

Pirarubicin, an Anthracycline Anticancer Agent, Induces Apoptosis Through Generation of Hydrogen Peroxide

HIDEKI MIZUTANI¹, SAKI HOTTA¹, AYANO NISHIMOTO¹, KENJI IKEMURA^{1,2}, DAISUKE MIYAZAWA¹, YOSHIAKI IKEDA¹, TOHRU MAEDA¹, MASAE YOSHIKAWA¹, YUSUKE HIRAKU³ and SHOSUKE KAWANISHI⁴

¹College of Pharmacy, Kinjo Gakuin University, Nagoya, Japan;

²Department of Pharmacy, Mie University Hospital, Tsu, Japan;

³Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine, Tsu, Japan;

⁴Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Suzuka, Japan

Abstract. *Background/Aim:* Pirarubicin (THP) has shown equal or superior cytotoxicity compared to doxorubicin. One of the main anticancer actions of doxorubicin is believed to be involved in ROS (reactive oxygen species) generation. Therefore, the anticancer mechanisms of THP may involve ROS generation. The aim of this study was to clarify the mechanisms of THP-induced apoptosis through ROS generation. *Materials and Methods:* We analyzed the apoptotic events induced by THP in HL-60 cells and HP100 cells, hydrogen peroxide (H₂O₂)-resistant cells derived from HL-60. *Results:* The apparent cytotoxicity could be detected at above 0.1 μ M in HL-60 cells after 24-h incubation, whereas it was suppressed under these conditions in HP100 cells. In HP100 cells, THP-induced apoptosis, evaluated by DNA ladder formation, H₂O₂ generation, mitochondrial membrane potential decrease and caspase-3/7 activity, was suppressed or delayed compared to those of HL-60 cells. *Conclusion:* These findings can be explained by the involvement of H₂O₂ generation in the THP apoptotic pathway. This is the first report on THP-induced apoptosis through the H₂O₂ generation.

Anthracyclines are anticancer drugs and are the most important drug categories in clinical usage. Doxorubicin (Figure 1), an anthracycline drug, shows potent effects on solid cancers as well as hematological malignancies (1-3).

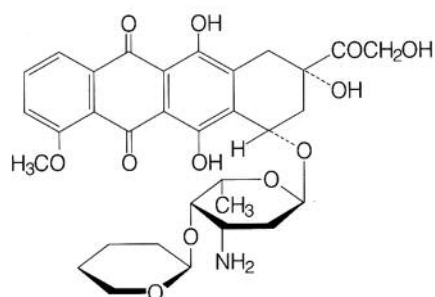
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Correspondence to: Hideki Mizutani, Ph.D., College of Pharmacy, Kinjo Gakuin University, Moriyama-ku, Nagoya 463-8521, Japan. Tel: +81 527987458, Fax: +81 527980754, e-mail: mizu@kinjo-u.ac.jp and Shosuke Kawanishi, Ph.D., Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Suzuka, Mie 513-8670, Japan. Tel: +81 593400568, Fax: +81 593681271, e-mail: kawanishi@suzuka-u.ac.jp

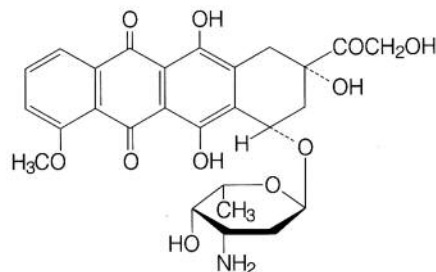
Key Words: Pirarubicin, apoptosis, hydrogen peroxide, ROS.

Pirarubicin (4'-O-tetrahydropyranyl doxorubicin, THP) (Figure 1), a tetrahydropyranyl-derivative doxorubicin, was found and developed by Umezawa *et al.* in 1979 (4). THP showed equal or superior cytotoxicity in cultured tumor cells, and less cardiotoxicity in hamsters compared to DOX (5, 6). THP is incorporated into cells about 170-times rapider than DOX in cultured tumor cells (5, 7). THP is clinically approved for head and neck cancer, stomach cancer, upper urinary tract cancer, uterus cancer, ovarian cancer, acute leukemia and malignant lymphoma in Japan. Recently, Hori *et al.* reported that THP appeared to be less cardiotoxic than daunorubicin on acute and late toxicities in the treatment of childhood acute lymphoblastic leukemia on a clinical trial by the Japan Association of Childhood Leukemia Study (8). On the other hand, THP has attracted increasing attention, because THP-DDS (drug delivery systems) developed by Maeda *et al.* have high tumor-targeting efficiency by EPR (enhanced permeability and retention) effect (9, 10). Maeda and his collaborators were also successful at treating the THP-DDS (HPMA copolymer-conjugated pirarubicin) for a patient with stage IV prostate cancer and extensive lung and bone metastases (11). Therefore, THP has once more attracted attention in the field of cancer chemotherapy.

