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## Novel Topoisomerase I and II Inhibitors, Parameritannins A-2 and A-3, are Selective Human Cancer Cytotoxins

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**Abstract:** Present study, isolated the parameritannins A-2 and A-3, new A-type tetrameric proanthocyanidins, from the bark of *Parameria laerigata* Moldenke and showed that they inhibited eukaryotic topoisomerase I, II or both activities *in vitro*. Although they are structurally isomeric compounds, their inhibitory effects against topoisomerases were different. Parameritannin A-2 only inhibited topoisomerase II activity with IC<sub>50</sub> value of 0.5 μM, while parameritannin A-3 inhibited both topoisomerase I and II activities with IC<sub>50</sub> values of 50 and 0.5 μM, respectively. The inhibitory actions of parameritannin A-3 against topoisomerase I and II were also different. Preincubation analysis suggests that parameritannin A-3 binds both topoisomerase I and substrate DNA and it has high binding affinity to DNA rather than topoisomerase I. Inhibitory activity against topoisomerase II was same for either pretreatment with enzyme or DNA. In addition, only parameritannin A-3 induced apoptosis although both parameritannins A-2 and A-3 arrested the cell cycle at G2/M phase. This is the first report that parameritannins act as topoisomerase inhibitors and present results further support its therapeutic potential as a leading anti-cancer compound that poisons topoisomerases.

**Key words:** Parameritannin A-2, parameritannin A-3, DNA topoisomerase I and II, cytotoxicity, cell cycle arrest, apoptosis, PARP

### INTRODUCTION

Topoisomerases (topos) are key enzymes that modulate the topological state of DNA. There are two classes of topoisomerases: type I acts by transiently nicking one of the two DNA strands and type II nicks both DNA strands and its ATP dependent and is involved in many vital cellular processes that influence DNA replication, transcription, recombination, integration and chromosomal segregation<sup>[1]</sup>. Topoisomerase inhibitors are very rare, the most widely studied and characterized inhibitor being camptothecin, a topoisomerase I poison<sup>[2]</sup> and etoposide and doxorubicin, topoisomerase II poisons<sup>[3-7]</sup>. In recent years, these enzymes have gained special interest because topoisomerase inhibitors have emerged as anti-cancer<sup>[8]</sup> and anti-parasitic agents<sup>[9,10]</sup>.

Topoisomerase inhibition can be achieved by two distinct mechanisms and the inhibitors are accordingly divided into two classes: class I and II inhibitors. Class I inhibitors

stabilize the enzyme-DNA covalent complex and block the subsequent rejoining of the DNA break. Class II inhibitors, also referred to as catalytic inhibitors, prevent the enzyme-DNA binding by interacting with either topoisomerase<sup>[11,12]</sup> or DNA<sup>[13]</sup>.

Parameritannins A-2 and A-3 (Fig. 1) were new A-type tetrameric proanthocyanidins isolated from the bark of *Parameria laerigata* Moldenke, which is a traditional medical plant widely distributed from southern China to Malaysia and Indonesia<sup>[14,15]</sup>. This plant has been traditionally used as an antiulcer and antidiarrheal medicine as well as to treat wounds. Some flavonoids inhibit topoisomerase I activity<sup>[16,17]</sup>, arrest cell cycle and lead to induction of apoptosis<sup>[18,19]</sup>. Further studies have also shown that both topoisomerase I and II activities are higher in colon cancer and in various colon carcinoma cell lines compared to normal tissues<sup>[20]</sup>. Based on these studies, inhibitors of these enzymes are effective agents against cancer. Since parameritannins have been shown to arrest

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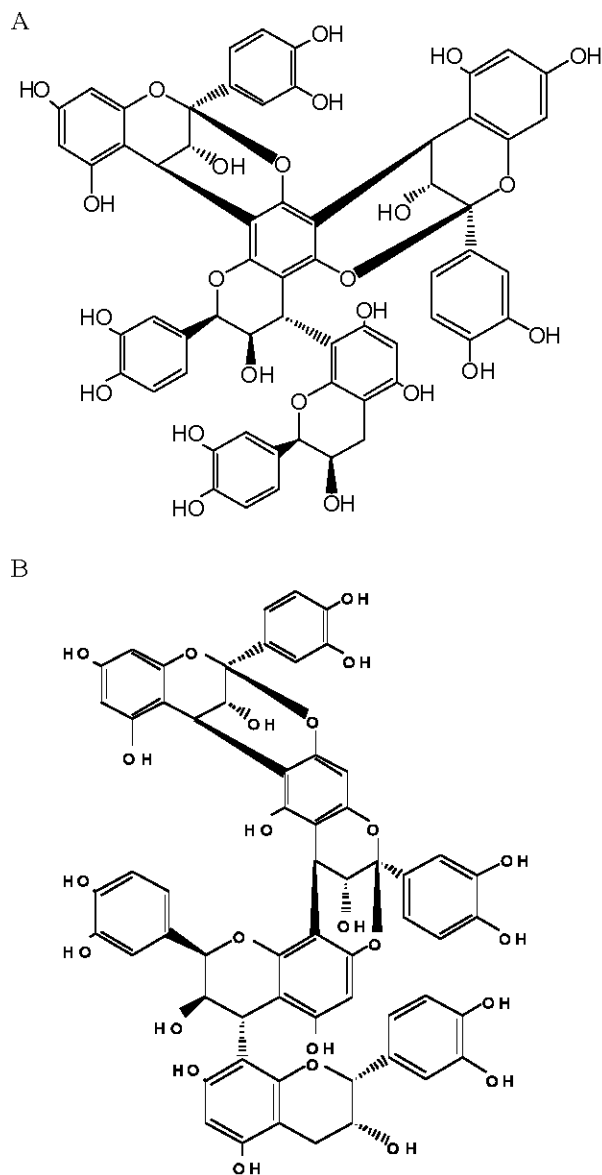


Fig. 1: Chemical structures of parameritannins A-2 and A-3. (A) parameritannin A-2; (B) parameritannin A-3

cell cycle and parameritannin A-3 especially has been shown to lead to induction of apoptosis, we considered the possibility that parameritannins may have a possible role in cancer therapy. However, their cytotoxic effect cannot yet be clearly assigned to a specific cellular target. Topo-mediated DNA damage seems to be a candidate mechanism, as well by which some flavonoids may exert their cytotoxic potential. Interestingly, parameritannin A-3 inhibited topoisomerase I and II activities, while parameritannin A-2 inhibited the activity of topoisomerase II but not topoisomerase I, although these two compounds are isomers. Thus, these

compounds seem to be an ideal model not only for the study of molecular mechanisms that inhibit topoisomerase I and II activities for the development of new anticancer drugs, but also of many cellular processes such as DNA replication, repair and recombination and acting in harmony with each other. In this study, we determined the inhibitory mechanisms of parameritannin A-3 against topoisomerase I and II activities *in vitro* and apoptosis induction in cultured cells.

