Bull. Environ. Contam. Toxicol. (2001) 67:12–19

DOI: 10.1007/s00128-001-0085-z

Bulletin of Environmental Contamination and Toxicology

Cytotoxic Effects of β-Thujaplicin on Rat Thymocytes and Prevention by the Compound in Tributyltin-Induced Thymocyte Damage

Y. Nakagawa

Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo 169-0073, Japan

Received: 14 July 2000/Accepted: 10 April 2001

β-Thujaplicin (4-isopropyl tropolone, hinokitiol), a derivative of aromatic seven-membered tropolone and a constituent of woody essential oils isolated from Cupressaceae, exerts antibacterial and antifungal activities. It is known that tropolone derivatives act as lipophilic chelators of divalent cations and that they form complexes with Fe^{2+}, Ca^{2+}, Mg^{2+} and other divalent cations (Bryant et al., 1953). Although the mechanism by which β-thujaplicin causes toxicity is unclear, cytotoxic effects may involve the chelation of metals necessary for the biological activity of critical enzymes in mammalian tumor cells or blastic cells (Inamori et al., 1993; Muto et al., 1995) and the impairment of mitochondrial function in rat hepatocytes (Nakagawa and Tayama, 1998). There is little information available on target organs of β-thujaplicin in experimental animals. However, we have found in a preminary study that thymus in rats may be a target for β-thujaplicin: this compound caused thymic atrophy (unpublished data, Nakagawa and Tayama). It is known that immature thymocytes are a highly sensitive to a number of chemicals including environmental toxins and reactive oxygen species, and further that the cells are extremely susceptible to undergoing activation of an endogenous suicide mechanism which has been termed apoptosis (programmed cell death) distinct from necrosis (McConkey et al., 1988; Cohen and Duke, 1984; Cohen et al., 1994; Sandstrom et al., 1994). Some trialkyltins, in particular tributyltin derivatives, cause acute cytotoxicity accompanied by apoptosis in rat thymocytes (Aw et al., 1990; Wyllie et al. 1980). In the present study, we have investigated i) the cytotoxic effects of β-thujaplicin on rat immature thymocytes and ii) the preventive effects of β-thujaplicin on tributyltin-induced thymocyte damage.

MATERIALS AND METHODS

The chemical compounds used were obtained from the following companies: β-thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one) and tri-*n*-butyltin chloride (tributyltin) (purities of >96%) from Tokyo Kasei (Tokyo, Japan); proteinase K and RNase (DNase-free) from Wako Chemicals (Osaka, Japan); and disodium ethylenediaminetetraacetate (EDTA), quin-2 AM, BAPTA AM and

Correspondence to: Y. Nakagawa

adenine nucleotides from Sigma (St. Louis, MO). All other chemicals were of the highest purity commercially available.

Male F344/DuCrj rats were obtained from Charles River Japan (Yokohama, Japan) and were allowed food (CE-2, Clea Japan Inc., Tokyo) and water ad libitum before thymocytes were prepared. Thymic glands were dissected 5- or 6-week-old rats and thymocytes were prepared by mincing the glands in ice-cold phosphate buffered saline (PBS; Ca^{2+}-free, pH 7.4). Thymocytes ($5x10^6$ cells/mL) were suspended in RPMI 1640 medium (pH 7.3) supplemented with 10 mM HEPES and 2% heat inactivated fetal calf serum. All incubations were performed in rotating, +round-bottomed flasks at 37°C, under an atmosphere of 95% air and 5% CO_2 during the experiments. Cell viability was assessed by exclusion of 0.16% Trypan blue, and initial cell viabilities were >95 %. Reactions were started by the addition of β-thujaplicin dissolved in dimethyl sulfoxide (DMSO; final concentration <1%). Aliquots of incubation mixture were taken at intervals for the assessment of cell death, concentration of adenine nucleotides and determination of DNA fragmentation. In another experiment with tributyltin, β-thujaplicin and some Ca^{2+} chelators were added to thymocytes in RPMI 1640 medium 20 min before the addition of tributyltin dissolved in PBS. The concentrations of these compounds used were based on previous works (Aw et al., 1990; Nakagawa and Tayama, 1998).

