Cardenolides from *Antiaris toxicaria* as Potent Selective Nur77 Modulators

Miao-Miao Jiang, Yi Dai, Hao Gao, Xue Zhang, Guang-Hui Wang, Jia-You He, Qiong-Ying Hu, Jin-Zhang Zeng, Xiao-Kun Zhang, and Xin-Sheng Yao

College of Traditional Chinese Medicine, Shenyang Pharmaceutical University; Shenyang 110016, China; Institutes of Traditional Chinese Medicine and Natural Products, Jinan University; Guangzhou 510632, China; Institute for Biomedical Research, Xiamen University; Xiamen 361005, China; and Burnham Institute for Medical Research; La Jolla, CA 92037, U.S.A.

Nur77 (also known as TR3 or NGFI-B) is perhaps the most potent apoptotic member of the nuclear receptor superfamily. The death effect of Nur77 in cancer cells was originally observed in cancer cells treated with the retinoid-related molecule AHPN. Rapid induction of Nur77 has since been observed in cancer cells after stimulation of apoptosis by a variety of apoptosis-inducing agents, including calcium ionophores, etoposide (VP-16), phorbol ester, synthetic thienoexoycholic acid derivatives, di-n-butylin dichloride, histone deacetylase inhibitors, cadmium, and 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes.

*Antiaris toxicaria* Lesch. (Moraceae) is known as “upas tree” and widespread over tropic rain forest of Southeast Asia. Previous investigations concentrated on the latex of *A. toxicaria* in addition to the effect of the cardenolides on the activity of the ubiquitous cell surface enzyme Na+/K+-ATPase, recent studies have demonstrated that cardiac glycosides could inhibit the growth of cancer cells and induce their apoptosis via activation of caspase-3, induction of cytochrome C release from mitochondria, and generation of reactive oxygen species.

In our present work searching for components with anticancer activity, 11 cardenolides (Fig. 1) were isolated from the stem of *A. toxicaria* by bioassay-guided fractionation. Their structures were determined on the basis of spectroscopic analysis. All the reported compounds effectively inhibited the growth of various cancer cell lines at nanomolar concentrations. Inhibition of cancer cell growth was accompanied with induction of the expression of Nur77, a potent apoptotic member of the steroid/thyroid hormone receptor superfamily.

Key words: *Antiaris toxicaria*; cardenolides; Nur77; anticancer

Results and Discussion

Toxicarioside D (1), a new cardenolide, was isolated from the stem of *Antiaris toxicaria* Lesch. by bioassay-guided fractionation. Their structures were determined on the basis of spectroscopic analysis. All the reported compounds effectively inhibited the growth of various cancer cell lines at nanomolar concentrations. Inhibition of cancer cell growth was accompanied with induction of the expression of Nur77, a potent apoptotic member of the steroid/thyroid hormone receptor superfamily.

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Results and Discussion

Toxicarioside D (1), a new cardenolide, was isolated from the bioassay-guided chromatographic fractionation of the 60% ethanol extract of the stem of *A. toxicaria*, of which the structure was elucidated by comprehensive analysis of 1D and 2D NMR spectra.

Compound 1 was obtained as white powder. The molecular formula was established as C₁₇₄H₂₄O₁₀ by HR-ESI-MS. In the ¹H- and ¹³C-NMR spectra (Table 1), the characteristic signals of a lactone (δC 174.4), an olefin (δH 6.21, δC 117.7, 175.6), an oxygenated methylene (δH 5.27, 5.01, δC 73.6), and an anomeric sugar signal (δH 4.80, δC 100.8) indicated that 1 was an α,β-unsaturated γ-lactone cardenolide with a sugar residue, which was consistent with UV and IR absorptions at 217 nm and 3383, 1744, and 1716 cm⁻¹. The signals in the ¹H-NMR spectrum at δ 10.41 (1H, s, H-19) and 1.00 (3H, s, H-18) suggested a cardenolide skeleton with 19-CHO and 18-CH₂. The aglycone of 1 was established on the basis of its key HMBC and ROESY correlations (Fig. 2), and the ¹H- and ¹³C-NMR spectra were similar to those reported for 3-O-substituted strophanthidins. Thus the aglycone of 1 was identified as strophanthinidin. In addition, the doublet at δ 1.57 (3H, d, J = 5.7 Hz, H-6') indicated a C-6'-deoxy hexose residue.