Anthracyclines generate ROS (reactive oxygen species) causing oxidative DNA damage that may be one of THP anticancer mechanisms (1-3). ROS also play an important role in cell signaling as well as in anticancer mechanisms (12-14), whereas the role of ROS in THP anticancer mechanisms remains to be clarified and is not reported. To clarify THP anticancer mechanisms, we investigated the mechanism of THP-induced apoptosis in HL-60 cells and HP100 cells, hydrogen peroxide (H₂O₂)-resistant cells derived from HL-60. The mechanism of apoptosis was analyzed by examining cytotoxicity, DNA ladder formation, H₂O₂ generation, mitochondrial membrane potential ($\Delta\Psi_m$) change and caspase-3/7 activity.



Pirarubicin (THP)



Doxorubicin (DOX)

Figure 1. Chemical structure of pirarubicin and doxorubicin.

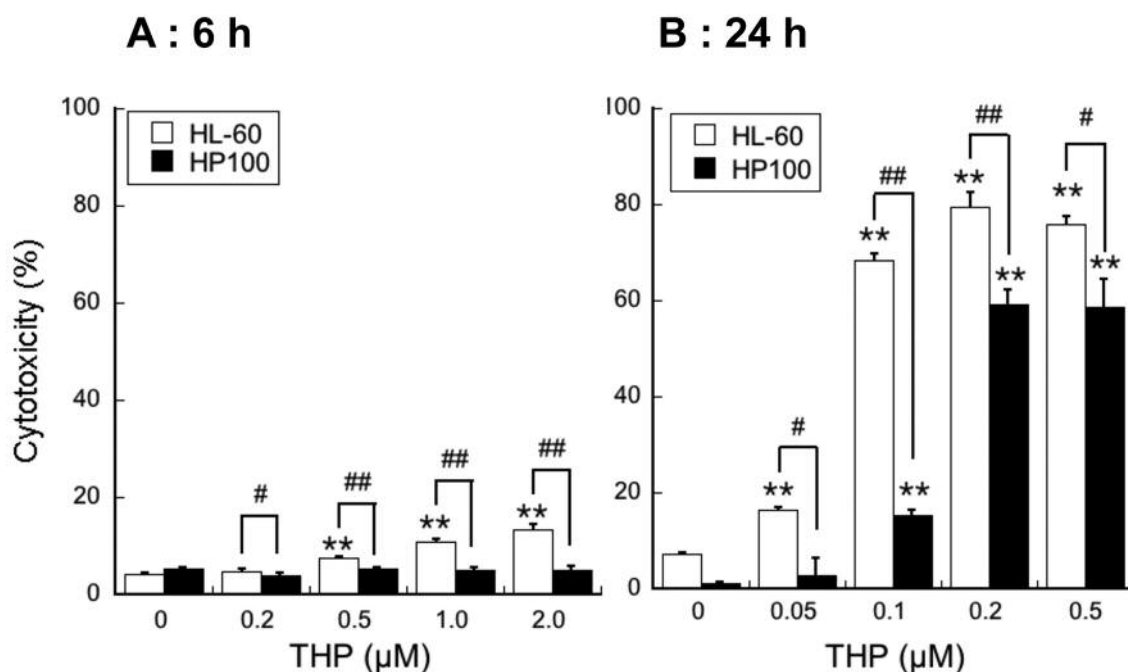


Figure 2. Detection of cytotoxicity in HL-60 and HP100 cells treated with THP. HL-60 and HP100 cells (0.5×10^6 cells/ml) were treated with THP at 37°C for 6 h (A) and 24 h (B). After treatment with THP, cytotoxicity was analyzed by the LDH activity assay kit (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega) according to the manufacturer's instructions. The data were expressed as means \pm SD ($n=3$). ** $p<0.01$, vs. control by ANOVA followed by Tukey's HSD test; # $p<0.05$ and ## $p<0.01$, HL-60 vs. HP100 by the Student's t -test.