Adenine nucleotides in thymocytes were determined by a modification of the HPLC methods, as described by Jones (1981). DNA fragments were analyzed using a slight modification of the procedure of Wyllie et al. (1980). Thymocytes ($1x10^7$ cells) were centrifuged at 3000 rpm for 5 min. The collected cells were resuspended in PBS and then recentrifuged. The rinsed cells were lysed with 200 μL of a solution of containing 20 mM Tris-HCl (pH 8.0), 20 mM EDTA and 0.2% Triton X-100. After lysis in ice-water for 10 min, the lysates were centrifuged at 12,000 rpm for 30 min to separate intact chromatin in the pellet fraction from fragmented DNA in the supernatant fraction. DNA contents in both fractions were assayed using diphenylamine reagent according to the procedure of Burton et al.(1956). The percentage of DNA fragmentation is expressed as a percentage of the amount of DNA in the supernatant fraction (fragments) to the total of the DNA in both pellet and supernatant fractions. An aliquot of the supernatant fraction obtained above was incubated with RNase (20 μg) for 1 hour at 37°C and then with proteinase K (20 μg) for another hour. After the incubation, the incubation mixture was added NaCl (final 0.5 M) and isopropyl alcohol (final 50%) and followed to precipitate DNA fragments at -20°C for overnight. The precipitated DNA fragments were redissolved in a solution containing 20 mM Tris-HCl (pH 8.0) and 20 mM EDTA and the solution was subjected to electrophoresis on 2% agarose gels. After the electrophoresis DNA fragments on the gel were visualized following reaction with ethidium bromide. One hundred base-pair ladder (Pharmacia Biotech AB, Uppsala, Sweden) was used as a molecular size standard.

Statistical analysis was performed by one-way analysis of variance, followed by a Dunnett's multiple comparison test. Statistical significance was assumed at $p < 0.05$.

RESULTS AND DISCUSSION

The incubation of thymocytes with β-thujaplicin (0- 2 mM) caused a concentration- and time-dependent cell killing accompanied by the loss of intracellular ATP (Fig. 1A and B). The pretreatment of the thymocyte suspension with EDTA (1 mM), a hydrophilic chelator, enhanced the cytotoxicity of β-thujaplicin at concentrations of 1 and 2 mM, accompanied by the depletion of intracellular levels of ATP. To investigate the possible involvement of apoptosis and/or necrosis in β-thujaplicin-induced cell death, the amount of DNA damage was determined, measured as the formation of soluble low-molecular weight fragments of double-stranded DNA in thymocytes (Fig.1C). Although the amount of spontaneous DNA fragments in untreated or EDTA-treated thymocytes increased during the incubation, β-thujaplicin decreased the amount of fragments during a 4.5 hr-incubation. In addition, the level of DNA fragments decreased with β-thujaplicin was enhanced by the pretreatment with EDTA during the incubation. These effects of β-thujaplicin at 0.5 mM were similar to those of thymocytes treated with EDTA alone (data not shown in Fig.1).

The fraction of DNA fragments isolated from thymocytes at 3 hr after exposure to β-thujaplicin was subjected to agarose gel electrophoresis (Fig. 2). The intranucleosomal cleavage of DNA during apoptosis produced a "ladder" pattern of DNA fragments on the gel comprising multiples of approximately 180 base-pairs (McConkey et al., 1988). Spontaneous DNA fragmentation was found in untreated thymocytes during a 3 hr-incubation. Despite this, the amounts of DNA fragments in the ladders derived from thymocytes treated with β-thujaplicin or β-thujaplicin plus EDTA were less than those derived from untreated thymocytes.

Treatment of thymocytes with 1 μM tributyltin caused a time-dependent cell death accompanied by an increase in the amount of DNA fragments , i.e., approximately 45% nonviable cells and 35% DNA fragmentation after 6 hr incubation (data not shown). To determine the effect of β-thujaplicin on tributyltin-induced cytotoxicity, thymocytes were preincubated with β-thujaplicin or known intracellular Ca^{2+} chelators, quin-2 AM and BAPTA AM. The result in Table 1 shows that β-thujaplicin (0.1 mM) at a non-cytotoxic concentration effectively prevented tributyltin (1μM)-induced cell killing (at 3 hr) accompanied by the inhibition of DNA fragmentation, showing integer multiples of approximately 180 base-pairs. The preventive effect of β-thujaplicin is more potent than that of both Ca^{2+} chelators.

