Xanthocidin Derivatives as Topoisomerase II α Enzymatic Inhibitors

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Few studies have examined xanthocidin, a biotic isolated from *Streptomyces xanthocidicus* in 1966, because its supply is limited. Based on its chemical structure, xanthocidin has the potential to become a lead compound in the production of agrochemicals and anti-cancer drugs; however, it is unstable under both basic and acidic conditions. We recently established the total synthesis of xanthocidin using the FeCl₃-mediated Nazarov reaction, and obtained two stable derivatives (#1 and #2). The results of the present study demonstrated that these derivatives exhibited the inhibitory activity of topoisomerase II α , known as a molecular target for cancer chemotherapy, and this was attributed to the respective *exo*-methylene ketone group without DNA intercalation. The results obtained also suggest that these derivatives may have value as lead compounds in the synthesis of topoisomerase II α inhibitors.

Key words xanthocidin; *Streptomyces xanthocidicus*; topoisomerase II α ; *exo*-methylene ketone group

Xanthocidin, isolated from *Streptomyces xanthocidicus* by Asahi *et al.* in 1966, was shown to have antibiotic activities against *Escherichia coli*, *Bacillus agri*, and *Xanthomonas oryzae*.¹⁾ Xanthocidin has a highly oxidized five-membered ring with contiguous *cis*-vicinal diol (–OH), carboxylic acid (–COOH), and conjugated *exo*-methylene group substituents (Fig. 1, upper panel).^{1,2)} Xanthocidin may be promising as a lead compound for the production of agrochemicals and anti-cancer drugs; however, it was shown to be unstable under both basic and acidic conditions.²⁾ In addition, few studies have assessed its bioactivity due to its limited supply.³⁾ We successfully established the total synthesis of xanthocidin using the FeCl₃-mediated Nazarov reaction,²⁾ and obtained two stable xanthocidin derivatives (#1 and #2) (Fig. 1, upper panel) that exhibited anti-proliferative effects on highly aggressive human breast cancer MDA-MB-231 cells.⁴⁾ Although the biological activities of these derivatives imply their possible use as anti-proliferative agents for cancer cells, their action point(s) have not yet been resolved.

Catalytic inhibitors of topoisomerase II α (Topo II α), without DNA intercalating potential, were previously shown to be useful in targeting tumors that expressed markedly higher levels of Topo II α (*i.e.*, MDA-MB-231 cells) than those of normal cells. Using biochemical analyses, we and other researchers demonstrated that the catalytic activity of Topo II α was sensitive to “Michael acceptors” due to the existence of nucleophilic thiol (–SH) residues in the active center.^{4–6)} Because xanthocidin derivatives (#1 and #2) contain an *exo*-methylene ketone group (a possible reactive electrophilic moiety, *see* Fig. 1), whether they behaved as an inhibitor of Topo II α through this group was investigated in the present study. We also analyzed their DNA intercalation potential. The results obtained

indicated that the xanthocidin derivatives have inhibitory activity of Topo II α as catalytic inhibitors of the enzyme, and that they did not have DNA intercalating potential.

MATERIALS AND METHODS

Reagents Xanthocidin derivatives (#1 and #2) were synthesized according to our previously established methods.²⁾ These synthesized compounds were purified by HPLC or column chromatography, and their purity (>98%) was confirmed by ¹H- and ¹³C-NMR spectroscopy. No ring-opened derivatives of the xanthocidins' lactones were detected in our analyses.^{2,4,5)} Amsacrine (m-AMSA) and etoposide (VP-16) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Ellipticine was purchased from ChromaDex Inc. (Irvine, CA, U.S.A.). All other reagents were of the highest grade commercially available.

Topo II α -Mediated DNA Relaxation and Cleavage Assays The human Topo II α enzyme was obtained from TopoGEN (Columbus, OH, U.S.A.) and negatively supercoiled pHOT1 DNA was used as a substrate for Topo II α (TopoGEN). The enzyme reaction and analysis of DNA relaxation/DNA cleavage were performed using the Topo II Drug Screening Kit according to the manufacturer's protocol (TopoGEN). In brief, to detect topoisomers that were relaxed DNA forms, reaction mixtures were subjected to 1% agarose gels cast in the absence of 0.5 μ g/mL ethidium bromide (EtBr) (non-EtBr gel) and electrophoresed in the absence of EtBr. When linear DNA was analyzed (Cleavage assay), 1% agarose gels were cast and run in the presence of 0.5 μ g/mL EtBr (EtBr gel). These gels were run at 50V for 60 min and either stained with EtBr (non-EtBr gel) or destained with water (EtBr gel).⁵⁾

DNA Intercalation Study An EtBr displacement fluorescence assay was employed to determine whether xanthocidin derivatives (#1 and #2) bind in the minor groove of DNA.^{5,7)} Experiments using DNA-intercalating Topo II inhibitors (m-AMSA, a positive control) were performed.