Materials and Methods

Materials. Pirarubicin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Proteinase K was obtained from Merck (Darmstadt, Germany). Fluorescent probes, 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and 3,3'-dihexyloxycarbocyanine iodide [DiOC₆(3)] were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals used were of the highest purity commercially available.

Cell culture and treatment with THP. HP100 cells have been derived from human leukemia HL-60 cells by repeated exposure to H₂O₂, followed by outgrowth of viable cells. HP100 cells were approximately 340-fold more resistant to H₂O₂ than HL-60 cells (15). Catalase activity of HP100 cells is 18-times higher than that of HL-60 cells (16). When scavengers such as catalase, superoxide dismutase and reduced glutathione are added in cell culture medium, these scavengers exist in the extracellular environment (17, 18).

Therefore, the effects of the scavengers are not evaluated correctly. On the other hand, the increased catalase of HP100 cells exist in the intracellular components, thus the effects of catalase can be evaluated correctly. HL-60 and HP100 cells were grown in RPMI 1640 supplemented with 6% FBS at 37°C under 5% CO₂ in a humidified atmosphere. The cells (0.5×10^6 or 1×10^6 cells/ml) were then treated with the indicated concentrations of THP.

Determination of cytotoxicity induced by THP in HL-60 and HP100 cells. A lactate dehydrogenase (LDH) activity assay kit (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega, USA) was used to measure cytotoxicity in cultures. 5×10^4 cells were seeded into each well of 96-well culture plates. After the treatment with THP for 6 or 24 h, cytotoxicity was analyzed according to the manufacturer's instructions (19).

Detection of apoptosis induced by THP in HL-60 and HP100 cells. For analyses of DNA ladder formation after the cells were treated with THP, cells were washed twice with PBS. Cells (2×10^6 cells), resuspended in 1 ml cytoplasm extraction buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM MgCl₂ and 0.5% Triton X-100], were centrifuged at $1,000 \times g$ for 5 min at 4°C. The pellet was resuspended in lysis buffer [10 mM Tris (pH 7.5), 400 mM NaCl and 1 mM EDTA] and centrifuged at $12,000 \times g$ for 10 min at 4°C. The supernatant was then treated with 0.2 mg/ml RNase overnight at room temperature, followed by treatment with proteinase K as described previously (20). DNA ladder formation was analyzed by conventional electrophoresis.

Imaging cytometric analysis of peroxide generation and $\Delta\Psi_m$ change in cells treated with THP. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with THP at 37°C for the indicated times. After the treatment, the cells were incubated with 5 μ M DCFH-DA for 30 min (peroxide generation) or 40 nM DiOC₆(3) for 15 min ($\Delta\Psi_m$) at 37°C. Cells were then washed twice with PBS. Following resuspension in PBS, the cells were analyzed on an imaging cytometer (Tali™ Image-based Cytometer; Invitrogen, USA). Dead cells and debris were excluded from the analysis by cell sizes (21).

Caspase-3/7 analysis of apoptosis induced by THP in HL-60 and HP100 cells. A caspase-3/7 activity assay kit (Apo-ONE® Homogeneous Caspase-3/7 Assay, Promega, USA) was used to measure caspase-3/7 activity in cultures. 5.0×10^4 cells were seeded into each well of 96-well culture plates. After the treatment with THP for 24 h, caspase-3/7 activity was analyzed according to the manufacturer's instructions.

Results

Cytotoxicity in HL-60 and HP100 cells induced by THP. We determined cytotoxicity in the cells treated with THP using an LDH release assay. As observed in Figure 2A and B, HL-60 cells were more sensitive to THP in comparison to HP100 cells at 6-h and 24-h incubation ($p < 0.05$). At 6 h, THP induced significant cytotoxicity at 0.5, 1.0 and 2.0 μ M in HL-60 ($p < 0.01$), but not in HP100 cells. The cytotoxicity of THP for 24-h incubation was higher than that of THP for 6-h incubation. The cytotoxicity at 0.1 μ M THP for 24-h incubation was about 68% in HL-60 cells, whereas that was