The results obtained in the present study have demonstrated that incubation of rat

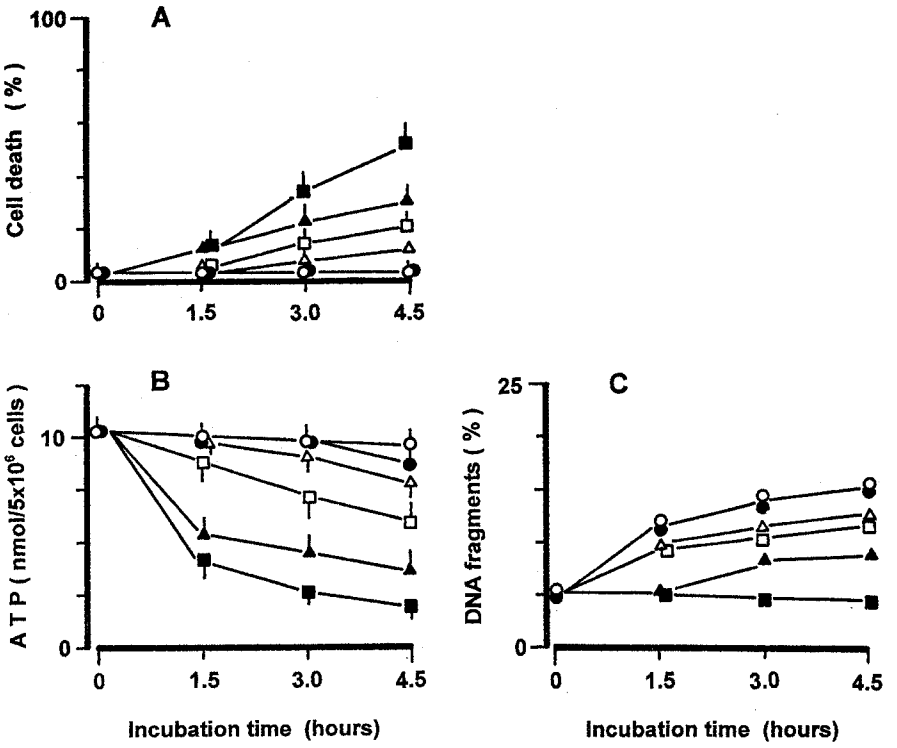

Figure 1. Effects of β-thujaplicin and/or EDTA on cell death (A), and intracellular levels of ATP (B) and DNA fragments (C) in isolated rat thymocytes (5 x 10^6 cells/mL). EDTA (1 mM) was added to the thymocyte suspensions 20 min prior to exposure of β-thujaplicin. Treatments are: no addition (○), 1 mM EDTA (●), 1 mM β-thujaplicin (Δ), 2 mM β-thujaplicin (□), 1 mM EDTA plus 1 mM β-thujaplicin (▲) and 1 mM EDTA plus 2 mM β-thujaplicin (■). Values in (A) and (B) are the means ± S.E. of three experiments, and values in (C) are the means of two experiments.

thymocytes with β-thujaplicin causes a concentration- and time-dependent cytotoxicity, which is accompanied by the loss of intracellular ATP. Because the mitochondria in thymocytes, as in other cells, are the main site of energy production, impairment of their function results in a decrease in the rate of cellular ATP synthesis and nucleotide levels. Mitochondrial dysfunction and its consequences are common mechanisms of cytotoxicity caused by a wide range of chemicals and a number of pathological conditions (Nieminen et al., 1990; Kehrer et al., 1990; Nakagawa and Moldéus, 1998).

The property of β-thujaplicin as a chelator of divalent cation may be important to the various cellular functions associated with the cations. Böhme et al. (1980)

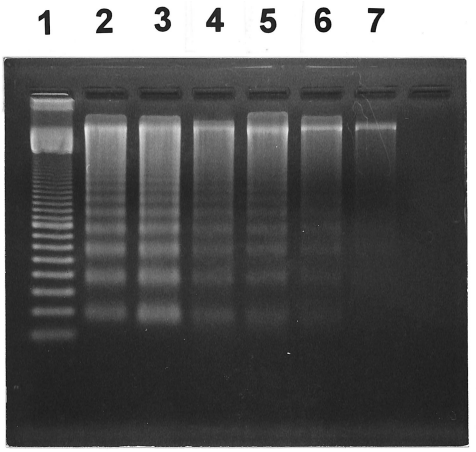

Figure 2. Agarose gel electrophoresis of DNA fragments isolated from thymocytes treated with β-thujaplicin and/or EDTA for 3 hr. EDTA (1 mM) was added to the thymocyte suspensions 20 min prior to exposure of β-thujaplicin (1 or 2 mM). Lane 1, DNA molecular weight calibration standard corresponding to multiples of 100 base pairs; lane 2, untreated; lane 3, 1 mM EDTA; lane 4, 1 mM β-thujaplicin; lane 5, 2 mM β-thujaplicin; lane 6, 1 mM EDTA plus 1 mM β-thujaplicin; lane 7, 1 mM EDTA plus 2 mM β-thujaplicin.