## MATERIALS AND METHODS

### Isolation of proanthocyanidine compounds:

Parameritannins A-2 and A-3 were isolated from the bark of *Parameria laerigata* Moldenke by the method described previously<sup>[14,15]</sup>. Briefly, they were obtained from proanthocyanidine fraction by repetitive column chromatography on Sephadex LH-20 using MeOH-H<sub>2</sub>O (2:1), Rp-18 using acetonitrile-H<sub>2</sub>O and MeOH-H<sub>2</sub>O systems and silica gel using EtOAc. The structures of these compounds were elucidated by spectroscopic analysis and chemical evidences. Chemical structures of parameritannins A-2 and A-3 were shown in Fig. 1A and B, respectively.

**Enzyme, DNA and chemicals:** Topoisomerase I and II (2 units/mL) were purchased from TopoGen Inc. (Columbus, OH). Supercoiled pBR322 were purchased from Takara (Tokyo, Japan). All other reagents were of analytical grade.

### Measurement of topo relaxation activity and topo-DNA interaction:

Relaxing activity of topoisomerase I was determined by detecting the supercoiled plasmid DNA in its relaxed form. Topoisomerase I reaction was performed in 20  $\mu$ L of reaction mixture containing 10 mM Tris-HCl (pH 7.9), pBR322 DNA (250 ng), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol and 2 units of topoisomerase I. Relaxing activity of topoisomerase II was analyzed in the same manner as described except that the reaction mixture contained 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM ATP, 0.5 mM DTT, supercoiled pBR322 DNA (250 ng), 2 units of topoisomerase II. The reaction mixtures were incubated at 37°C for 30 min followed by 1% SDS and 1 mg mL<sup>-1</sup> proteinase K digestion and then 2  $\mu$ L of loading buffer was added consisting of 5% sarkosyl, 0.0025% bromophenol blue and 25% glycerol. For the study of binding of enzyme and DNA by mobility shift, SDS denaturation and proteinase K digestion were omitted. The mixtures were subjected to 1% agarose gel electrophoresis in TBE buffer. The agarose gel was stained with ethidium bromide (EtBr) and DNA was visualized under UV light.

**DNA intercalating measurement:** The intercalation profiles of double-stranded DNA (dsDNA) with or without parameritannins were determined with a fluorescence emission spectra using RF-1500 (Shimadzu, Kyoto). Calf thymus dsDNA ( $2 \mu\text{g mL}^{-1}$ ) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing  $0.5 \mu\text{M}$  EtBr and 1% dimethylsulfoxide (DMSO) at  $25^\circ\text{C}$ . The emission spectra were measured upon excitation of 520 nm. Any change in the absorbance of the compound at each wavelength point (550-630 nm) was automatically subtracted from that of DNA plus the compound in the fluorescence meter.

**Investigation of cytotoxicity on cultured cells:** For investigation of the effects of parameritannins in cultured cells, a human gastric cancer cell line NUGC-3 derived from a patient with cancer was used. NUGC-3 was obtained from Health Science Research Bank (Osaka, Japan). The cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum,  $100 \mu\text{g mL}^{-1}$  streptomycin, 100 unit/mL penicillin and  $1.6 \text{ mg mL}^{-1}$   $\text{NaHCO}_3$ . The cells were routinely cultured at  $37^\circ\text{C}$  in standard medium in a humidified atmosphere of 5%  $\text{CO}_2$ -95% air. The cytotoxicity of parameritannins was investigated as follows. High concentrations (10 mM) of the compounds were dissolved in DMSO and stocked. Approximately  $1 \times 10^4$  cells per well were inoculated in 96 well micro plates, then the compound stock solution was diluted to various concentrations and applied to each well. After incubation for 48 h, the survival rate was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay<sup>[21]</sup>.

**Cell cycle analysis:** Cellular DNA content for cell cycle analysis was determined by flow cytometry. The cells ( $3 \times 10^5$  cells in a 35 mm dish) were collected by trypsinization and washed with ice-cold PBS by centrifugation. The cells were suspended in PBS, fixed with 70% ethanol (v/v) and stored at  $-20^\circ\text{C}$ . The cells were collected by centrifugation and stained with 4'-6-diamidino-2-phenylindole (DAPI,  $2 \mu\text{g mL}^{-1}$ ) for at least 20 min at room temperature in the dark. The DNA content was analyzed using a cell counter analyzer (Partec, CCA model, Münster, Germany) with Multicycle 3.11 software (Phoenix Flow Systems, San Diego, CA). The cell debris and fixation artifact were gated out.

**DNA fragmentation:** DNA fragmentation was determined by electrophoresis in 1.5% agarose gel. The cells were lysed with 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 0.5% Triton X-100 and RNase A ( $0.2 \text{ mg mL}^{-1}$ ) and incubated at  $37^\circ\text{C}$  for 1 h followed by digestion with

proteinase K ( $0.5 \text{ mg mL}^{-1}$ ) at  $50^\circ\text{C}$  for 30 min. After the addition of  $\frac{1}{2}$  vol. 10 M ammonium acetate, the DNA was precipitated with 2.5 vol. ethanol, dissolved in gel loading buffer (40 mM Tris-5 mM sodium acetate-1 mM EDTA, pH 7.8) and separated by electrophoresis in a 1.5% agarose gel. The gel was stained with EtBr and the DNA bands were visualized under UV light.

**Western blot analysis:** The cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM leupeptin). The lysate was fractionated on SDS-PAGE then blotted on a PVDF membrane. The blots were subsequently incubated with the desired primary antibody. After rinsing, the membranes were incubated with horseradish peroxidase-linked secondary antibody. The proteins were then detected with an enhanced chemiluminescence detection system (Perkin-Elmer Life Science, Boston, MA). Zero-D scan (version 1.0, M and S Instruments Trading) was used for densitometric quantification.

**Immunoprecipitation:** For immunoprecipitation, protein G sepharose beads were incubated with monoclonal anti-PARP antibody and then washed with TBS-T. The beads were resuspended TBS-T containing 0.1% BSA. Cell lysates was combined with protein G preequilibrated with lysis buffer and then incubated with constant rotation at  $4^\circ\text{C}$  for 16 h. After extensive washing, proteins were eluted from the beads with SDS sample buffer and then separated by SDS-PAGE. Proteins were detected by immunoblotting.

## RESULTS

**Inhibition of topo activity by parameritannins:** For the screening of topo-targeted drug effects, we used a strategy based on the alteration of the electrophoretic mobility of pBR322 plasmid DNA by DNA relaxation assay *in vitro*. As shown in Fig. 2, the mobility of the naturally supercoiled closed circular double-stranded plasmid DNA increases upon topo-mediated relaxation. Among parameritannins tested, only parameritannin A-3 was able to inhibit both topoisomerase I- and II-mediated relaxation (Fig. 2A, lane 3-7, Fig. 2B, lane 3-6). Since the  $\text{IC}_{50}$  values of parameritannin A-3 with topoisomerase I and topoisomerase II activities were 50 and  $0.5 \mu\text{M}$ , respectively, the inhibitory effect of parameritannin A-3 against topoisomerase II activity was stronger than that against topoisomerase I. In addition, parameritannin A-2 inhibited topoisomerase II-mediated relaxation of supercoiled DNA as well as parameritannin A-3.