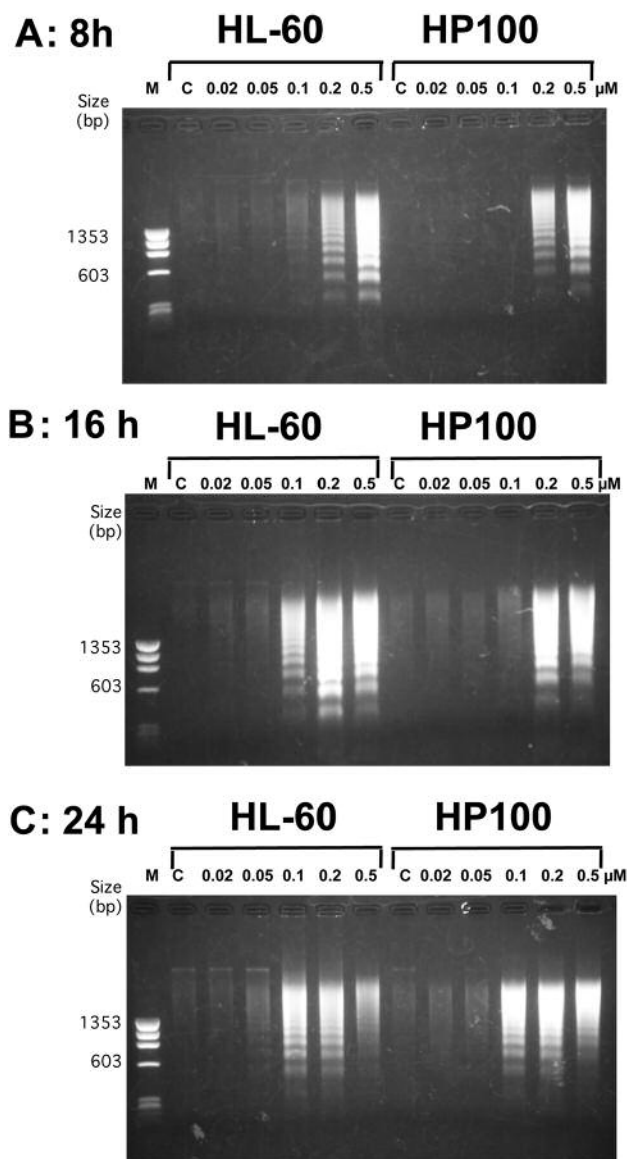


Figure 3. Detection of DNA ladder formation in HL-60 and HP100 cells treated with THP. HL-60 and HP100 cells (0.5×10^6 cells/ml) were treated with THP at 37°C for 8 h (A), 16 h (B) and 24 h (C). The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis. Marker lane: size marker DNA (Φ X174/Hae III digest).

only approximately 15% in HP100 cells. Therefore, HL-60 cells were about 4.5-fold sensitive to THP than HP100 cells at 0.1 μ M THP for 24-h incubation.

DNA ladder formation in HL-60 and HP100 cells treated with THP. We have analyzed DNA ladder formation, which is characteristic for apoptosis, in the cells treated with THP using conventional electrophoresis (Figure 3). The DNA

