RESEARCH ARTICLE

Apoptosis Induction in Human Leukemic Promyelocytic HL-60 and Monocytic U937 Cell Lines by Goniothalamin

Ploingarm Petsophonsakul[1], Wilart Pompimon[2], Ratana Banjerdpongchai[1]*

Abstract

Goniothalamin is an active compound extracted from *Goniothalamus griffithii*, a local plant found in northern Thailand. Goniothalamin inhibits cancer cell growth but is also toxic to normal cells. The aims of this study were to identify the cytotoxic effect of goniothalamin and the mechanism of cell death in human HL-60 and U937 cells. Cytotoxicity was determined by MTT assay and cell cycle profiles were demonstrated by staining with propidium iodide (PI) and flow cytometry. Apoptosis was confirmed by staining with annexin V-FITC/propidium iodide (PI) and flow cytometry. Reduction of mitochondrial transmembrane potential was determined by staining with dihexyloxacarbocyanine iodide and flow cytometry and expression of Smac, caspase-8 and -9 was demonstrated by Western blotting. Goniothalamin inhibited growth of HL-60 and U937 cell lines. An increase of SubG1 phase was found in their cell cycle profiles, indicating apoptosis as the mode of cell death. Apoptosis was confirmed by the flip-flop of phosphatidylserine using annexin V-FITC/PI assay in HL60 and U937 cells in a dose response manner. Furthermore, reduction of mitochondrial transmembrane potential was found in both cell types while expression of caspase-8, -9 and Smac/Diablo was increased in HL-60 cells. Taken together, our results indicate that goniothalamin-treated human leukemic cells undergo apoptosis via intrinsic and extrinsic pathways.

Key words: Goniothalamin - apoptosis - *Goniothalamus griffithii* – caspase - human leukemic cells

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Introduction

Among constituents from the stems of *Goniothalamus griffithii* is goniothalamin (GTN), a plant bioactive styryl-lactone (Mu et al., 2003). GTN can also be extracted from several other species of Goniothalamus such as *Goniothalamus macrophyllus* (Alabsi et al., 2012), *Goniothalamus tapisoides* Mat Salleh (Kim et al., 2012) and *Goniothalamus andersonii* (Inayat-Hussain et al., 1999).

It is cytotoxic to and induces apoptosis in cancer cells such as cervical cancer (HeLa), breast carcinoma MCF-7 cell lines and colon cancer HT29 cells (Alabsi et al., 2012); Ca9-22 oral cancer cells (Yen et al., 2012); hepatoblastoma HepG2 cells (Al-Qubaisi et al., 2011); human lung cancer cells (Chiu et al., 2011); Jurkat T leukemia cells (Inayat-Hussain et al., 2010); and invasive breast carcinoma MDA-MB231 cell lines (Chen et al., 2005). It is also toxic to normal mouse fibroblast 3T3 cells (Alabsi et al., 2012), coronary artery smooth muscle cells (Chan et al., 2010), vascular smooth muscle cells (Chan et al., 2006). GTN induces apoptosis and autophagy in renal cancer cells and inhibits the nitric oxide synthase (NOS) activity and expression (de Fátima et al., 2008). Furthermore, goniothalamin induces genotoxicity in Chinese ovary hamster (CHO) cells by inducing chromosome aberration (Umar-Tsafe et al., 2004). GTN, a plant styrylpyrone derivative, induces apoptosis in Jurkat T-cells as assessed by the externalization of phosphatidylserine via caspase-3 and -7 activation (Inayat-Hussain et al., 1999). It induces leukemic HL-60 cell apoptosis via the loss of mitochondrial transmembrane potential and caspase-9 activation (Inayat-Hussain et al., 2003). Even though there are several reports of the apoptotic induction of GTN in many cancer cell lines, the involvement of extrinsic pathway remains elusive.

The aims of this study were to determine the cytotoxic effect of GTN on HL-60 and U937 leukemic cell lines and the mechanisms of cell death involving both mitochondrial (intrinsic) and death receptor (extrinsic) pathways. The differences between the U937 and HL60 subclones are pronounced, the latter expressing fucose residues, which might be part of the CD15 cell adhesion molecules. The differences of the carbohydrate residues between the two cell lines can attribute to their differentiation within the myelomonocytic cell lineage (Schumacher et al., 1996).

*[1]Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, [2]Faculty of Science, Lampang Rajabhat University, Lampang, Thailand *For correspondence: ratana.b@cmu.ac.th*

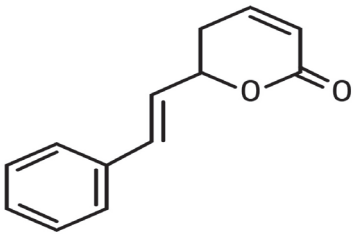

Figure 1. The Structure of Goniothalamin

Materials and Methods

Chemicals

Goniothalamin was purified from the stem of *Goniothalamus griffithii* as previously reported (Mu et al., 2003) and the structure is shown in Figure 1. RPMI-1640 medium was obtained from Invitrogen, USA. MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide), propidium iodide (PI) and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody to caspase-8, rabbit monoclonal antibody to caspase-9 and rabbit monoclonal antibody to Smac/Diablo and horseradish peroxidase (HRP) conjugated secondary antibody were purchased from Abcam, Cambridge, UK. SuperSignal West Pico Chemiluminecent Substrate was obtained from Pierce, Rockford, IL, USA. Complete mini protease inhibitor cocktail and annexin V-fluos staining kit were purchased from Roche, Indianapolis, IN, USA.

