β -Thujaplicin Zinc Chelate Induces Apoptosis in Mouse High Metastatic Melanoma B16BL6 Cells

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The cytotoxic effects of β -thujaplicin and five kinds of metal chelates were examined on mouse melanoma B16BL6 cells by cell viability and lactate dehydrogenase (LDH) release assay. β -Thujaplicin-zinc chelate and β -thujaplicin-copper chelate had higher cytotoxic effects than β -thujaplicin, and the 50% effective doses (ED₅₀) of these metal chelates were 12.5 and 25 μ M, respectively. In addition, the zinc chelate induced DNA ladder formation in B16BL6 cells, as shown by the DNA fragmentation assay, suggesting that cell death induced by the zinc chelate is apoptosis. The zinc chelate also had a cytotoxic effect and induced DNA fragmentation on other tumor cell lines: HeLa, Meth A, and B16F1 cells, but not on normal human diploid fibroblasts FS-4. These results suggest that β -thujaplicin-zinc chelate induces apoptotic cell death in various tumor cell lines and is a potent antitumor agent for tumor cells including malignant melanomas.

Key words β -thujaplicin; β -thujaplicin-zinc chelate; DNA ladder formation; apoptosis; B16BL6 melanoma

β -Thujaplicin (2-hydroxy-4-(1-methylethyl)-2,4,6-cycloheptatrien-1-one) is a tropolone-related compound found in the heartwood of the cupressaceous plants, western red cedar (*Thuja plicata*), eastern white cedar (*Thuja occidentalis*) and hinoki cypress (*Chamaecyparis obtusa*).^{1,2} The compound has been reported to be effective as an antimicrobial agent²⁻⁶ and a plant growth stimulator.⁷ Recent reports have also demonstrated that β -thujaplicin exhibits an inhibitory effect on the growth of a broad range of tumor cell line: mouse lymphoid neoplastic cell line P388,⁸ mouse leukemic cell RL δ -1, mouse hepatic cell MH134, human promyelocytic cell HL-60, human leukemic cell K562 and human stomach cancer cell KATO-III.⁹ β -Thujaplicin has a β -diketone structure in the molecule and forms metal chelates in the presence of various metal ions, such as ferric ions (Fe³⁺), copper ions (Cu²⁺) and zinc ions (Zn²⁺).^{10,11} Akers *et al.*¹² reported that β -thujaplicin serves as a red iron ion chelator for *Salmonella thymurium*, and Yamato *et al.*¹³ proposed the hypothesis that tropolone and its derivatives chelate with iron ions present in the catalytic moiety to inactivate ribonucleotide reductase, an obligatory enzyme in DNA synthesis. Thus, the property of β -thujaplicin as a chelator of metal ions may play an important role in the various biological functions of the compound. However, the cytotoxic effects of the metal chelates on tumor cells have not yet been examined.

In this study, we examined the cytotoxic effects of five kinds of β -thujaplicin metal chelates: zinc chelate, copper chelate, ferrous chelate, ferric chelate and manganese chelate, on mouse high metastatic melanoma B16BL6 and other tumor cells, and found that the zinc chelate was strongly cytotoxic to several tumor cells. Furthermore, this chelate induced apoptotic cell death in the tumor cells, suggesting that this compound would be a more potent antitumor reagent than β -thujaplicin.

MATERIALS AND METHODS

Reagents Natural β -thujaplicin was generously provided

by Takasago International Co., Ltd. (Tokyo, Japan). All the β -thujaplicin metal chelates: those for zinc, copper, ferrous, ferric and manganese were prepared as follows. Briefly, β -thujaplicin was dissolved in methanol at a concentration of 150 mM, and then each metal salt was added at a final concentration of 80 mM, respectively. After mixing for 5 h followed by paper filtration, the mixtures were washed three times with Milli Q water to eliminate free metal ions, and dried under vacuum. β -Thujaplicin and the metal chelates were dissolved in dimethyl sulfoxide (DMSO) (Sigma) at 40 mM, and stocked in the dark at -40 °C until used. Final concentrations (0.0025—0.05%) of DMSO did not influence cell growth or viability of any of the cells used in this study.