Table 1. Effects of β-thujaplicin and Ca^{2+} chelators on tributyltin-induced cytotoxicity in rat thymocytes.

Treatments	Cell death (%)	ATP (nmol/5x10^6 cells)	DNA fragments (%)
None	6.2 ± 0.7	9.8 ± 0.4	12.0 ± 1.7
tributyltin (1 μM)	31.4 ± 3.6*	5.1 ± 1.0*	25.1 ± 2.9*
+ β-thujaplicin (0.1mM)	7.7 ± 2.1	10.3 ± 0.3	8.2 ± 1.0*
+ quin-2 AM (0.1 mM)	14.9 ± 2.0*¶	8.9 ± 0.5*	12.8 ± 0.9¶
+ BAPTA AM (0.1 mM)	17.5 ± 2.8*¶	8.0 ± 0.6*¶	14.3 ± 2.4¶

Thymocytes (5 x 10^6 cells/mL) suspended in RPMI 1640 medium (pH 7.3) supplemented with 10 mM HEPES and 2 % heat inactivated fetal calf serum were incubated with tributyltin (1 μM) for 3 hr. β-Thujaplicin and Ca^{2+} chelators at the concentration of 0.1 mM added the cell suspension 20 min prior to addition of tributyltin. Values are the means ± S.E. of three experiments.

* Significant difference from values for untreated thymocytes ($p<0.05$).

¶ Significant difference from values for β-thujaplicin-treated thymocytes ($p<0.05$).

have reported that the ability as a lipophilic chelator of β-thujaplicin to extract mitochondrial Mg^{2+} is related to an impairment of mitochondrial respiration. Although tropolones containing β-thujaplicin form a complex with Ca^{2+} and Mg^{2+} in Krebs-Henseleit buffer, the affinity of tropolones for the divalent cations is less than that of EDTA. In addition, tropolones bound to cations are easily substituted for EDTA, which is a highly hydrophilic chelator that does not easily enter into cells (Nakagawa and Tayama, 1998). Therefore, the hydrophobicity of β-thujaplicin was dependent on the presence of Ca^{2+} and Mg^{2+} in the incubation medium. Unchelated β-thujaplicin may easily pass through the cell membrane and access intracellular targets such as mitochondria and some metal containing proteins. This is supported as the cytotoxicity of β-thujaplicin in thymocytes is enhanced by the presence of EDTA (Fig. 1).

The damage of thymocytes can be triggered experimentally by a variety of extra- and intra-cellular stimuli including environmental toxins (McConkey et al., 1988), hormones (Cohen and Duke, 1984), and synthetic derivatives (Raffray and Cohen, 1991; Nakagawa et al., 1997; Lutz et al., 1998). Immature thymocytes are capable of activating an endogenous suicide mechanism which has been termed "apoptosis" (McCabe et al., 1993; Raffray and Cohen, 1993). Despite this, the incubation of immature normal thymocytes with β-thujaplicin inhibited DNA fragmentation, a biochemical hallmark of cells undergoing apoptosis (Montague and Cidlowski, 1996), but induced cell death accompanied by the loss of intracellular ATP (Fig. 1). The β-thujaplicin-induced cytotoxicity enhanced by the addition of EDTA may be associated with necrotic death rather than apoptotic death, because induction of DNA fragmentation in thymocytes was inhibited by EDTA plus β-thujaplicin.

The toxic effects of tributyltin have been attributed to a disturbance of macromolecular synthesis, reduction of DNA synthesis, and direct interaction with the cell membranes and elevation of the intracellular Ca^{2+} level (McCabe et al., 1993). DNA fragmentation is catalyzed by an endonuclease that is stimulated by Ca^{2+} : many agents that elevate intracellular Ca^{2+} induce thymocyte apoptosis, and Ca^{2+} chelators which buffer intracellular Ca^{2+} prevent the apoptosis (McConkey et al., 1998). Aw et al. (1990) have suggested that tributyltin-induced toxicity in rat thymocytes is involved in apoptosis and/or DNA fragmentation via the activation of a Ca^{2+}- and Mg^{2+}-dependent endogenous endonuclease by elevation of the intracellular Ca^{2+} level. These results indicate that β-thujaplicin, a hydrophobic chelator, as well as other intracellular Ca^{2+} chelators could significantly prevent tributyltin-induced thymocyte killing accompanied by the inhibition of DNA fragmentation.