Data Analysis IC₅₀ values were determined using Sig-

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Fig. 1. Chemical Structures of Xanthocidin and Xanthocidin Derivatives (#1 and #2) and Three Established Topo II α Inhibitors (Etoposide, Also Known as VP-16, m-AMSA, and Ellipticine)

The *exo*-methylene ketone moiety, indicated with a gray inclusion, has been suggested to play a critical role in the inhibitory effects of these derivatives on MDA-MB-231 cell growth.⁴⁾

maPlot 11[®] software (Systat Software, Inc., San Jose, CA, U.S.A.), according to analyses described previously.^{4,5)} Differences were considered significant when the *p* value was calculated as less than 0.05. Significant differences between two groups were calculated by the Student's *t*-test. Other statistical analyses were performed by Scheffé's *F* test, a *post-hoc* test for analyzing the results of the ANOVA. Calculations were performed using Statview 5.0J software (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

We recently reported that xanthocidin derivatives (#1 and #2), used in the present study at 100 μ M (Fig. 1, upper panel), significantly abrogated the proliferation of MDA-MB-231 breast cancer cells.⁴⁾ In addition, a concentration of 100 μ M etoposide, a typical Topo II α inhibitor known as Topo II α poison, was required to significantly inhibit Topo II α .^{5,8)} Thus, we used the xanthocidin derivatives in combination with established Topo II α inhibitors including etoposide at 100 μ M. Topo II α has been shown to catalyze DNA relaxation after its transient introduction into DNA double-strand breaks (*i.e.*, linear DNA). After incubation with the two xanthocidin derivatives in the absence of EtBr, the reaction products were subjected to agarose gel electrophoresis under conditions that allowed the topoisomers (*i.e.*, relaxed DNA forms) characteristic of Topo II α catalytic activity to be detected. As shown in Fig. 2A (lanes 2 vs. 3), supercoiled DNA (indicated as SC) was converted to the relaxed form (indicated as topoisomers) by Topo II α -mediated enzymatic conversion. No significant effects were seen with vehicle alone (lanes 3 vs. 4). When experiments were performed in the presence of etoposide, m-

AMSA, and ellipticine (*see* structures and chemical natures in Fig. 1, lower panel), the relaxation of DNA from its SC conformation was inhibited by these inhibitors (lanes 5–7), although, as expected, ellipticine, which has the potential to bind covalently to DNA (lane 7), displayed a different profile from those of etoposide and m-AMSA. The inhibitory potentials of the two xanthocidin derivatives were similar to those of the established inhibitors (lanes 8 and 9 vs. 5–7). Low concentrations (10 μ M) of xanthocidins (#1 and #2) exhibited similar inhibitory potentials (data not shown) (*see* Fig. 2C, lane 5). We conducted Topo II α -mediated DNA relaxation assays using a method that distinguished the appearance of linear DNA (indicated as LIN) from more complex forms to clarify the inhibitory mode of the two xanthocidin derivatives. Given that xanthocidins act as a catalytic inhibitor of Topo II α and not a Top II poison, such as etoposide and m-AMSA, xanthocidin treatments were not expected to yield a "LIN" species. Reaction products were analyzed with agarose gel electrophoresis following incubation with the xanthocidins, etoposide, m-AMSA, or ellipticine in the presence of EtBr. The appearance of LIN DNA was only detected with etoposide+Topo II α and m-AMSA+Topo II α , but not with the xanthocidins+Topo II α and ellipticine+Topo II α , respectively (Fig. 2B, lanes 5 and 6 vs. 7–9 and *see* also the enlarged image placed below). Although etoposide exhibited its concentration-dependent production of LIN species (100 μ M, 500 μ M) (Fig. 2C, lanes 12 and 13), no LIN species were detected with xanthocidin derivative (#1) at concentrations up to 500 μ M (Fig. 2C, lanes 3–8 and *see* also the enlarged image placed below) (not shown for #2). In the absence of the Topo II α enzyme, LIN was not detected either in the presence of etoposide/m-AMSA or xanthocidins/ellipticine (data not shown), which suggested that LIN DNA was produced as a result of the interaction between Topo II α and etoposide or m-AMSA, and that the two xanthocidins may have an inhibition mode that differs from that of etoposide/m-AMSA (Topo II poisons) and ellipticine.