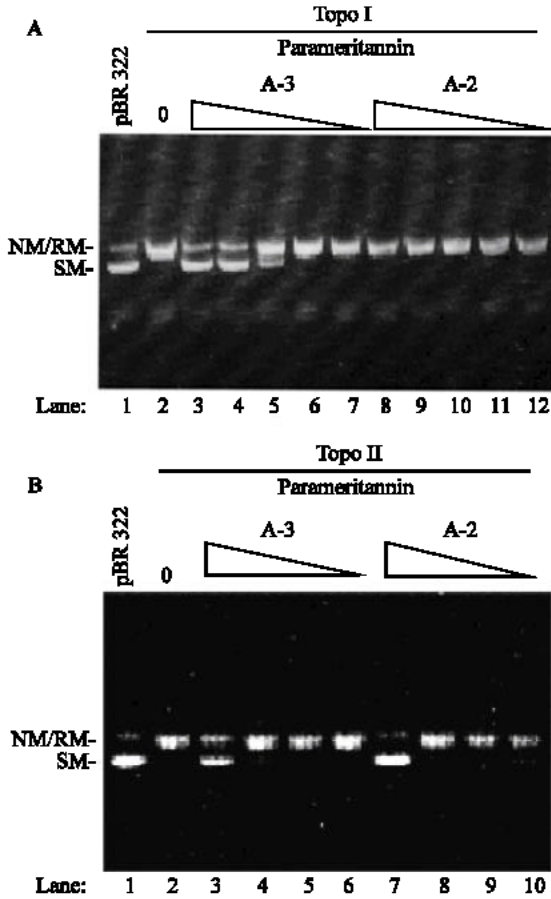


Fig. 2: Inhibition of catalytic activity of human topoisomerase I and II by parameritannins A-2 and A-3. Simultaneous addition of enzyme, parameritannins and DNA. (A) Lane 1, supercoiled pBR322 DNA; lanes 2-12, DNA with 2 units of purified human topoisomerase I; lanes 3-7, inhibition of catalytic activity in the presence of 250, 100, 25, 5, 1  $\mu\text{M}$  parameritannin A-3; lanes 8-12, inhibition of catalytic activity in the presence of 250, 100, 25, 5, 1  $\mu\text{M}$  parameritannin A-2. (B) Lane 1, supercoiled pBR322 DNA, lanes 2-10, DNA with 2 units of purified human topoisomerase II; lanes 3-6, inhibition of catalytic activity in the presence of 1, 0.2, 0.04, 0.008  $\mu\text{M}$  parameritannin A-3; lanes 7-10, inhibition of catalytic activity in the presence of 1, 0.2, 0.04, 0.008  $\mu\text{M}$  parameritannin A-2. Positions of supercoiled monomer (SM), nicked monomer (NM) and relaxed monomer (RM) are indicated

(Fig. 2B, lane 7-10), but not that of topoisomerase I activity (Fig. 2A, lane 8-12). Although parameritannins A-2 and A-3 were tetrameric polyphenols and the chemical structures of them were very similar, it was found that the inhibitory activity of topoisomerase I by parameritannin A-3 was different from that by parameritannin A-2.

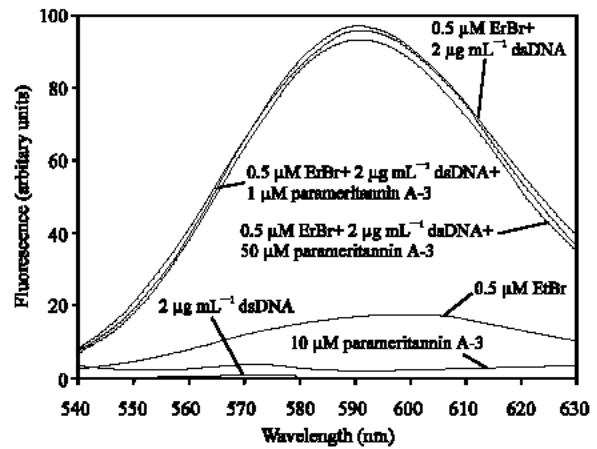
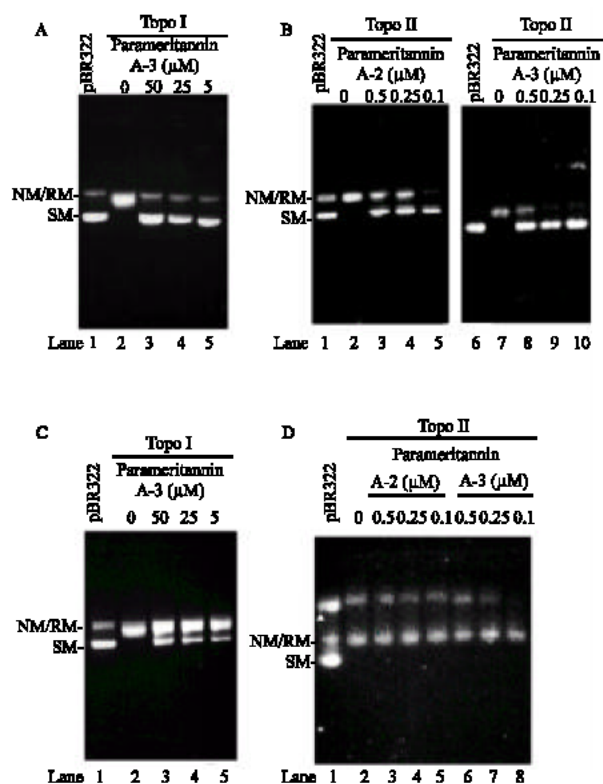


Fig. 3: Fluorescence emission spectra of EtBr-dsDNA complex in the presence of parameritannin A-3. Zero, 1 and 50  $\mu\text{M}$  parameritannin A-3 were incubated with 2  $\mu\text{g mL}^{-1}$  of calf thymus dsDNA, 0.5  $\mu\text{M}$  ethidium bromide (EtBr) in 0.1 M Na-phosphate buffer (pH 7.0). The excitation wavelength was 520 nm

**Influence of parameritannin A-3 on the binding of double-stranded DNA:** To determine whether the parameritannin A-3 binds to DNA, the fluorescence emission spectra of ethidium bromide (EtBr) in the presence of dsDNA and the compound was measured (Fig. 3). As described in Materials and Methods, calf thymus dsDNA at 2  $\mu\text{g mL}^{-1}$  was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% DMSO. When EtBr was intercalated with dsDNA, the fluorescence of EtBr-dsDNA complex increased and the maximum emission wavelength was 595 nm. At high concentrations (i.e., 10  $\mu\text{M}$ ) of parameritannin A-3, no decrease of the fluorescence of EtBr was observed. Thus, parameritannin A-3 could not replace EtBr, suggesting that it did not bind or intercalate with the dsDNA. Parameritannin A-3 must inhibit the enzyme activities by interacting with the enzymes directly. The same results were obtained with parameritannin A-2 instead of parameritannin A-3 (data not shown).