According to the HMBC correlations of H-3/C-1’ and H-1/C-3, the sugar moiety was connected to C-3. The large coupling constant of anomeric proton H-1’ (1H, d, J = 8.0 Hz) indicated a β-configuration of the hexasaccharide moiety. In the HMBC spectrum, H-2’ correlated with the carbon signal of...
The growth inhibitory activity of all the compounds was examined in a panel of cancer cell lines, including NIH-H460, A549, and Calu-6 lung cancer, LNCaP prostate cancer, MCF-7 breast cancer, SW480 colon cancer, and HeLa ovarian cancer cell lines. The results demonstrated that these compounds exhibited significant inhibitory activities against the proliferation of these human cancer cell lines, with IC_{50} in nanomolar range (Table 2). The research on antitumor activity of these compounds provided more data on structure–activity relationships (SAR) of cardenolides. Compounds 1, 5, 6, 7, and 9 were more active than their corresponding aglycones: 2 and 3, respectively. Thus 3-glycosidation seems crucial for high cell-growth inhibition, which is consistent with literature data. Cytotoxic activities also could be remarkably altered after 12β-hydroxylation of aglycones (compounds 5/11 and 7/10), but the influence of the cytoxic activity of 5β-hydroxylation was complex. Additionally, activity against cancer cell-growth increased after hydrogenation at 19-CHO of aglycones (compounds 3/8 and 7/9) and the activity decreased after 2-O-methylation of sugar moieties (compounds 5/7 and 10/11). After all, aglycone glycosidation and 12β-hydroxylation seem necessary in terms of high cancer cell-growth inhibition. However, more experiments are needed to clarify this point.

The growth inhibitory effect of cardenolides might be attributed to their induction of apoptosis. Cardenolides promote apoptosis of cancer cells by inducing release of cytochrome C from mitochondria. To explore the potential molecules involved in mediating the apoptotic action of cardenolides, their effect on expression of a number of protein factors was examined. Because NIH-H460 is sensitive cell line to cardenolides compared with the other cell lines tested, NIH-H460 cells were used in our subsequent experiments. The most potent compounds 1, 4, 6, 7, and 8 were tested for their regulation of Nur77 expression at mRNA and protein levels. As shown in Fig. 3A, 4, 6, and 7 potently induced expression of Nur77 protein in NIH-H460 lung cancer cells. The inducing effect was observed when cells were treated with nanomolar concentrations of the compounds for as short as 6 h. Consistently, we found that the Nur77 transcripts were strongly upregulated by 4 and 6 in the same cells within nanomolar concentrations. At higher concentrations (10 μM),
Nur77 mRNA could also be elevated by 1 and 8 (Fig. 3B). Compound 7 appeared to stabilize Nur77 protein rather than to regulate Nur77 expression at the mRNA level. As a positive control, upregulation of Nur77 mRNA expression could also be seen in NIH-H460 cells treated with the phorbol ester 12-O-tetradecanoyl-13-phorbol acetate (TPA) at 100 ng/ml with extensively expressed Nur77 mRNA. Since Nur77 induction plays a critical role in mediating the death effect of a variety of apoptotic stimuli,1,2 our results suggest that induction of Nur77 may be a mechanism by which cardenolides exert their antitumor effects, and more investigations needed to be conducted to ascertain.

**Experimental**

**General** Optical rotations were measured by a JASCO P-1020 digital polarimeter. UV spectra were determined by JASCO V-550 UV/vis spectrophotometer in MeOH. IR spectra were acquired by JASCO FT/IR-480 polarimeter. UV spectra were determined by JASCO V-550 UV/vis spectrophotometer in MeOH. MS spectra were recorded on a VG Autospec Me spectrometer. ESI-mass spectra were taken on a Finnigan LCQ Advantage MAX mass spectrometer. HR-mass spectra were recorded on a Micromass Q-TOF mass spectrometer. 1D and 2D NMR spectra were measured on a Bruker AM-400 spectrometer (400 MHz for 1H, 100 MHz for 13C). Prep-HPLC was performed on a Varian chromatograph equipped with a Prostar 215 pump and a Prostar325 UV–vis detector with a C18 reversed-phase column (Cosmosil, 20×250 mm, 5 μm). Preparative high-performance liquid chromatography (HPLC) was run on a Varian chromatograph equipped with a Prostar 215 pump and a Prostar325 UV–vis detector with a C18 reversed-phase column (Cosmosil, 20×250 mm, 5 μm). Column chromatography was performed with silica gel (200–300 mesh, Qingdao Haiyang Chemical Group Corp., Qingdao, China), Sephadex LH-20 (Pharmacia) and ODS (60–80 mesh, Merck).