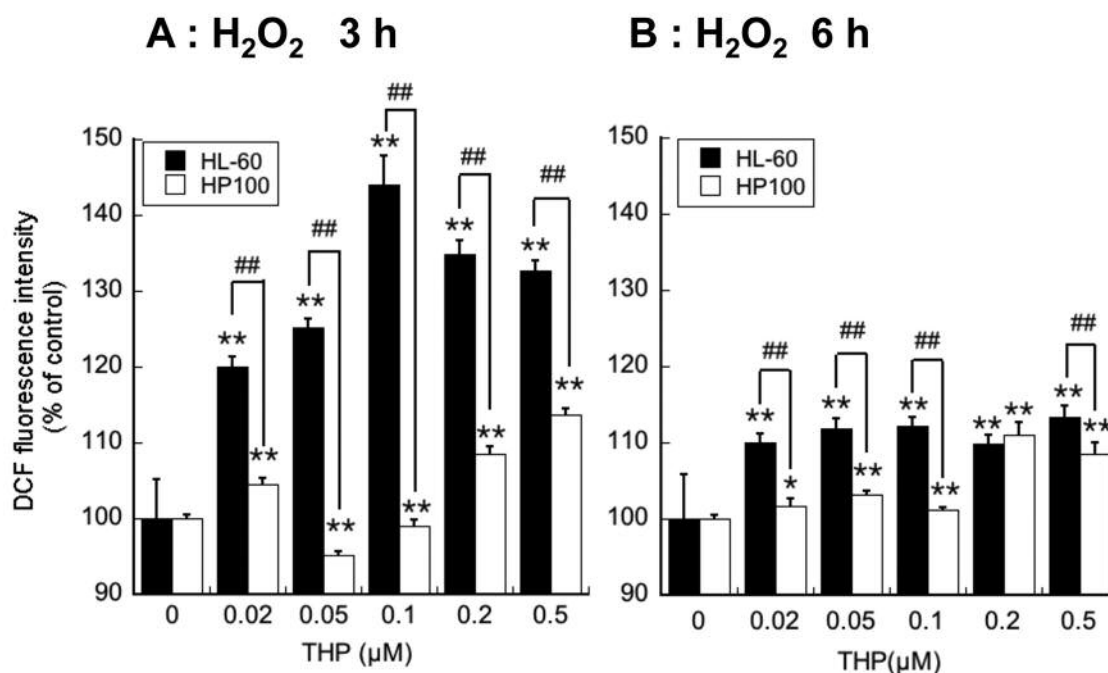


Figure 4. Generation of H_2O_2 in HL-60 and HP100 cells treated with THP. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with THP at 37°C for 3 h (A) and 6 h (B). After the treatment, the cells were incubated with $5 \mu\text{M}$ DCFH-DA for 30 min at 37°C . The cells were analyzed with an imaging cytometer (Tali™ Image-based Cytometer; Invitrogen, USA). The data were expressed as means \pm SD ($n=9$ capture fields). * $p<0.05$ and ** $p<0.01$, vs. control by ANOVA followed by Tukey's HSD test; ## $p<0.01$, HL-60 vs. HP100 by the Student's t -test.

ladder formation by THP could be detected at 0.2 and 0.5 μM in HL-60 and HP100 cells after 8-h incubation (Figure 3A), and it could be detected at 0.1, 0.2 and 0.5 μM in HL-60 and HP100 cells after 24-h incubation (Figure 3C). After 16-h incubation, the DNA ladder by THP could be detected at 0.1, 0.2 and 0.5 μM in HL-60 cells, whereas it could not be detected at 0.1 μM in HP100 cells (Figure 3B). On the other hand, after 6-h incubation, the DNA ladder by THP could not be detected at in both HL-60 and HP100 cells (data not shown).

Generation of peroxide and change of $\Delta\Psi_m$ in HL-60 and HP100 cells treated with THP. As shown in Figures 4A and B, peroxide generation in HL-60 and HP100 cells was observed at 3 and 6 h. The peroxide generation in HL-60 cells remarkably increased at 3 h after the treatment with THP ($p<0.01$), whereas its generation in HP100 cells was significantly suppressed at 3 and 6 h compared with HL-60 cells ($p<0.01$). These results indicate that the THP-induced peroxide is mainly H_2O_2 , because catalase activity in HP100 cells is much higher than that in HL-60 cells (16). Figure 5A and B show change of $\Delta\Psi_m$ in HL-60 and HP100 cells treated with THP. $\Delta\Psi_m$ was apparently decreased at above

0.05 μM THP in HL-60 and HP100 cells at 16-h incubation ($p<0.01$), whereas the decrease of $\Delta\Psi_m$ in HP100 cells was significantly suppressed in comparison with HL-60 cells at above 0.2 μM THP ($p<0.01$).

Assessment of THP-induced apoptosis by caspase-3/7 activity in HL-60 and HP100 cells. We assessed THP-induced apoptosis by activation of caspase-3/7. The activation of caspase-3/7 in THP-treated cells was measured using a caspase-3/7 activity assay kit at 24-h incubation. The caspase-3/7 activity was remarkably increased in treatment with THP at above 0.1 μM in HL-60 and HP100 cells ($p<0.001$). Caspase-3/7 activity in HL-60 was significantly higher than that observed in HP100 at 0.1, 0.2 and 0.5 μM (Figure 6) ($p<0.05$).

