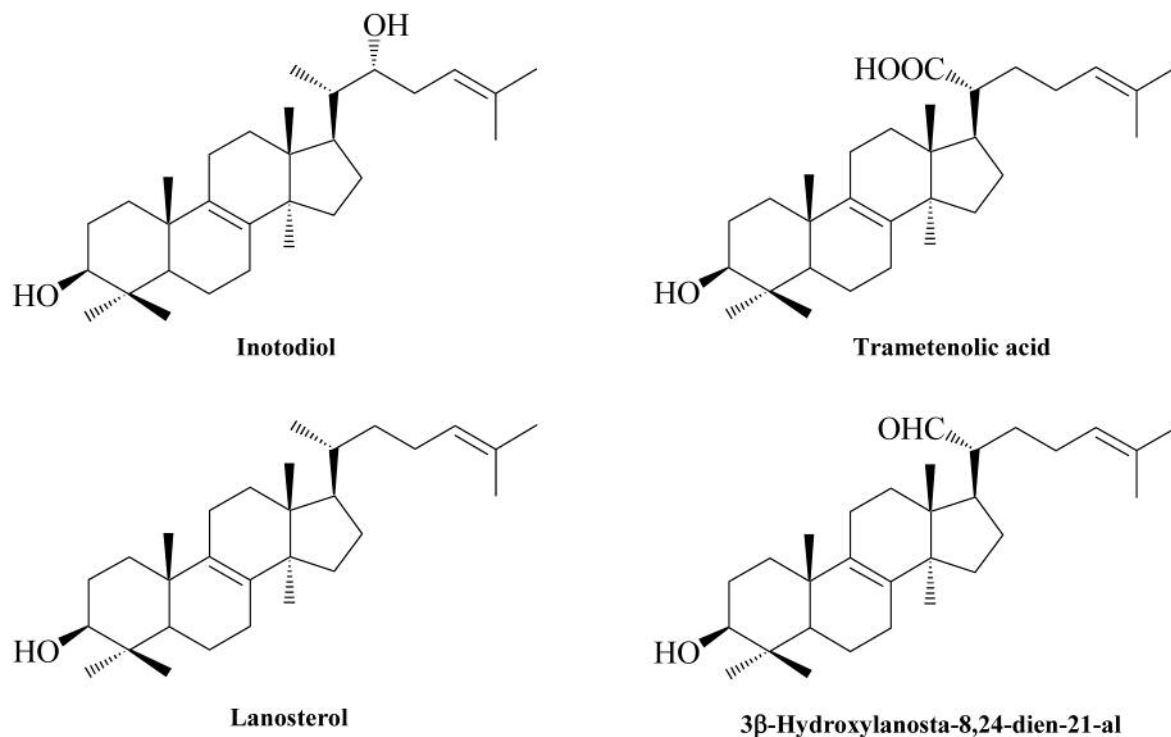


Figure 1. Chemical structures of lanostane triterpenoids from the chloroform extract of *I. obliquus*.

Materials and Methods

Materials. Ribonuclease A, proteinase K, ethidium bromide, *N*-acetyl-Asp-Glu-Val-Asp-al (DEVD-CHO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). The Caspase-Glo® 3/7 Assay was obtained from Promega Corp. (Madison, WI, USA).