**Acting irreversibly of parameritannins against the enzyme:** Since parameritannins A-2 and A-3 inhibited the catalytic activity of human topoisomerase I, topoisomerase II or both, the next and most important issue was to understand the mechanism of inhibition. It is not clear whether parameritannins A-2 and A-3 are acting reversibly or irreversibly against the enzyme. This critical matter has been sorted out by dilution experimentation. Figure 4A proves that under non-denaturing conditions a non-covalent complex of topoisomerase I or II and pBR322 DNA is



**Fig. 4:** Irreversible interaction of topoisomerase enzymes and parameritannins A-2 and A-3. (A) Topo I (2 units) was preincubated with or without 50  $\mu\text{M}$  parameritannin A-3 for 5 min at 37°C (lanes 3 and 2, respectively), then diluted 2 and 10 fold at the final drug concentrations to 25  $\mu\text{M}$  (lane 4) and 5  $\mu\text{M}$  (lane 5), respectively and lane 1 was supercoiled pBR322 DNA alone. Reactions were performed at 37°C for 15 min after addition of 250 ng of supercoiled pBR322 DNA. (B) Topo II (2 units) was preincubated with 0.5  $\mu\text{M}$  parameritannins A-2 and A-3 for 5 min at 37°C (lanes 3 and 8, respectively), then diluted 2- and 5-fold at the final drug concentrations to 0.25  $\mu\text{M}$  (lanes 4 and 9) and 0.1  $\mu\text{M}$  (lanes 5 and 10) respectively. © Relaxation of supercoiled pBR322 DNA (lane 1) in the presence of 2 units of topo I (lane 2) and additional incubation in the presence of 50  $\mu\text{M}$  (lane 3), 25  $\mu\text{M}$  (lane 4) and 5  $\mu\text{M}$  (lane 5) parameritannin A-3, respectively. (D) Relaxation of supercoiled pBR322 DNA (lane 1) in the presence of 2 units of topo II (lane 2) and additional incubation in the presence of 0.5  $\mu\text{M}$  (lane 3), 0.25  $\mu\text{M}$  (lane 4) and 0.1  $\mu\text{M}$  (lane 5) parameritannin A-2 and 0.5  $\mu\text{M}$  (lane 6), 0.25  $\mu\text{M}$  (lane 7) and 0.1  $\mu\text{M}$  (lane 8) parameritannin A-3, respectively. Positions of supercoiled monomer (SM), nicked monomer (NM) and relaxed monomer (RM) are indicated

formed, which is immobile in agarose gel electrophoresis. To show this, DNA and topo I or topo II were incubated together and subsequently subjected to agarose gel electrophoresis carried out in TBE buffer. Topos I (Fig. 4A) and II (Fig. 4B) were preincubated with 50  $\mu\text{M}$  and 0.5  $\mu\text{M}$  parameritannins, respectively. The reaction mixtures were diluted 2- and 10 or 5 fold so that the final drug concentration became 25 and 5  $\mu\text{M}$  and 0.25 and 0.1  $\mu\text{M}$ , respectively. Drug-control reactions showed relief of inhibition on 5  $\mu\text{M}$  parameritannin A-3 against topo I activity, however, complete inhibition showed continuously on dilution, suggesting that parameritannin A-3 acts irreversibly against topo I (Fig. 4A, lane 4 and 5). Moreover, the inhibition by parameritannins A-2 (Fig. 4B, lane 3-5) and A-3 (Fig. 4B, lane 8-10) against topo II also acts irreversibly.

In addition, to extrapolate the mode of interaction between parameritannins and DNA and its effect, the ability of parameritannins to intercalate with DNA was determined by topo-catalyzed unwinding assay (Fig. 4C and D). The relaxed substrate DNA was induced by treatment with topoisomerases. In the presence of parameritannins, generation of a negative supercoiling of relaxed substrate DNA was observed. At a concentration which inhibited topo I activity, parameritannin A-3 induced a negative supercoiling DNA (Fig. 4C, lane 3-5). On the other hand, parameritannins A-2 and A-3 against topo II did not induce negative supercoiling DNA (Fig. 4D lane 3-5 and lane 6-8, respectively). These results suggest that parameritannin A-3 intercalates with DNA-topo I complex and neither parameritannin A-2 nor A-3 intercalates with DNA-topo II complex.

**The inhibitory effect of parameritannin-enzyme direct interaction:** The data shown in Fig. 2 give a clear indication of the inhibition of the catalytic activities of topoisomerases I and II. This effect of parameritannins A-2 and A-3 could be likewise obtained by interaction with partially relaxed plasmid DNA or by a direct interaction of the drug with the enzyme itself. In order to distinguish between these possibilities we carried out the experiments of electrophoretic mobility shift assay (EMSA) shown in Fig. 5. When pBR322 plasmid DNA was first completely relaxed by topo I or topo II and then incubated with parameritannin A-2 or A-3, the electrophoretic mobility of the relaxed plasmid DNA did not change, indicating that parameritannins A-2 and A-3 do not intercalate into the DNA. On the other hand, the inhibitory effect of parameritannin A-3 on topo I-mediated DNA relaxation can be enhanced by preincubation with the enzyme before addition of the DNA plasmid. Upon preincubation with enzyme, effective inhibition of topo I-catalyzed pBR322 DNA relaxation could already be observed at 5  $\mu\text{M}$  concentrations, in contrast with 25  $\mu\text{M}$  without