Cell Culture Mouse melanoma cell lines B16BL6 and B16F1 were maintained in Dulbecco's modified eagle medium (DMEM)/HAM's F12 medium (pH 7.2) containing 60 mg/l of kanamycin sulfate and 10% (v/v) fetal calf serum (FCS) at 37 °C in a 5% CO₂ atmosphere. Human cervix epithelial-like cell line HeLa, mouse sarcoma cell line Meth A, and normal human diploid fibroblast cell FS-4 were maintained in Eagle's minimal essential medium (EMEM) (pH 7.2) containing 60 mg/l of kanamycin sulfate and 10% (v/v) FCS at 37 °C in a 5% CO₂ atmosphere. Human monoblastic cell line U937 and human promyelocytic cell line HL-60 were maintained in RPMI 1640 medium (pH 7.2) containing 60 mg/l of kanamycin sulfate and 10% (v/v) FCS at 37 °C in a 5% CO₂ atmosphere. All cells were used for the experiment at passages 3 to 7.

Lactate Dehydrogenase (LDH) Release Assay Cells (1 $\times$ 10⁴/well) were incubated in 96 well flat-bottom microplates for 1 d at 37 °C in culture medium, and then each sample (0.01—0.2 mM) was added to cultured cells. After incubation for 16 h, the culture supernatants of the treated cells were examined by LDH release assay. The activity of LDH released from tumor cells as a measurement of the cytotoxicity of test samples was determined according to the modified protocol of the colorimetric assay as described previously.¹⁴ The cells were further examined under a light microscope for

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morphological changes and cell viability by trypan blue exclusion, followed by DNA fragmentation assay. The assays were carried out in triplicate, and morphological changes were denoted as follows: —, none; $\pm$, slight morphological changes without cell rounding; +, less than 50% of cells rounded; ++, more than 50% of cells rounded and many cells afloat.

DNA Preparation and Gel Electrophoresis Electrophoresis of total DNA was performed as described previously with slight modifications.¹⁵⁾ Briefly, after the monolayer cells treated with samples were rinsed twice with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 (pH 7.4)), PBS containing 0.05 mM EDTA was added to the dishes and then incubated for 15 min at 37°C. After the incubation, the cells were collected and washed twice more with PBS. Harvested cells were centrifuged, and then 1×10^6 cells were resuspended in 20 μl of lysis buffer [10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% (w/v) sodium lauryl sarkosinate (SDS), 0.5 mg/ml proteinase K] followed by incubation for 1 h at 50°C. After the incubation and phenol/chloroform extraction, RNase A was added to obtain a final concentration of 0.5 mg/ml, and the sample was subsequently incubated at 50°C for 1 h. Thereafter all treated samples were applied and run on a 1.6% agarose gel. After the electrophoresis, the gel was stained with ethidium bromide and photographed.

RESULTS

Cytotoxic Effects of β -Thujaplicin on Tumor Cell Lines

In a preliminary experiment, we examined the cytotoxic effects of β -thujaplicin on several tumor cell lines: human cervix epithelial-like cell line HeLa, mouse sarcoma cell line Meth A, human monoblastic cell line U937, human promyelocytic leukemia cell line HL-60, and mouse high metastatic melanoma cell line B16BL6, by LDH release assay and cell viability. B16BL6 cells were resistant to the cytotoxic effects of β -thujaplicin at a concentration below 200 μM . In addition, various metal salts, including Cu^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+} and Zn^{2+} , at concentrations of 1 to 100 μM , had no toxic effect on B16BL6 cells. On the basis of these results, we selected the cell line to test the cytotoxic effects of the five β -thujaplicin metal chelates.

Cytotoxic Effects of β -Thujaplicin Metal Chelates on Mouse Melanoma B16BL6 Cells We examined the cytotoxic effects of β -thujaplicin and the five metal chelates referred to above on B16BL6 cells by LDH release assay and cell viability. β -Thujaplicin-zinc chelate and β -thujaplicin-copper chelate had a cytotoxic effect on B16BL6 cells, as assessed by LDH release assay (Fig. 1A). Cell viability after the addition of the metal chelates decreased in a dose dependent manner (data not shown). β -Thujaplicin and the ferrous, ferric and manganese chelates showed no cytotoxicity on the cells. The zinc chelate exhibited a cytotoxic effect in a dose dependent manner in a concentration range from 5 to 50 μM , while the copper chelate exhibited cytotoxicity in the concentration range from 5 to 25 μM (Fig. 1B). The ED_{50} of the zinc chelate and the copper chelate was about 25 and 12.5 μM , respectively.

Synergistic Effect of β -Thujaplicin and Zinc Ions on Cytotoxicity to B16BL6 Cells To examine whether the cy-

A.

B.