Cultured *Inonotus obliquus* (pers: Fr.) Pilat (*I. obliquus*) was obtained from Salada Melon Co. Ltd., Nayoro, Hokkaido, Japan. The sclerotia (4 kg) of *I. obliquus* were extracted with chloroform (10 l) in an automatic percolator for 7 days at 50°C. The chloroform solution was evaporated under reduced pressure, and a brown residue (chloroform extract) was obtained. Preliminary column chromatography of the chloroform extract (150.0 g) of the sclerotia of *I. obliquus* and the isolation of triterpenoid constituents, inotodiol (lanosta-8,24-diene-3β,22R-diol; 25.2 g), trametenolic acid (3β-hydroxylanosta-8,24-diene-21-oic acid; 11.8 g) and lanosterol (2.2 g), has already been reported (10). Rechromatography of the filtrate (17.1 g) of inotodiol (Residue C) on MPLC (230-400 mesh silica gel, 500 g) using *n*-hexane/EtOAc (5:1) yielded inonotsuoxides A and B from fraction Nos. 28-42 (10). Elution was continued with the same solvent to yield a crystalline solid (3.2 g, fraction Nos. 50-59), which was recrystallized from MeOH/chloroform to yield 3β-hydroxylanosta-8,24-dien-21-al (2.4 g). The chemical structures of these triterpenoid constituents are shown in Figure 1. The physical and spectral data of the constituents showed good agreement with published data, and the constituents had over 99.5% purity. The chloroform extract and triterpenoids were each dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the test cell culture medium was below 0.2%.

Cell culture and in vitro growth-inhibitory assay. Mouse leukemia P388 cells were kindly supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. The cells were passaged weekly through intraperitoneal transplantation in female CDF₁ mice (Nippon SLC, Hamamatsu, Japan). The cells were suspended in RPMI-1640 (Nakalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS), 20 μM 2-mercaptoethanol and 100 μg/ml kanamycin at a density of 2×10³ cells/0.1 ml and cultured with or without the chloroform extract or a triterpenoid in the presence or absence of a caspase-3 inhibitor (DEVD-CHO) at 37°C for 72 h in a CO₂ incubator. The viability of the cells was measured by means of MTT assay, and the 50% growth-inhibitory concentration (IC₅₀) was calculated.

DNA fragmentation. The cells (5×10⁵) were cultured in RPMI-1640 with 10% FBS, 20 μM 2-mercaptoethanol and 100 μg/ml kanamycin in the presence or absence of a triterpenoid (30 μM) and/or caspase-3 inhibitor (DEVD-CHO; 100 μM) at 37°C for 12 h or 48 h in a CO₂ incubator. The cells were lysed with 0.1 ml lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM EDTA, 0.5% Triton X-100) at 4°C for 10 min. The supernatant fraction was collected by centrifugation at 12,000 g for 20 min. Ribonuclease A (20 μg) was added and the solution was incubated at 37°C for 1 h, then further incubated with proteinase K (20 μg) at 37°C for 30 min. After centrifugation at 12,000 g for 5 min, the supernatant (15 μl) was electrophoresed on 2% agarose gel in TBE buffer (100 mM Tris-HCl, pH 8.0; 100 mM boric acid and 1.5 mM EDTA). The agarose gel was stained with ethidium

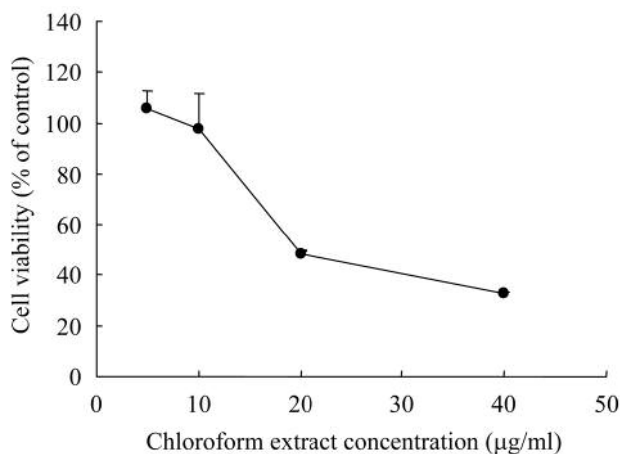


Figure 2. Antiproliferative effect of the chloroform extract from *I. obliquus sclerotia* against mouse leukemia P388 cells. Cells (2×10^3 cells/well) were seeded into a 96-well plate and cultured with different concentrations of the chloroform extract for 72 h in a CO_2 incubator. The cell viability was evaluated by MTT assay. Each point represents the mean \pm SE ($n=12$).