In conclusion, the present study shows that in immature rat thymocytes, a hydrophobic chelator β-thujaplicin elicits a dose-dependent cell death with intracellular ATP loss, and that the cytotoxicity without DNA fragmentation is enhanced in the presence of a hydrophilic chelator EDTA. In contrast, β-thujaplicin

at a nontoxic concentration (0.1 mM) effectively prevents tributyltin-induced cell killing, accompanied by the inhibition of DNA fragmentation of thymocytes.

REFERENCES

Aw TE, Nicotera P, Manso L, Orrenius S (1990) Tributyltin stimulates apoptosis in rat thymocytes. Arch Biochem Biophy 283: 46-50

Böhme G, Schönfild P, Kiüster U, Kunz W, Lyr H (1980) Multifunction action of β-thujaplicin on the oxidative energy transformations as a consequence of its lipophilic and chelating properties. Acta Biol Med Ger 39:1153-1163

Bryant BE, Fernelius, WC, Douglas, BE (1953) Formation constants of metal complexes of tropolone and its derivatives. J American Chem Soc 75: 3486-3784

Burton K (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62: 315-323

Cohen JJ, Duke RC (1984) Glucocorticoid activation of a calcium-dependent endonuclease in the thymocyte nuclei leads to cell death. J Immunol 132: 38-42

Cohen GM, Sun XM, Fearnhead H. MacFarlane M, Brown DG, Snowden RT, Dinsdale D (1994) Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. J Immunol 153: 507-516

Inamori Y, Tsujibo H, Ohishi H, Ishii F, Mizugaki M, Sano H, Ishida N (1993) cytotoxic effect of hinokitiol and tropolone on the growth of mammalian cells and blastogenesis of mouse splenic T-cells. Biol Pharm Bull 16: 521-523

Jones DP (1981) Determination of pyridine dinucleotides in cell extracts by high-performance liquid chromatography. J Chromatogr 225: 446-449

Kehrer JP, Jones DP, Lemasters JJ, Farber JL, Jaeschke H (1990) Contemporary issue in toxicology: mechanisms of hypoxic cell injury. Toxicol Appl Pharmacol 106: 165-178

Lutz CT, Browne G, Petzold RC (1998) Methylcholanthrene causes increased thymocyte apoptosis. Toxicol 128: 151-167

McCabe MJ, Jiang SA, Orrenius S (1993) Chelation of intracellular zinc triggers apoptosis in mature thymocytes. Lab Investig 69: 101-110

McConkey DJ (1998) Biochemical determinants of apoptosis and necrosis. Toxicol Lett 99: 57-168

McConkey DJ, Hartzell P, Duddy SK, Håkansson H, Orrenius S (1988) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. Science 242: 256-259

Montague JW, Cidlowski JA (1996) Cellular catabolism in apoptosis: DNA degradation and endonuclease activity. Experimentia 52: 957-962

Muto N, Dota, A Tanaka T, Itoh N, Okabe M, Inada A, Takanishi K, Tanaka K (1995) Hinokitiol induces differentiation of teratocarcinoma F9 cells. Biol Pharm Bull 18: 1576-1579

Nakagawa Y, Moldéus P (1998) Mechanism of *p*-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. Biochem Pharmacol 55: 1907-1914

Nakagawa Y, Moldéus P, Moore G (1997) Propyl gallate-induced DNA fragmentation in isolated rat hepatocytes. Arch Toxicol 72: 33-37

Nakagawa Y, Tayama K (1998) Mechanism of mitochondrial dysfunction and cytotoxicity induced by tropolones in isolated rat hepatocytes. Chemico-Biol Interact 116: 45-60

Nieminen AL, Gores GJ, Dawson TL, Herman B, Lemasters JJ (1990) Toxic injury from mercuric chloride in rat hepatocytes. J Biol Chem 265: 2399-2408

Raffray M, Cohen GM (1991) Bis(tri-n-butyltin)oxide induces programmed cell death (apoptosis) in immature rat thymocytes. Arch Toxicol 65: 135-139

Raffray M, Cohen GM (1993) Thymocyte apoptosis as a mechanism for tributyltin-induced thymic atrophy in vivo. Arch Toxicol 67: 231-236

Sandstrom PA, Tebbey PW, Van Cleave S, Buttke TM (1994) Lipid peroxidations induce apoptosis in T-cells displaying a HIV-associated glutathion peroxidase deficiency. J Biol Chem 269: 798-801

Wyllie AH, Kerr JFR, Cumi AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68: 251-306