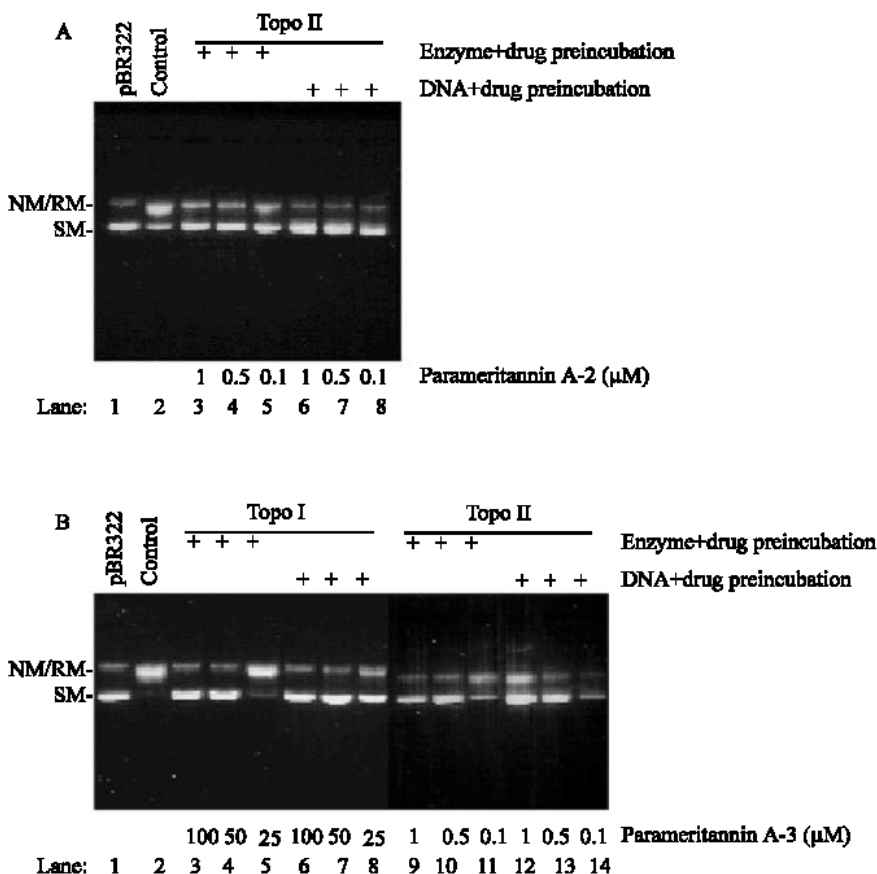


Fig. 5: Preincubation experiment by electrophoretic mobility shift assay (EMSA). (A) Relaxation of supercoiled pBR322 DNA (lane 1) in the presence of topo II 2 units (lane 2). Preincubation of parameritannin A-2 with topo II (lanes 3-5) and with DNA (lanes 6-8), respectively. (B) Relaxation of supercoiled pBR322 DNA (lane 1) in the presence of topo I 2 units (lane 2). Preincubation of parameritannin A-3 with topo I (lanes 3-5) and DNA (lanes 6-8), with topo II (lanes 9-11) and DNA (lanes 12-14), respectively. Positions of supercoiled monomer (SM), nicked monomer (NM) and relaxed monomer (RM) are indicated

preincubation. Recently, quercetin inhibited topo I inhibition by stabilizing the covalent topo I-DNA intermediate and binding with the free enzyme<sup>[22]</sup>. In addition, it was already known to act on topo II relegation activity in a similar way, as shown for topo I<sup>[23]</sup>. Moreover, luteolin inhibited topo I activity by binding to both enzyme and DNA individually<sup>[16]</sup>. Therefore, it was suggested that parameritannin A-3 binds to both DNA and enzyme and may bind to enzyme primarily because of the data of influence of parameritannin A-3 binding of dsDNA (Fig. 3).

**Effects of parameritannins on cultured mammalian cells:** The topoisomerases have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. The parameritannins from *P. laerigata*

could, therefore, be useful as a new anti-cancer chemotherapy agent. We tested the cytotoxic effect of parameritannins A-2 and A-3 against a human stomach cancer cell line, NUGC-3 *in vitro*.

As shown in Fig. 6, the cell growth inhibition of parameritannins A-2 and A-3 were dose-dependent and the concentrations required for the LD<sub>50</sub> were 15.0 and 12.5  $\mu$ M, respectively. The inhibitory activity for cell growth by parameritannin A-3 was slightly stronger than that by parameritannin A-2. The LD<sub>50</sub> value of parameritannin A-3 was approximately 25 fold higher than the IC<sub>50</sub> values *in vitro* for topo II. The inhibition curves of the enzymes and cell growth showed parallel dose-dependent reductions. Parameritannins were, therefore, suggested to inhibit the activities of topoisomerases in the intact cell. These observations suggested that the cell

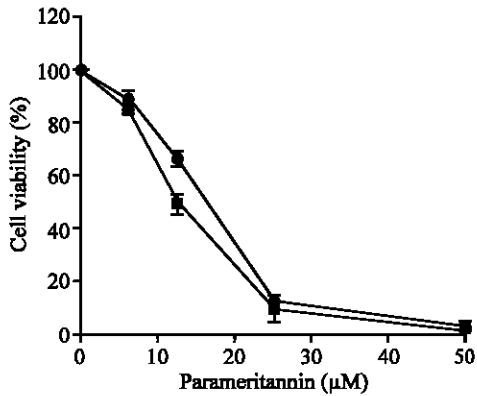


Fig. 6: Dose-response curves of cancer cell growth inhibition. Parameritannins A-2 (closed circle) and A-3 (closed square). Inhibitory assay of cell growth of the human stomach cancer cell line NUGC-3 were carried out under the conditions with compounds at the indicated concentrations. Survival rate was determined by MTT assay<sup>[21]</sup>. Values are shown as means  $\pm$  SEM for three independent experiments

growth inhibition occurred in a manner dependent on the enzyme inhibition and that inhibition of the enzymes influenced cell growth *in vivo*.

To confirm whether cell cycle events mirrored topo inhibition, we determined the effect of parameritannins on cell cycle distribution. As shown in Fig. 7, the cells accumulated primarily in S phase and then increased G2/M and G1 phase accumulation was observed after 24 h by both parameritannins A-2- and A-3-treatment. In the case of parameritannin A-2, the percentage of the cells in the S phase increased from 34.4 to 47.7% after 4 h incubation. The percentage of the cells in the G1 decreased from 37.0 to 22.9%. After 24 h, the percentage of the cells in the G2/M and G1 phases increased from 28.6 to 34.8% and 37.0 to 46.3%, respectively. On the other hand, the percentage of the cells in the S phase decreased from 34.4 to 18.9%, mirroring the increase of G1 and G2/M phase. In the case of parameritannin A-3, the percentage of the cells in the S phase increased from 34.4 to 48.6% after 4 h incubation. The percentage of the cells in the G1 was decreased from 37.0 to 18.0% and that in the G2/M phase was increased from 28.6 to 33.4%. After 48 h, the percentage of the cells in the G2/M and G1 phases increased from 28.6 to 44.3% and 37.0 to 44.7%, respectively. On the other hand, the percentage of the cells in the S phase decreased from 34.4 to 11.0%. Topo I poison increased S phase population reflecting S phase slowing because of activation of the checkpoint by replication-induced conversion of the stabilized

DNA-topo I complexes into frank DNA double strand breaks (dsb)<sup>[24-27]</sup>. On the other hand, topo II poisons arrested the cells in the G2/M phase reflecting the higher expression of topo II in S and G2/M phase cells<sup>[28,29]</sup>. These results indicated that the cell cycle events mirrored topo II inhibition fairly well.