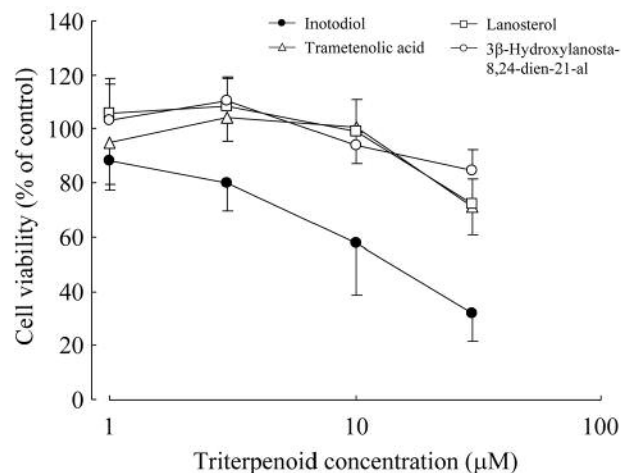


Figure 3. Antiproliferative effects of lanostane triterpenoids against P388 cells. Cells (2×10^3 cells/well) were seeded into a 96-well plate and cultured with different concentrations of each triterpenoid for 72 h in a CO_2 incubator. The cell viability was evaluated by MTT assay. Each point represents the mean \pm SE ($n=12$).

bromide, and DNA fragmentation was detected using a Typhoon 9410 imaging analyzer (GE Healthcare Biosciences, Little Chalfont, Buckinghamshire, UK). DNA fragmentation indicates apoptotic forms in a laddering pattern consisting of segments differing by around 200 bp (15).

Caspase-3/7 activity. The caspase-3/7 activity in the P388 cells was assessed by using a Caspase-Glo[®] 3/7 assay kit according to the manufacturer's instructions. Briefly, the cells (2×10^3 cells/0.1 ml) were cultured in RPMI-1640 with 10% FBS, 20 μ M 2-mercaptoethanol and 100 μ g/ml kanamycin in the presence or absence of a triterpenoid (30 μ M) at 37°C for 12 h or 48 h in a CO_2 incubator. An equal volume of Caspase-Glo[®] 3/7 reagent was added and the culture was further incubated for 3 h at 37°C in a CO_2 incubator. The luminescence was measured using a FLUOstar OPTIMA multiple microplate reader (BMG Labtech Japan Ltd., Saitama, Japan).

In vivo effect. All the animal experiments were performed in accordance with the guidelines of the Committee on Animal Experiments in Hokuriku University. Female CDF₁ mice (nine in a group) were intraperitoneally inoculated with P388 cells (1×10^6), and inotodiol was intraperitoneally administered at doses of 3 and 10 mg/kg in saline at days 1 and 4 after the tumor cell inoculation. The number of survival days of the mice were recorded, and the percentage increase in lifespan (% ILS) was calculated from the following equation: % ILS = $[(T-C)/C] \times 100$, where T and C represent the mean survival days of the treated group and the mean survival days of the vehicle control group, respectively.

Statistical analysis. All the data were expressed as mean \pm SE. Two means were compared by an unpaired Student's *t*-test.

Results

Antiproliferative effects of chloroform extract and triterpenoid constituents of *I. obliquus sclerotia*. The chloroform extract inhibited P388 cell proliferation in a concentration-dependent manner (Figure 2). After exposure to the 20 μ g/ml of the chloroform extract for 72 h, the cell viability was reduced by about 50% as compared with the control. Among the triterpenoids examined, only inotodiol had an antiproliferative effect on the P388 cells at concentrations up to 30 μ M, and the IC₅₀ was 13.9 μ M (Figure 3).

Induction of apoptosis by inotodiol in P388 cells. As shown in Figure 4, DNA fragmentation was observed in the P388 cells treated with 30 μ M inotodiol for 12 h or 48 h. In addition, caspase-3/7 activity, which plays a key role in apoptosis, also significantly increased in the P388 cells treated with 30 μ M inotodiol for 12 h or 48 h (Figure 5).