Fig. 1. Cytotoxic Effects of β -Thujaplicin Metal Chelates on B16BL6 Melanoma Cells

A. Cytolytic effects of five kinds of β -thujaplicin metal chelates on B16BL6 cells. Cells (1×10^4 /well) were cultured in 96 well flat-bottom microplates for 1 d at 37°C in culture medium, and then each sample (5–50 μM) was added to the culture supernatant. After incubation for 16 h, each supernatant was collected and LDH activity was examined. B. Induction by β -thujaplicin zinc chelate and β -thujaplicin copper chelate of cytotoxicity. After treatment with each sample (5–200 μM) for 16 h, the supernatants of the treated cells were collected and the LDH activity was examined. ●, β -thujaplicin zinc chelate; ■, β -thujaplicin copper chelate; ○, zinc sulfate; □, copper sulfate; △, β -thujaplicin. Values are the means of three independent determinations; bars, S.D.

tototoxic effect of β -thujaplicin-zinc chelate is due to the chelate alone, which is formed by chelating of β -thujaplicin with zinc ions, 5 to 200 μM of zinc sulfate (ZnSO_4) solution was added to culture media containing 50 μM of β -thujaplicin. As shown in Fig. 2A, the cytotoxic effect increased in parallel with the concentration of zinc ions and reached a plateau at about 25 μM . This result indicated that the molar ratio of β -thujaplicin to zinc ions is about 2 to 1 at that point where the cytolytic effect reached a plateau, suggesting that the zinc chelate itself has a cytotoxic effect on B16BL6 cells. Furthermore, the zinc chelate showed the effect in a time dependent manner (Fig. 2B). LDH release was observed within 3 h of the addition of the zinc chelate and reached a plateau between 6 to 12 h. We also examined whether the zinc chelate irreversibly induces the cytotoxic effect in B16BL6 cells.

A.

B.

Fig. 2. Induction of Cytotoxicity by β -Thujaplicin Zinc Chelate in B16BL6 Melanoma Cells

A. Synergistic effects of β -thujaplicin and zinc ions on cytotoxicity. B16BL6 cells (1×10^5 /well) were incubated for 16 h in a medium containing β -thujaplicin ($50 \mu\text{M}$) and zinc sulfate (5 – $200 \mu\text{M}$) (●), or in a medium containing zinc sulfate alone (○). After incubation, each culture supernatant was collected and the LDH activity was examined. B. Induction of cytotoxicity after treatment with β -thujaplicin zinc chelate. The cells (1×10^5 /well) were incubated for the indicated time with β -thujaplicin zinc chelate (○). After the incubation, each culture supernatant was collected and the LDH activity was examined. Values are the means of three independent determinations; bars, S.D.

The cells were incubated with the zinc chelate for 1, 2, 3, 6, 8, and 12 h, and cultured in fresh culture medium without the sample up to 16 h. LDH release was observed after treatment with the zinc chelate for more than 3 h (data not shown), indicating that the chelate irreversibly induced the cytotoxic effect on B16BL6 cells.

DNA Ladder Formation in B16BL6 Cells Induced by Treatment with β -Thujaplicin-Zinc Chelate We examined DNA fragmentation in B16BL6 cells induced by the zinc chelate and the copper chelate. As shown in Fig. 3, DNA ladder formation was observed in B16BL6 cells treated with the zinc chelate at concentrations of more than $25 \mu\text{M}$, suggesting that the cell death it induces is apoptosis. The DNA from B16BL6 cells treated with the copper chelate, on the other hand, showed the same pattern as that treated with sodium azide which induced necrosis. This indicates that the zinc chelate might induce apoptosis in B16BL6 cells and the copper chelate might induce necrosis.

Cytotoxic Effects of β -Thujaplicin Zinc Chelate on Various Tumor and Normal Cell Lines The cytotoxic ef-

Fig. 3. Induction of DNA Fragmentation in B16BL6 Cells by β -Thujaplicin Zinc Chelate

B16BL6 cells (1×10^5 /ml) were incubated for 16 h with each sample and examined under a light microscope for morphological change and cell viability by the trypan blue exclusion test. DNAs of the treated cells were prepared as described in Materials and Methods and analyzed by gel electrophoresis. DNAs (1×10^6 cells equivalent) were separated by 1.6% agarose gel electrophoresis followed by staining with ethidium bromide. The morphological changes were denoted as follows: -, none; ±, slight morphological changes without cell rounding; +, less than 50% of cells rounded; ++, more than 50% of cells rounded and many cells afloat. 1, β -thujaplicin zinc chelate ($25 \mu\text{M}$); 2, β -thujaplicin zinc chelate ($50 \mu\text{M}$); 3, β -thujaplicin copper chelate ($25 \mu\text{M}$); 4, β -thujaplicin copper chelate ($50 \mu\text{M}$); 5, β -thujaplicin copper chelate ($100 \mu\text{M}$); 6, β -thujaplicin ($100 \mu\text{M}$); 7, zinc sulfate ($100 \mu\text{M}$); 8, copper sulfate ($100 \mu\text{M}$); 9, medium alone; 10, 0.1% NaN_3 .