Discussion

The present study showed that THP induced apoptosis in both HL-60 cells and the H_2O_2 -resistant clone, HP100 cells. The apparent cytotoxicity could be detected at above 0.1 μM in HL-60 cells after 24-h incubation, whereas it was suppressed under these conditions in HP100 cells. The

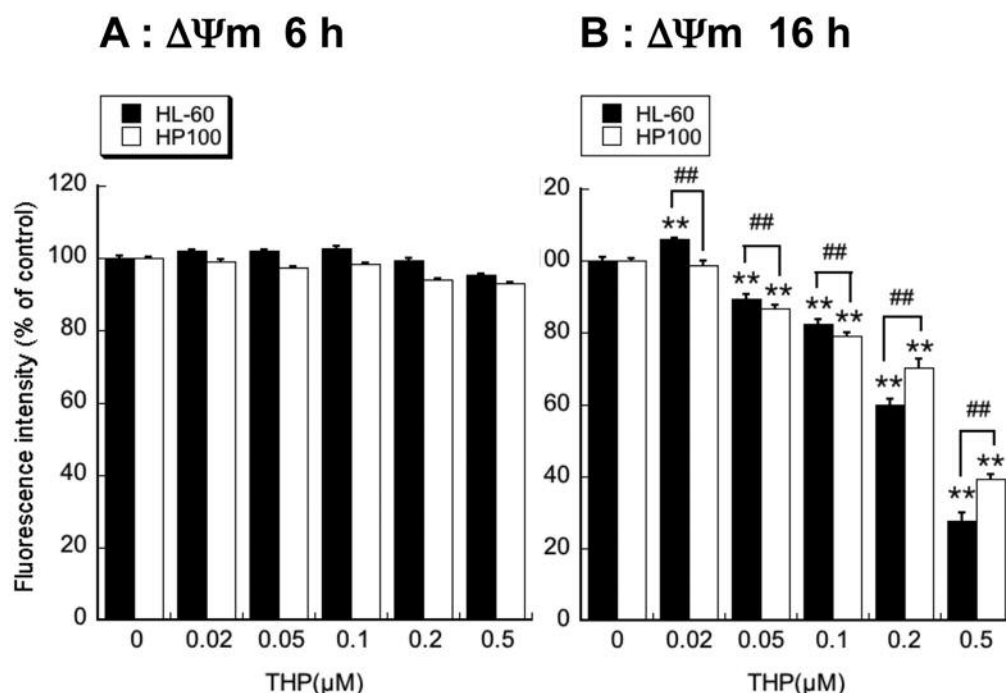


Figure 5. Change of mitochondrial membrane potential ($\Delta\Psi_m$) in HL-60 and HP100 cells treated with THP. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with THP at 37°C for 6 h (A) and 16 h (B). After the treatment, the cells were incubated with 40 nM DiOC₆(3) for 15 min at 37°C. The cells were analyzed with an imaging cytometer (Tali™ Image-based Cytometer; Invitrogen, USA). The data were expressed as means \pm SD ($n=9$ capture fields). ** $p<0.01$, vs. control by ANOVA followed by Tukey's HSD test; ## $p<0.01$, HL-60 vs. HP100 by the Student's *t*-test.

apparent DNA ladder formation could be detected at above 0.2 μ M after 8-h incubation and at above 0.1 μ M after 24-h incubation in HL-60 and HP100 cells, whereas it could be detected at above 0.1 μ M in HL-60 cells and at above 0.2 μ M in HP100 cells after 16-h incubation. In HP100 cells, assessment of THP-induced apoptosis by peroxide generation, $\Delta\Psi_m$ decrease and caspase-3/7 activity were suppressed, compared to those of HL-60 cells. Peroxide formation preceded the apparent cytotoxicity, DNA ladder, caspase-3/7 activation and $\Delta\Psi_m$ decrease in THP-induced apoptosis; in HL-60 cells. The peroxide formation occurred at 3 h and 6 h in HL-60 cells, whereas the peroxide formation was suppressed in HP100 cells. It was reported that HP100 cells are about 340-fold more resistant to H₂O₂ than the parent cells, HL-60 (15). Therefore, these findings can be explained by the involvement of H₂O₂ generation in the THP apoptotic pathway.

It has been considered that the anticancer mechanisms of anthracyclines are due to DNA intercalation, topoisomerase II inhibition and ROS generation (1-3). The DNA intercalation inhibits DNA and RNA synthesis, and results in double-strand DNA breaks (1-3). The topoisomerase II inhibition results in double-strand DNA breaks, and ROS generated by the redox-cycle of anthracyclines cause