Caspase-3 activation in the antiproliferative effect of inotodiol. The specific caspase-3 inhibitor DEVD-CHO (100 μ M) partially inhibited the DNA fragmentation in the P388 cells treated with 30 μ M inotodiol for 12 h (Figure 6). Furthermore, the reduction of cell proliferation by inotodiol was inhibited by about 30% and 35% by DEVD-CHO (100 μ M) at 12 h and 72 h, respectively (Figure 7).

Antitumor effect of inotodiol in P388-bearing mice. When inotodiol was administered *in vivo* at 10 mg/kg, the number

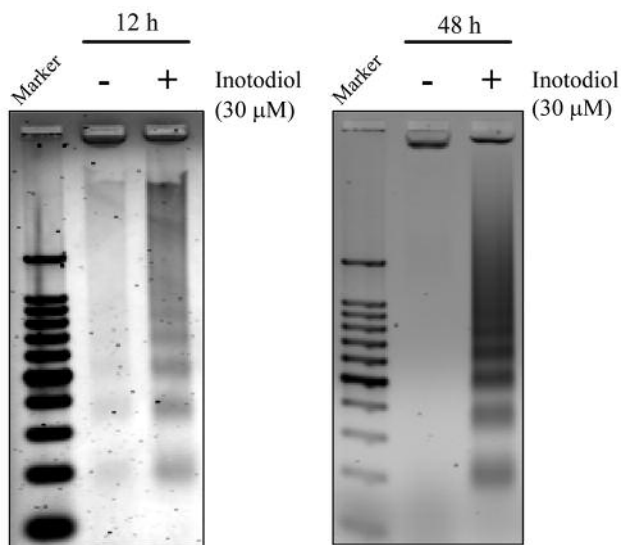


Figure 4. DNA fragmentation in P388 cells treated with inotodiol. The DNA fragment samples were extracted from P388 cells treated with inotodiol (30 μ M) for 12 h or 48 h. The samples were then electrophoresed on a 2% agarose gel and stained with ethidium bromide. Marker: 100 bp DNA ladder.

of survival days (ILS: 20.8%) of the P388-bearing mice was significantly prolonged (Table I), without marked side-effects, such as body weight loss and diarrhea, in the tumor-bearing mice.

Discussion

In this study, a chloroform extract from *I. oblituus* sclerotia inhibited proliferation of mouse leukemia P388 cells at a concentration of 20-40 μ g/ml. The antiproliferative effect was much stronger than that of aqueous extracts of *I. oblituus* sclerotia in human hepatoma cells (11) and human cervical cancer cells (13). Our preliminary data also indicated that the chloroform extract had an antiproliferative effect on human cervical cancer HeLa cells at the same concentration as for the P388 cells. Nakata *et al.* (10) have shown that lanostane triterpenoids, such as inotodiol and trametenolic acid, are the main constituents of the chloroform extract. These triterpenoids had significant antiproliferative effects against Walker 256 carcinosarcoma cells and human mammary adenocarcinoma MCF-7 cells (12). It is likely that the antiproliferative effect of the chloroform extract is due to the triterpenoids. Among the triterpenoids examined in the present study, inotodiol had an antiproliferative effect at a concentration of 30 μ g/ml. Inotodiol (mw: 442.7) comprises about 26% (25.2 g) of the residues (96.3 g) eluted with various eluents from the chloroform extract (150 g) by the column chromatographic method (10).