fect of the zinc chelate was examined by the LDH release assay on several kinds of tumor and normal cell line: HeLa, Meth A, mouse low metastatic melanoma B16F1, and normal human diploid fibroblasts FS-4. As shown in Fig. 4, no cytotoxic effects of β -thujaplicin on B16F1, Meth A, and FS-4 cells were observed at concentrations below $100 \mu\text{M}$. The zinc chelate had highly cytotoxic effects on HeLa, Meth A, and B16F1 cells at concentrations of more than $25 \mu\text{M}$, respectively, and the DNA fragmentation assay showed that the zinc chelate induced DNA ladder formation in these cell lines when $25 \mu\text{M}$ of the compound was added to each cell culture (data not shown). These results suggest that this chelate has a cytotoxic effect on a broader range of tumor cell lines than β -thujaplicin and induces apoptosis in the tumor cells. The zinc chelate, however, showed no cytotoxicity on normal diploid fibroblasts FS-4 derived from human foreskin.

DISCUSSION

β -Thujaplicin (hinokitiol) is a tropolone-related compound with a β -diketone structure in the molecule,^{1,2)} and has been reported to possess various biological activities, including anti-bacterial,^{2,4,6)} anti-fungal,³⁾ anti-tumor^{8,9)} and antioxidant actions.^{16,17)} The biological effects are thought to be due to action of the compound as a chelator of metal ions.^{8,9,12,13,16–19)} With respect to its anti-tumor activity, Yamato *et al.*⁸⁾ suggested that tropolone and its derivatives including β -thujaplicin may inhibit ribonucleotide reductase, an obligatory enzyme in DNA synthesis, and proposed a hypothesis that β -thujaplicin chelates ferric ions present in the catalytic moiety of the enzyme leading to its inactivation. Recent reports also speculated that the cytotoxic effect of β -thujaplicin on sev-

Fig. 4. Induction of Cytotoxicity by β -Thujaplicin Zinc Chelate in Tumor and Normal Cell Lines

Cells (1×10^4 /well) were cultured in 96 well flat-bottom microplates for 1 d at 37°C in culture medium, and then incubated with the medium containing each sample (5–200 μM) for 16 h. After the incubation, each culture supernatant of the treated cells was collected and the LDH activity was examined. ●, β -thujaplicin zinc chelate; ○, zinc sulfate; △, β -thujaplicin. Values are the means of three independent determinations; bars, S.D.

eral tumor cell lines may be due to the inhibition of DNA synthesis.^{9,16} Thus, the property of β -thujaplicin as a chelator of metal ions may play an important role in the effects of the compound on proliferation and cytotoxicity of tumor cells. However, the cytotoxic effects of the metal chelates themselves on tumor cells have not yet been examined. Therefore, in this study, we prepared five kinds of metal chelates and examined their cytotoxic effect on several tumor cell lines, as compared with that of β -thujaplicin. Among several tumor cell lines tested, B16BL6 cells were resistant to the cytotoxic effect of β -thujaplicin, and β -thujaplicin and the five metal ions had no effect on growth inhibition or morphological change of the cells. On the basis of these results, we selected B16BL6 cells to examine the cytotoxic effects of five kinds of β -thujaplicin metal chelates. The zinc chelate and the copper chelate had a cytotoxic effect on B16BL6 cells (Fig. 1A), and their ED_{50} was about 25 and 12.5 μM , respectively (Fig. 1B). Inamori *et al.*⁹ reported that β -thujaplicin effectively inhibited cell growth of several tumor cells and blastic splenocytes at 1.8 to 3.6 μM . Compared with these values, relative high concentrations were required for the cytotoxic effects of the zinc and copper chelates on B16BL6 cells and other tumor cell lines (Fig. 4).