**Apoptosis induction by parameritannins:** Topo I inhibitor, camptothecin (CPT) and topo II inhibitors, teniposide (VM-26) and etoposide (VP-16), were reported to induce apoptosis in a cancer cell line<sup>[8,30,31]</sup>. Next, we determined apoptosis induction by parameritannins A-2 and A-3. When cells were treated with only parameritannin A-3, DNA fragmentation was detected (Fig. 8A).

To determine the apoptotic pathway induced by parameritannin A-3, we examined the expression of p53, bcl-2 and bax proteins by Western blotting using their specific antibodies (Fig. 8B and C). Bax is a member of the bcl-2 gene family, acting as a promoter of cell death and present predominantly in the cytosol. However, partial translocation to the mitochondria could occur on activation of caspase cascade, resulting in apoptosis induction<sup>[32,33]</sup>. The p53 expression increased in parameritannin A-3-treated cells but did not change in parameritannin A-2-treated cells (Fig. 8B). After 24 h treatment with parameritannin A-3, the level of the anti-apoptotic protein bcl-2 slightly decreased. On the other hand, apoptotic protein bax increased significantly (Fig. 8C). The parameritannin A-3-induced apoptosis is suggested to be associated with the stabilization of the p53 protein and subsequently, the expression of the bax protein.

**Induction of PARP expression by parameritannins:** Topos I and II play critical roles in the removal of superhelical tensions generated during transcription and DNA replication<sup>[16,17]</sup>. Topo drugs involve the formation of a stabilized DNA cleavage complex<sup>[4-7, 34]</sup>. Because these intermediates contain strand breaks, they need to be repaired by base excision repair machinery<sup>[35,36]</sup>. Poly (ADP-ribose) polymerase (PARP) is a repair molecule based on the observation that enzymatic activity is strongly enhanced following DNA damage. PARP is activated by DNA strand breaks to play a key role in single strand break (ssb) repair by its rapid binding to ssb and subsequent activation<sup>[37]</sup>. To determine PARP expression with topoisomerase I and II poisons, cells were treated with parameritannins A-2 and A-3 for 24 h, respectively. Western blot analyses were carried out to assess PARP levels in the cells (Fig. 9). The cells treated with parameritannin A-2 increased PARP expression, but not



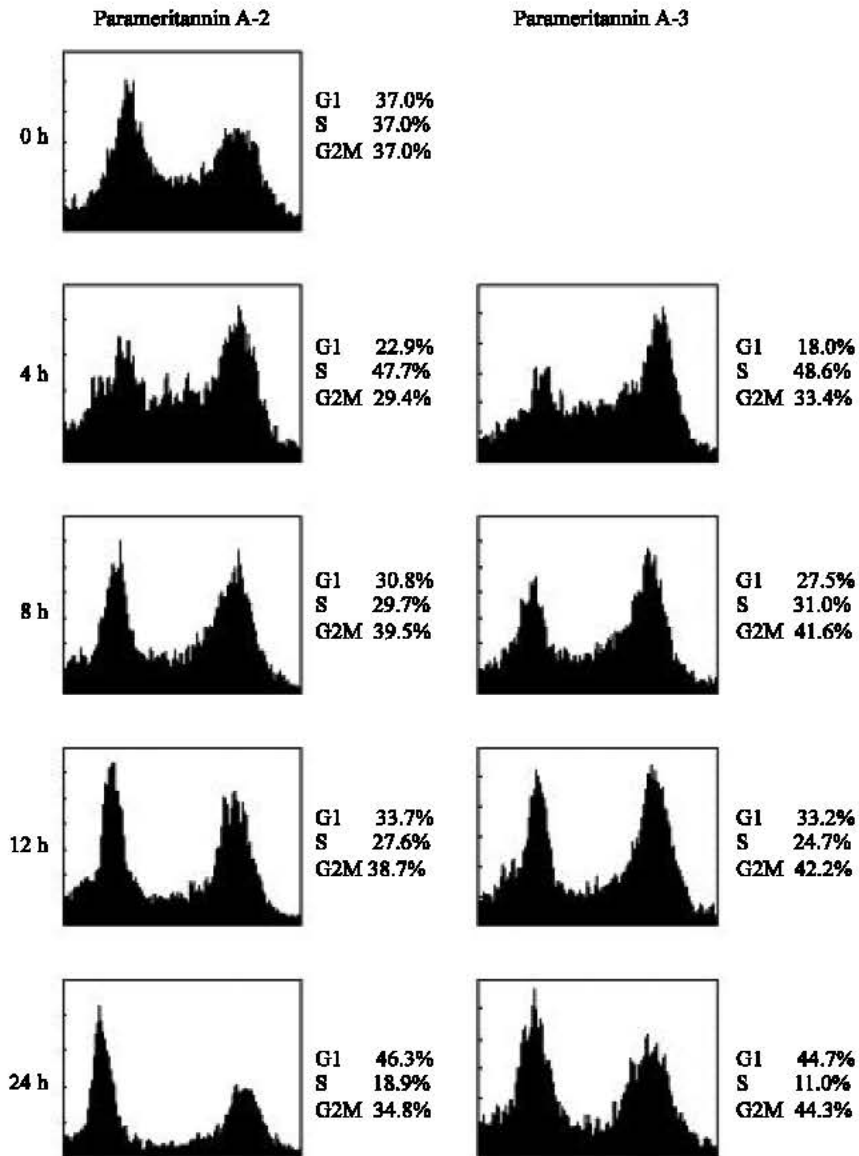


Fig. 7: Effect of parameritannins A-2 and A-3 on cell cycle. Flow cytometric analysis of NUGC-3 cells were treated with 15.0  $\mu$ M parameritannin A-2 and 12.5  $\mu$ M parameritannin A-3 for 24 h. The cell cycle distribution was calculated as the percentage of cells containing G1, S and G2M phase

with parameritannin A-3, almost the same as control cells (Fig. 9A). XRCC1 is another protein that plays a central role in the repair of ssb. In biochemical studies, association of PARP with XRCC1 activated PARP in Base Excision Repair (BER)<sup>[38-40]</sup>. Next, we determined the accumulation of XRCC1 in the cells treated with parameritannins A-2 and A-3, respectively (Fig. 9A). XRCC1 also accumulated in the cells treated with parameritannin A-2. On the other hand, parameritannin A-3 did not change XRCC1 compared to untreated cells as well as PARP (Fig. 9A). Furthermore, in

immunoprecipitation with anti-PARP antibody in the cells treated with parameritannin A-2, PARP showed formation of the complex with XRCC1 (Fig. 9B). Since the majority of DNA ssb will not have ligatable termini, it is likely that end processing enzymes, such as DNA polymerase  $\beta$  (pol  $\beta$ ), are recruited to the DNA ligase III-XRCC1-DNA strand break complex via their interaction with XRCC1<sup>[41-44]</sup>. Quantification of pol  $\beta$  accumulation was analyzed by Western blotting (Fig. 9A). Pol  $\beta$  also accumulated in the cells treated with parameritannin A-2 as well as PARP and XRCC1. These results suggest that