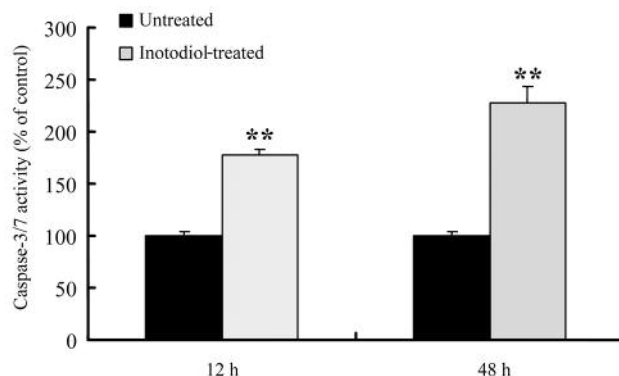


Figure 5. Caspase-3/7 activity in P388 cells treated with inotodiol. Cells were treated with inotodiol (30 μ M) for 12 h or 48 h. Caspase-3/7 activity was then measured by measuring luminescence generated by cleavage of a proluminescent substrate containing the DEVD sequence. Data represents the mean \pm SE (n=4). **Significantly different from the untreated control at $p < 0.01$.

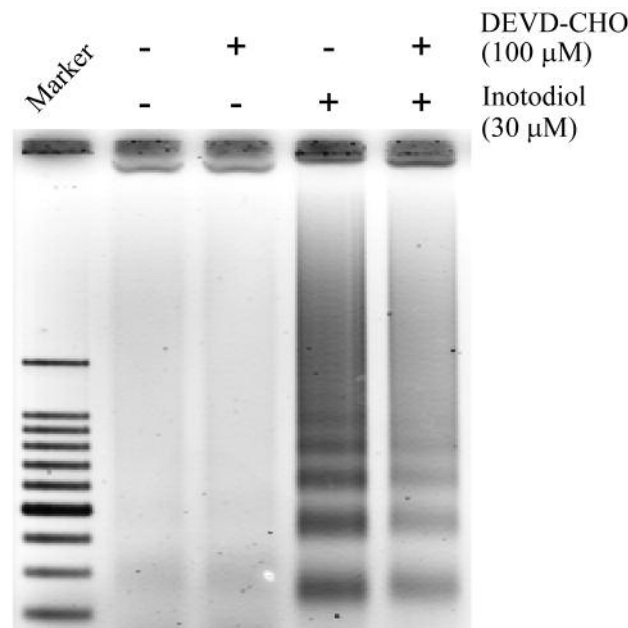


Figure 6. Effect of caspase-3 inhibitor on inotodiol-induced DNA fragmentation in P388 cells. The DNA fragment samples were extracted from P388 cells treated with inotodiol (30 μ M) in the presence or absence of a caspase-3 inhibitor, DEVD-CHO (100 μ M) for 12 h. The samples were then electrophoresed on a 2% agarose gel and stained with ethidium bromide. Marker: 100 bp DNA ladder.

The cytotoxic concentration of inotodiol was similar to that of the chloroform extract. It is therefore suggested that the antiproliferative effect of the chloroform extract is mainly dependent on the effect of inotodiol. As shown in Figure 1, inotodiol has an OH-group at position 22 (C-22) of lanosterol. Therefore, the OH-group substitution at

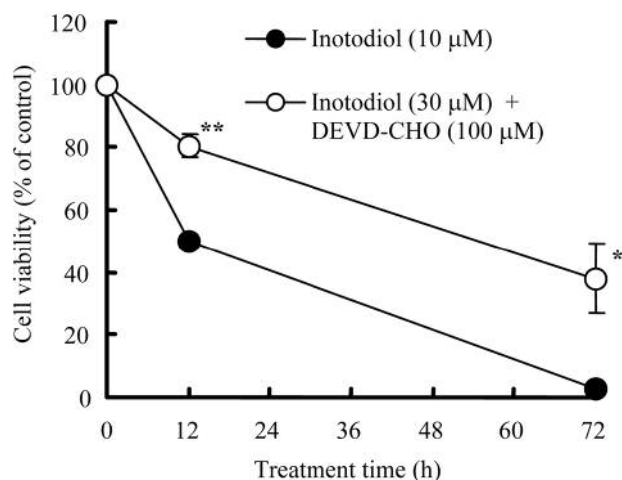


Figure 7. Effect of caspase-3 inhibitor on the reduction of cell proliferation by inotodiol in P388 cells. Cells (2×10^3 cells/well) were seeded into a 96-well plate and cultured with inotodiol (30 μ M) in the presence or absence of a caspase-3 inhibitor, DEVD-CHO (100 μ M), for 12 h or 72 h in a CO₂ incubator. The cell viability was evaluated by MTT assay. Each point represents the mean \pm SE (n=6). *, **Significantly different from the untreated control at $p < 0.05$ and $p < 0.01$, respectively.