**Plant Materials** The stem of *A. toxicaria* was collected from Yunnan province of PR. China in August 2005 and identified by Prof. Hao Zhang, West China School of Pharmacy, Sichuan University. A voucher specimen (YANTO2005-08) was deposited in the Institute of Traditional Chinese Medicine & Natural Products, Jinan University.

**Extraction and Isolation** Air-dried plant material (6 kg) was minced finely then refluxed with 60% ethanol (50×1×2h) three times. The combined extract was concentrated under reduced pressure to afford a brown residue (300 g). The residue was suspended in water then partitioned successively with EtOAc and n-BuOH. The n-BuOH-soluble fraction exhibited significant cytotoxicity activity (IC50=0.625 μg/ml) against human lung carcinoma cells (NIH-H460), while the EtOAc-soluble fraction showed only weak activity. Then the n-BuOH extract (35 g) was subjected to open silica gel column (65.0×60 cm) and eluted with CHCl3/MeOH/H2O (75 : 1 : 0.05) to obtain compounds 4 (10 mg), 6 (20 mg), or 7 (20 mg) for the indicated time. Cell lysates were prepared and analyzed for expression of Nur77 protein by Western blotting using anti-Nur77 antibody. Expression of β-actin was used for loading control. (B) NIH-H460 lung cancer cells were treated with vehicle, TPA (100 ng/ml), or various cardenolides (10 μM, 1 μM, 0.1 μM) for 3 h. Nur77 mRNA expression was examined by RT-PCR method.
and 100 μg/ml streptomycin. By the standard trypsinization method, the cells were transferred into 96-well plates (1×10^4 cells per well) and incubated at 37°C in a 5% CO₂ incubator for 24 h. The medium was replaced by the growth medium containing various concentrations of each sample. The cells were incubated for a further 72 h, then cell proliferation was evaluated by an MTT assay procedure as previously described. Twenty milliliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (4.14 mg/ml) was added to each well and incubated at 37°C for 4 h. The formed formazan crystals were dissolved in 100 mM DMSO for 10 min with shaking. The UV/Vis absorbance at 570 nm was measured by microplate reader. Cytotoxicity was expressed as the concentration of the extract required to inhibit cellular growth 50% (IC_{50})±standard deviation (S.D.).

**Western Blotting Analysis for Nur77 Expression**
Nur77 expression was determined by Western blotting using polyclonal anti-Nur77 antibody as previously described. Briefly, cell lysates were boiled in SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide), and transferred to nitrocellulose. After transfer, the membranes were blocked in 5% milk in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing the anti-Nur77 antibody. The membranes were then washed three times with TBST, and incubated at room temperature for 1 h in TBST containing horseradish peroxidase-linked anti-immunoglobulin. After 3 washes in TBST, immunoreactive products were detected by chemiluminescence with an enhanced chemiluminescence system (ECL, Amer sham).

**RT-PCR Analysis**
NIH-H460 lung cancer cells seeded in six-well plate (5×10^3 cells/well) were allowed to adhere overnight and were starved for 12 h before treatment. After treatment with the compounds at desired concentrations, total RNA was isolated with Trizol LS (Invitrogen) and first-strand synthesis was performed with SuperScript II (Invitrogen) according to the manufacturer’s instructions. Two microliters of cDNA was used for PCR reactions in Eppendorf AG 22331 Hamburg (Eppendorf, U.S.A.) with primers specific to Nur77 (forward primer: 5′-TCA TGG AGC GCT ACA CAG-3′; reverse primer: 5′-GTA GGC ATG GAA TAG TTC-3′) and β-actin (forward primer: 5′-CTG GAG AAC TAC GAG-3′; reverse primer: 5′-TGA TGG AGT TGA AGG TAG-3′). Cycling conditions were 30 s denaturation at 94°C, 30 s annealing at 55°C, and 30 s elongation at 72°C for 35 cycles. In all, 5 μl of PCR reactions were electrophoresed on 2% agarose gels containing 0.1 mg/ml ethidium bromide in Tris-Borate-EDTA. Gel images were captured with a Gel Logic 200 system (Kodak, U.S.A.).

**Statistical Analysis**
Statistical significance of differences between groups was analyzed by Student’s t-test. Values of p<0.05 were considered significant.

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**References**