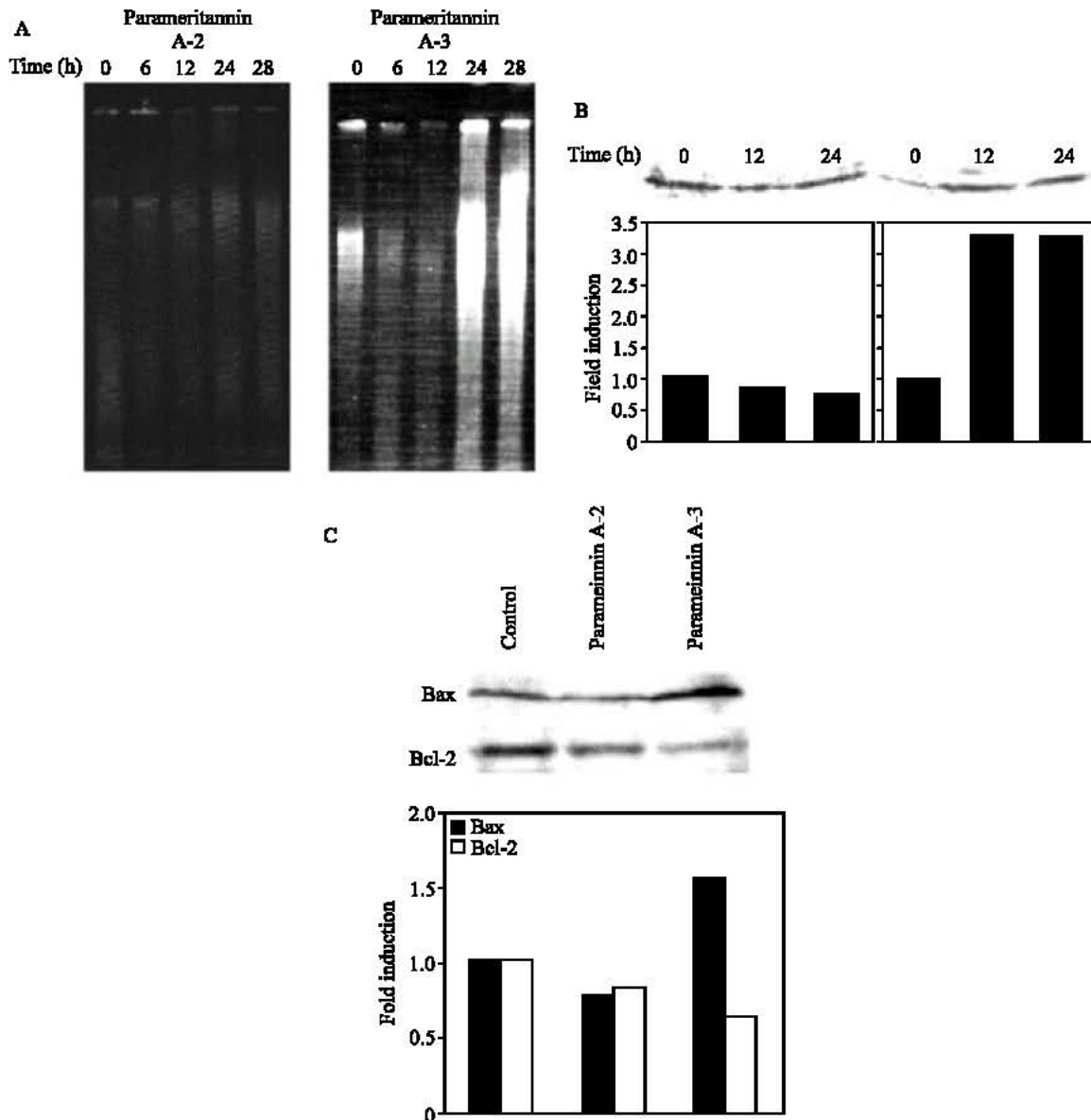


Fig. 8: Effect of parameritannins A-2 and A-3 on apoptosis. (A) NUGC-3 cells were treated with or without 15.0  $\mu$ M parameritannin A-2 and 12.5  $\mu$ M parameritannin A-3 for indicated times. The formation of fragment DNA was analyzed by agarose gel electrophoresis. (B and C) NUGC-3 cells were treated with or without 15.0  $\mu$ M parameritannin A-2 and 12.5  $\mu$ M parameritannin A-3 for 24 h. p53, Bax and Bcl-2 expressions were analyzed by Western blotting. Densitometric analysis of the proteins was performed and fold induction was calculated

topo II poison is probably associated with the formation of complex including at least PARP, XRCC1 and pol  $\beta$  in the BER pathway.

#### DISCUSSION

In this study, it was determined whether parameritannins A-2 and A-3 inhibited human topoisomerase activities and arrested cell cycle. Parameritannin A-2

selectively inhibited topoisomerase II activity, however, parameritannin A-3 inhibited the activities of both topoisomerase I and II (Fig. 2). Cell cycle was arrested at the G2/M phase with both parameritannins A-2 and A-3 (Fig. 7). The enzyme-parameritannin interaction was irreversible, as studied by the dilution experiment with both parameritannins A-2 and A-3 (Fig. 4A and B). In addition, interaction of parameritannins A-2 and A-3 with DNA was determined by two independent DNA-binding assays.

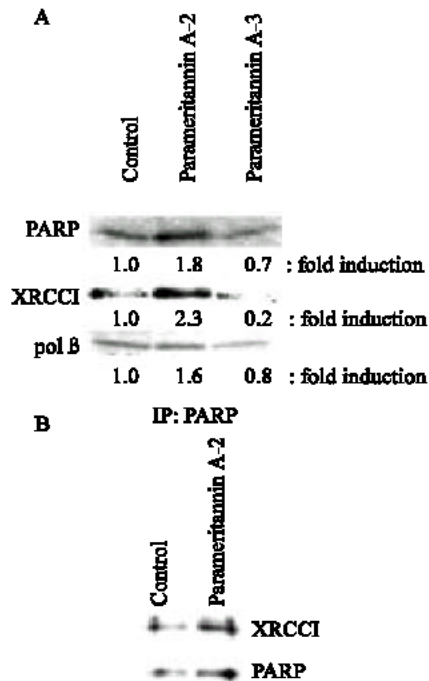


Fig. 9: Effect of parameritannins A-2 and A-3 on DNA repair related protein expression. (A) NUGC-3 cells were treated with or without 15.0  $\mu$ M parameritannin A-2 and 12.5  $\mu$ M parameritannin A-3 for 24 h. PARP, XRCC1 and pol  $\beta$  protein expressions were analyzed by Western blotting. Densitometric analysis of the proteins was performed and fold induction was calculated. (B) NUGC-3 cells were treated with 15.0  $\mu$ M parameritannin A-2 for 24 h. Whole cell extracts were immunoprecipitated (IP) with anti-PARP antibody and then detected with the indicated antibody