C-22 is suggested to be important for the antiproliferative effect of inotodiol in the P388 cells. On the other hand, oxygen substitution at C-21, as in trametenolic acid and 3 β -hydroxylanosta-8,24-dien-21-al, may not increase the anti-proliferative effect of the lanostane triterpenoids. However, Kahlos *et al.* (12) have reported that lanosterol and 3 β -hydroxylanosta-8,24-dien-21-al, as well as inotodiol, inhibited cell proliferation of MCF-7 adenocarcinoma cells. Evaluation of differences in the cell-line dependency of the cytotoxicity of the lanostane triterpenoids may be important for clarifying the antiproliferative and anticancer effects.

As well as the induction of apoptosis, several reports have shown that lanostane triterpenoids inhibited cancer cell proliferation (16, 17). In this study, inotodiol induced DNA fragmentation and caspase-3/7 activation, which are the biochemical hallmarks of apoptosis (15, 18, 19), in the P388 cells. Caspase-3 is activated by any of the initiator caspases (caspase-8, -9 or 10), and is considered to be the most important of the executioner caspases (19). It directly activates the enzymes that are responsible for DNA fragmentation in the intrinsic apoptosis pathway (20, 21). The caspase-3 inhibitor, DEVD-CHO, inhibited the inotodiol-induced DNA fragmentation and reduction of cell proliferation, suggesting that the antiproliferative effect of inotodiol, at least in part, resulted from induction of apoptosis *via* activation of caspase-3. This is the first report to show that the triterpenoids from *I. obliquus* induce apoptosis. However, the mechanisms through which

Table I. Antitumor effect of inotodiol in P388-bearing mice.

	Survival days	% ILS
Control	7.2 \pm 0.4	-
Inotodiol		
3 mg/kg	7.9 \pm 0.1	9.7
10 mg/kg	8.7 \pm 0.5*	20.8

P388 cells were intraperitoneally inoculated into mice (9 mice in a group) on day 0, and inotodiol (3 or 10 mg/kg) was intraperitoneally administered to the mice on day 1 and 4. % ILS: Percentage increase in lifespan of the experimental group over the control group. Data represents mean \pm SE (n=9). *Significantly different from the control group at $p < 0.05$.

inotodiol induces caspase-3 activation and DEVD-CHO-independent apoptosis have not been identified in this study, but are currently under investigation in our laboratory.

Kahlos *et al.* (12) reported that trametenolic acid and 3 β -hydroxylanosta-8,24-dien-21-al did not have any *in vivo* anti-tumor effect against P388-bearing mice. In the present study, trametenolic acid and 3 β -hydroxylanosta-8,24-dien-21-al also did not show *in vitro* antiproliferative effect against P388 cells. Inotodiol exhibited an antiproliferative effect against the P388 cells was selected for *in vivo* testing. The intraperitoneal administration of inotodiol at 10 mg/kg significantly prolonged the number of survival days of P388-bearing mice, without any apparent side-effects. The intraperitoneal administration of 10 mg/kg inotodiol in saline three times a week for 1 month also did not cause any apparent side-effects, including body weight loss and diarrhea, in normal male ddY mice (data not shown).

In the present study, inotodiol, a lanostane triterpenoid, from a chloroform extract of *I. obliquus* sclerotia exhibited an *in vitro* antiproliferative effect through apoptosis induction *via* activation of caspase-3 in mouse leukemia P388 cells. The OH-group substitution at C-22 is suggested to be important for the antiproliferative effect of inotodiol against P388 cells. Inotodiol also had an *in vivo* antitumor effect against P388-bearing mice. Inotodiol may therefore be an interesting compound for the development of a novel anticancer drug.

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