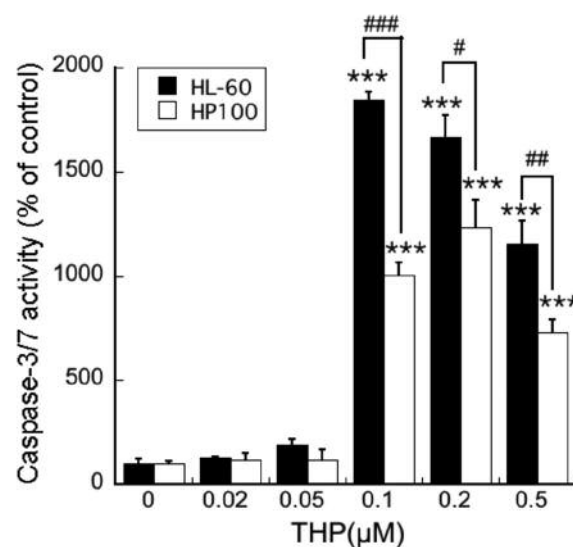


Figure 6. Caspase-3/7 analyses of apoptosis induced by THP in HL-60 and HP100 cells. HL-60 and HP100 cells (0.5×10^6 cells/ml) were treated with THP at 37°C for 24 h. After treatment with THP, caspase-3/7 was analyzed by the caspase-3/7 activity assay kit (Apo-ONE® Homogeneous Caspase-3/7 Assay, Promega, USA) according to the manufacturer's instructions. The data were expressed as means \pm SD ($n=3$). *** $p<0.001$, vs. control by ANOVA followed by Tukey's HSD test; # $p<0.05$, ## $p<0.01$ and ### $p<0.001$, HL-60 vs. HP100 by the Student's *t*-test.

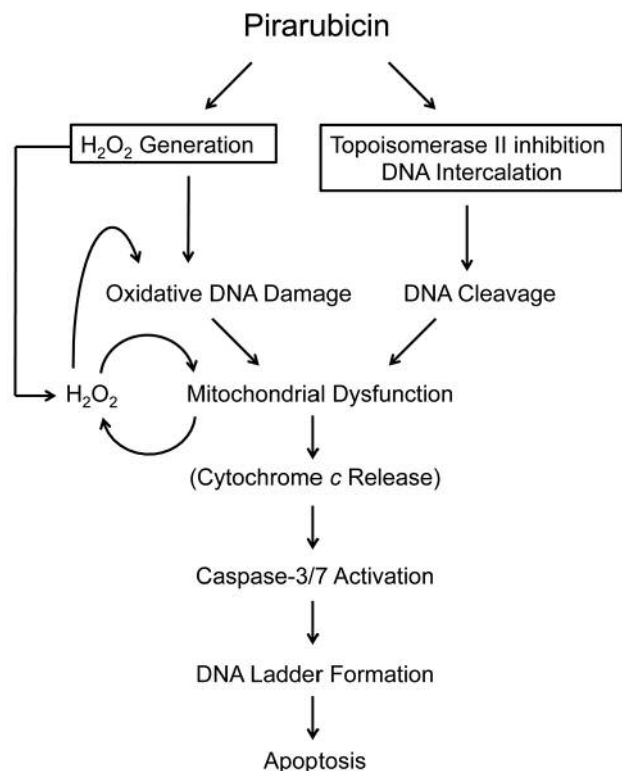


Figure 7. Proposed mechanisms of THP-induced apoptosis via H_2O_2 generation.

oxidative DNA damage (1-3). The main mechanism is considered to be the topoisomerase II inhibition, however it is considered that ROS generation is also an important mechanism. We reported that DOX induced apoptosis through H_2O_2 generation (22), and also reported that DOX caused site-specific oxidative DNA damage in the presence of copper(II) (23). In addition, many antioxidants attenuate apoptosis and cardiotoxicity induced by anthracyclines (24-28). These reports suggest that H_2O_2 formation plays important roles in apoptosis induced by anthracyclines. Moreover, mitochondrial dysfunction also induces H_2O_2 production that induces more DNA damage (29-31). Thus, we conclude that the apoptotic pathways of DNA damage-induced THP are as follows: THP induces oxidative DNA damage through H_2O_2 generation, DNA cleavage through topoisomerase II inhibition and DNA intercalation. Oxidative DNA damage and DNA cleavage cause mitochondrial dysfunction, inducing cytochrome c release that activates caspase-3/7, leading to apoptosis (Figure 7).

In conclusion, we demonstrated that H_2O_2 generation was involved in the THP apoptotic pathway. This H_2O_2 generation may be a useful clinical biomarker for anticancer

activity or cardiotoxicity of THP in comparison to other anthracyclines. This is the first report on THP-induced apoptosis through H_2O_2 generation.

Acknowledgements

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