Parameritannin direct interaction with enzyme of DNA was determined by fluorescence spectrometry (Fig. 3) and electrophoretic mobility shift assay (EMSA) (Fig. 5). In the inhibitory effect of parameritannin A-2 which is preincubated with enzyme, topo II was the same as preincubated with substrate DNA. It was also found that parameritannin A-3 showed the same inhibitory effect as different preparations. However, parameritannin A-3 indicated the reduction of inhibitory effect when it was preincubated with DNA compared to the preincubation with topo I. It was deduced from these results that these agents inhibited topoisomerase activities by binding to both enzymes and DNA individually. Topoisomerase reaction has three general mechanistic steps, i.e. (I) binding of the enzyme to the substrate DNA, (ii) strand breakage and subsequent strand passage through the break leading to change in

linking number and (iii) strand re-ligation. The binding of parameritannin A-2 and topo II does not prevent this enzyme from its subsequent DNA binding, i.e. mechanistic step I of the topo-catalyzed reaction, as is evident from EMSA. Parameritannin A-2 did not intercalate with topo II and substrate DNA *in vitro* and *in vivo*. On the other hand, parameritannin A-3 intercalated with topo I and substrate DNA *in vivo* and did not intercalate with topo II and substrate DNA *in vitro* and *in vivo*. Parameritannin intercalated with substrate DNA, even though enzyme-DNA binary complex was cleaved. These results confirmed that parameritannins A-2 and A-3 bind to topo II and prevent the access of this enzyme to DNA, on the contrary, parameritannin A-3 interacts with DNA primarily rather than topo I and prevents the access of this enzyme to DNA. The effectiveness of these compounds to topo I activity inhibition was completely different. Natural flavonoid, quercetin<sup>[22]</sup> and luteolin<sup>[45]</sup> also inhibited topo activity by stabilizing the covalent enzyme-DNA cleavage complex. In order to stabilize the catalytic intermediate, these flavonoids need to form a ternary complex with topo-DNA substrate during the cleavable reaction, which does not affect the cleavage itself, but prevents a subsequent relegation reaction. It was suggested from these evidence that parameritannins A-2 and A-3 do not inhibit the formation of ternary complex and an essential space of hydrophobic pocket in the topo I molecule is affected by these differences of inhibition of topo I activity.

Inhibition of topo I and II activities by substances that stabilize the covalent enzyme-DNA intermediate is a longstanding and important therapeutic concept in cancer therapy<sup>[8,46-48]</sup>. It was prompted that drug-induced stabilization of covalent topo-DNA complex is not sufficient to insure cell death. Several discrete steps along the drug-induced death pathway have been delineated, including (a) the processing of stabilized cleavage complexes into frank DNA strand breaks; (b) sensing of DNA damage, leading to activation of stress-associated signaling pathways and cell cycle arrest and (c) activation of a preexisting group of enzymes and enzyme precursors, typified by caspase, which then catalyze the relatively orderly biochemical cascade of terminal events known as apoptosis. Camptothecin (CPT), etoposide, or actinomycin D (which stabilizes cleavage complexes involving both topoisomerase I and II) results in arrest of cells in the G1 and G2/M phases of the cell cycle. Genomic integrity and a proficient DNA repair process are essential for normal development and suppression of tumorigenesis. Topoisomerase poisons have been reported to activate a number of different signaling pathways. Some of the resulting signals appear to reflect

DNA damage and others appear to be intimately involved in the response to stress. PARP is an abundant 116 kDa zinc finger protein that recognizes and is activated by certain types of DNA damage<sup>[49,50]</sup>. PARP has been proposed to have roles in transcription, DNA replication and DNA repair through interactions with transcription initiation activators<sup>[51]</sup>, DNA polymerase  $\alpha$ <sup>[52,53]</sup> and DNA ligase III-XRCC1 complexes<sup>[54-57]</sup>, respectively. In addition, PARP has been proposed to be functionally linked to topo I and enhanced this activity<sup>[58]</sup>. The down regulation of topo I activity is inhibited by treatment of cells with a known PARP inhibitor, 3-aminobenzamide<sup>[59]</sup>. Recently it was reported that PARP has a supercoiled DNA relaxation activity by CPT like topo I and PARP seems to be a target for topo I inhibitor, CPT<sup>[60]</sup>. CPT inhibits topo I activity by stabilizing intermediate DNA-topo I complexes. On the other hand, topo I activity of PARP may not disrupt DNA replication and transcription in a manner similar to that of topo I inhibitory activity. In contrast CPT, topo II inhibitor VP16 had no effect on the relaxation activity<sup>[60]</sup>. Indeed, present results showed that parameritannin A-2 accumulated PARP, XRCC1 and pol  $\beta$  and detected direct interaction between PARP and XRCC1, but this phenomenon was not observed by parameritannin A-3. This result supported this evidence and it is hypothesized that parameritannin A-3 may inhibit tight association of topo I and PARP. Moreover, it was previously indicated that topo II poison generated an abasic site which is the initial step of BER pathway<sup>[61]</sup>. After base loss, abasic residues ultimately are converted to their original nucleotides by BER. Although not all forms of base damage enhance enzyme-mediated DNA cleavage, a variety of lesions that are generated by endogenous or environmental insults act as topo II poisons<sup>[62]</sup>. Irrespective of their initial effects on the enzyme, many of these lesions are repaired by BER pathways<sup>[63-65]</sup>. It was proposed that parameritannin A-2 induced cytotoxicity mediated through different from parameritannin A-3 and intermediate formed by parameritannin A-2 induced the activation of BER pathway. Parameritannin A-3 inhibited both topoisomerases I and II activities, inducing cell cycle arrest and apoptosis. These results suggest that cytotoxicity mediated by topo inhibitor are involved in multiple factors. Direct action toward the enzyme of parameritannins A-2 and A-3 remained unclear, but an indirect approach leading to the elaboration of putative models might help in our understanding of molecular drug-DNA and drug-enzyme interactions and to design new inhibitor.

In addition, we could investigate whether these agents could be a useful drug to develop a design

strategy for cancer chemotherapy agents. Indeed, since parameritannins could prevent the growth of NUGC-3 cancer cells and since the cells were halted at G2/M phase in the cell cycle, the agent should also be considered as the lead compound of a group of potentially useful cancer chemotherapy agents. These findings also suggest that because single ancient civilizations had both temperate and tropical zones, ancient Asian civilizations such as China and Indonesia found and succeeded in cultivating many medicinal plants and consequently, not only *Parameria laerigata* but many other traditional Asian medicinal plants could be very useful for screening novel cancer chemotherapy agents.

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