Next, we examined whether the cytotoxic effect of the zinc chelate is due to free β -thujaplicin or to the chelate itself in culture medium. The cytotoxicity increased in parallel with the concentration of zinc ions and reached a plateau at about 25 μM (Fig. 2A). β -Thujaplicin has been reported to chelate with divalent metal ions at 2 to 1 as a molar ratio.^{10,11} This indicates that the molar ratio of β -thujaplicin to zinc ions was about 2 to 1 at that point where the cytotoxic effect

reached a plateau, suggesting that this effect might be due to the function of the chelate itself on B16BL6 cells, but not to chelation of intercellular metal ions by free β -thujaplicin in culture medium. Similar results were also obtained with copper ions in the presence of β -thujaplicin (data not shown). Furthermore, zinc chelate showed the cytotoxic effect in a time dependent manner (Fig. 2B); the cytotoxicity was observed within 3 h of chelate addition and reached a plateau between 6 to 12 h. When the zinc chelate was removed from culture medium after treatment with the compound for 3 h, its cytotoxic effect on B16BL6 cells was also detected, indicating that the zinc chelate irreversibly induces a cytotoxic effect on the cells. This effect was not inhibited in the presence of 5 $\mu\text{g/ml}$ of cycloheximide (CHX) (data not shown), suggesting that new protein synthesis may not be required for the rapid and strong cytotoxic effect of zinc chelate.

There are two major morphologically and biochemically distinct modes of death in eukaryotic cells: apoptosis and necrosis.^{20–22} As the zinc and copper chelates have a cytotoxic effect on several tumor cells, we examined whether or not they induce apoptotic cell death in B16BL6 cells. As shown in Fig. 3, DNA ladder formation was observed in B16BL6 cells treated with the zinc chelate, while the DNA from cells treated with the copper chelate showed the same pattern as that treated with sodium azide, which induces necrosis in the cells. These results indicate that the zinc chelate might induce apoptosis in B16BL6 cells and the copper chelate might induce necrosis in the cells. Arima *et al.*¹⁶ recently demonstrated that β -thujaplicin has an inhibitory effect on hydroxyl radicals ($\cdot\text{OH}$) generated by the Fenton reaction, while Baba *et al.*¹⁷ reported that β -thujaplicin in-

hibits ultraviolet (UV) B-induced apoptosis in keratinocytes, suggesting that the inhibitory mechanism is due to the antioxidant activity of metallothionein induced by the compound. Zinc ions are also a well-defined inhibitor of the endonucleases responsible for the DNA degradation observed in apoptosis.²³⁾ In these regards, β -thujaplicin and zinc ions are potent inhibitors of apoptosis, but the zinc chelate induces apoptotic cell death in tumor cell lines including B16BL6 cells. Although the mechanism of the zinc chelate for induction of apoptosis in tumor cells is still unclear, the fact that β -thujaplicin is a scavenger for hydroxyl radicals and hydrogen peroxide (H_2O_2)¹⁶⁾ led us to hypothesize that the cell death induced in B16BL6 cells by this chelate may be involved in intercellular generation of reactive oxygen species (ROS), which has been reported to mediate apoptotic signaling.^{24–27)} Therefore, we tested the effect of *N*-acetylcysteine (NAC), which is known to scavenge H_2O_2 ,^{24,25)} on the zinc chelate- and copper chelate-induced cell death. The addition of NAC abolished the cell death induced by the zinc chelate, but not by the copper chelate (unpublished data). This result suggests that the mechanisms of cell death induced by these chelates differ, and that apoptotic cell death induced in B16BL6 cells by the zinc chelate may be involved in intercellular generation of H_2O_2 .

Figure 4 shows that the zinc chelate had a much higher cytotoxic effect on HeLa, Meth A, and B16F1 cells at concentrations of more than $25\ \mu\text{M}$, respectively, than that of β -thujaplicin. The DNA fragmentation assay also showed that this chelate induced DNA ladder formation in these cell lines when $25\ \mu\text{M}$ of the compound was added to each cell culture (data not shown). These results suggest that the zinc chelate has a cytotoxic effect on a broader range of tumor cell lines than β -thujaplicin and might induce apoptosis in the tumor cells. However, this chelate showed little or no cytotoxicity on normal diploid fibroblasts FS-4 derived from human foreskin, suggesting that it, as well as β -thujaplicin, has little or no toxicity on normal skin.^{8,9)}

In this study, we found that β -thujaplicin zinc chelate might induce apoptosis in several tumor cells including metastatic melanoma B16BL6 cells, but not in normal fibroblast cells derived from skin; this indicated that the mechanism of cell death induced by this chelate may be different from that induced by the copper chelate and β -thujaplicin. These findings suggest that the zinc chelate could be used as a potent antitumor reagent for malignant melanomas.

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