Certain chemicals that alter the gross structure of DNA were shown to markedly affect the catalytic activity of Topo II α , while some –SH interacting agents had the ability to bind directly to DNA.^{9,10)} Therefore, whether the two xanthocidins (#1 and #2) inhibition of Topo II α may occur through direct interactions with DNA was examined. The ability of xanthocidins at 100 μ M to displace EtBr from the minor groove of DNA was determined by an established fluorescence emission assay,^{5,7)} as DNA-bound EtBr has been shown to exhibit markedly stronger fluorescence emission than that of free EtBr. Although the DNA intercalator, m-AMSA (mA) at 100 μ M, was capable of displacing EtBr, neither xanthocidin (#1) nor xanthocidin (#2) significantly displaced EtBr (<1%) (Fig. 2D).

Taken together, these findings suggest that xanthocidins (#1 and #2) may be a catalytic inhibitor of Topo II α , which is not mediated by an interaction with DNA. Compounds containing an *exo*-methylene ketone group/*exo*-methylene lactone group are widely considered to be toxic to humans and animals, and this has been attributed to these substances reacting non-selectively with cellular macromolecules¹¹⁾; however, recent evidence strongly suggested that, for example, (–)-xanthatin, a component of *Xanthium strumarium* (Cocklebur) exhibited little or no toxicity to animals, with an LD₅₀ value of *ca.* 800 mg/kg.¹²⁾ Furthermore, parthenolide, a molecule containing an *exo*-methylene lactone group, which was identified as

Fig. 2. Xanthocidin Derivatives Inhibit Topo IIα without Direct Interaction with DNA

(A: DNA relaxation assay) The effects of xanthocidin derivatives (#1 and #2) and established Topo IIα inhibitors (etoposide, m-AMSA, and ellipticine) (100 μM) on DNA relaxation (pHOT-1) catalyzed by human Topo IIα (Lanes 1–9). Control incubation contained equivalent addition of vehicle (dimethyl sulfoxide; DMSO). (B: DNA cleavage assay) The effects of xanthocidin derivatives (#1 and #2) and established Topo IIα inhibitors (etoposide, m-AMSA, and ellipticine) (100 μM) on the human Topo IIα-mediated cleavage of DNA (pHOT-1) (Lanes 1–9). To better visualize linear DNA (indicated as LIN), an enlarged view of the upper panel is also presented (Lanes 5 and 6 with arrows). Control incubation contained equivalent addition of vehicle (DMSO). (C: DNA cleavage assay) The effects of the xanthocidin derivative (#1) (1, 10, 100, 250, 500 μM) and etoposide (100, 500 μM) on the human Topo IIα-mediated cleavage of DNA (pHOT-1) (Lanes 1–13). To better visualize linear DNA (indicated as LIN), an enlarged view of the upper panel is also presented (Lanes 12 and 13 with arrows). NC, nicked open circular DNA; LIN, linear DNA; SC, supercoiled DNA; topoisomers (RLX), relaxed forms of DNA. (D: DNA intercalation assay) The ability of 100 μM of the two xanthocidin derivatives (#1 and #2) or m-AMSA (mA) to interact with the minor groove of DNA was determined by a fluorescence-based ethidium displacement assay. Samples contained 1 μM EtBr and 5 nM double-stranded 40-mer DNA oligonucleotide. Ethidium fluorescence at 605 nm (λ_{max}) was monitored (510 nm excitation wavelength). Data are expressed as the percent of the vehicle-treated group (Control), as the mean ± S.D. ($n=8$). *Significantly different ($p<0.05$) from the two xanthocidins.

the major active component in Feverfew (*Tanacetum parthenium*), exhibited selective toxicity to human leukemic stem cells, but not to normal cells¹³; however, its anti-proliferative mechanism(s) have not yet been fully resolved. Since Topo IIα is known to be highly expressed in rapidly proliferating human cancer cells, xanthocidins (#1 and #2) may cause selective toxicity to cancer cells by inhibiting Topo IIα. Further studies are needed to establish the biological effects of the xanthocidins *in vivo*.

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