Cell culture

Human leukemic promyelocytic HL-60 and human leukemic monocytic U937 cells were gifts from Dr. Sukhathida Ubol and Dr. Watchara Kasinroek, respectively. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin G (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. Goniothalamin was dissolved in dimethyl sulfoxide (DMSO) as a vehicle and the maximal volume used did not exceed 10 μl/ml of media. The human leukemic cells ($1x10^6$) were treated with goniothalamin at indicated concentration and duration.

MTT assay for cytotoxicity

HL-60 and U937 ($3x10^5$ cells/ml) were cultured and incubated with goniothalamin (0, 10, 20, 40 and 80 μM) at 37°C in 5% CO_2 atmosphere for 24 h. The cell viability was determined by using MTT assay (Banjerdpongchai et al., 2011). Briefly, MTT dye solution was added and incubated in CO_2 incubator for 4 h. Then, 100 μl of DMSO was added to dissolve the violet dye crystals. Absorbance was measured by using a microtiter plate reader (Biotek, USA) at 570 nm. The percentage of cell viability was calculated and 10, 20 and 50% inhibitory concentrations (IC_{10}, IC_{20} and IC_{50}) were determined and used for further experiments. Since HL-60 cells were more sensitive to GTN than U937, the doses of GTN were varied lower as follows: 0, 2, 4, 6, 8, 10 μM.

Determination of cell cycle distribution

For flow cytometric assessment of DNA fragmentation and cell cycle distribution, $1x10^6$ cells were harvested and re-suspended in a solution containing PI (50 μg/ml), 0.1% Triton X-100 and 0.1% sodium citrate in PBS. Cells then were analyzed in a FACScan equipped with a 488 nm argon laser using CellQuest software (Becton-Dickinson, USA) (Banjerdpongchai et al., 2010). Data were depicted as histograms and the percentage of cells displaying hypodiploid DNA content was indicated.

Determination of phosphatidylserine externalization in apoptotic cells

Goniothalamin-treated cells were washed once in phosphate-buffered saline solution, centrifuged at 200 x g and the cell pellet was suspended in 100 μl of binding buffer provided by the annexin V-fluos staining kit. Annexin V-FITC (20 μl) and PI (10 μl) were added and the cell suspension was left at room temperature for 15 min in the dark. Finally, 970 μl of binding buffer were added. Analysis was conducted using FACScan (Becton Dickinson, USA). Cells that were stained with annexin V-FITC, and annexin V-FITC together with PI, were designated as early and late apoptotic cells, respectively (Prommaban et al., 2012).

Determination of mitochondrial transmembrane potential (MTP)

For MTP determination, $5x10^5$ cells were treated with the GTN at IC_{10}, IC_{20} and IC_{50} for 24 h, harvested and re-suspended in a PBS containing 40 nM of $DiOC_6$ (Wudtiwai et al., 2011). The cells were incubated for 15 min at 37°C and then subjected to flow cytometer (Becton Dickinson, USA).

Western blot analysis

The goniothalamin-treated cells were washed once in ice cold PBS and incubated at 4°C for 10 min with ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, 0.25% Triton X-100 in PBS containing complete mini protease inhibitor cocktail). Following centrifugation at 20,000 x g for 20 min, supernatant (50 μg, determined by Bradford method) was separated by 10% SDS-PAGE and 4% SDS-PAGE for stacking gel and then transferred onto nitrocellulose membrane. After treating with 5% non-fat milk in PBS containing 0.2% Tween-20, membrane was incubated with rabbit polyclonal antibody to caspase-8, rabbit monoclonal antibody to caspase-9 or rabbit monoclonal antibody to Smac/Diablo, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000). Protein bands were visualized on X-ray film with SuperSignal West Pico Chemiluminescent Substrate (Prommaban et al., 2012).

Statistical analysis

Results are expressed as mean±S.D. Statistical

Table 1. Inhibitory Concentrations at 10, 20 and 50 Percent (IC_{10}, IC_{20} and IC_{50}) of Goniothalamin on Human Leukemic Cell Lines

Cell types	HL-60	U937
IC_{10} (μM)	1.95±1.4	1.15±0.1
IC_{20} (μM)	2.94±1.7	2.71±0.1
IC_{50} (μM)	5.67±2.0	11.5±1.1

*The cytotoxicity effects of goniothalamin on human leukemic HL-60 and U937 cells were determined by MTT assay

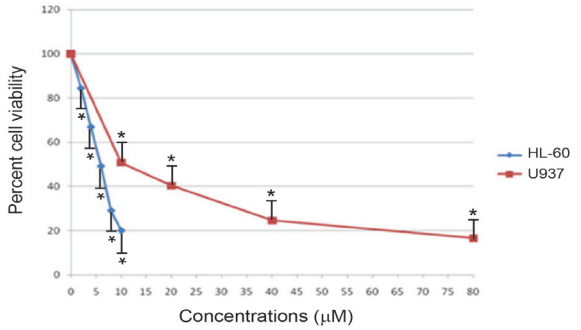

Figure 2. Cytotoxic Effect of Goniothalamin on Human Leukemic HL-60 and U937 Cell Lines. HL-60 or U937 cells were treated with GTN at various concentrations for 24 h, then the cell viability was determined by the MTT assay. *p<0.05, compared to control

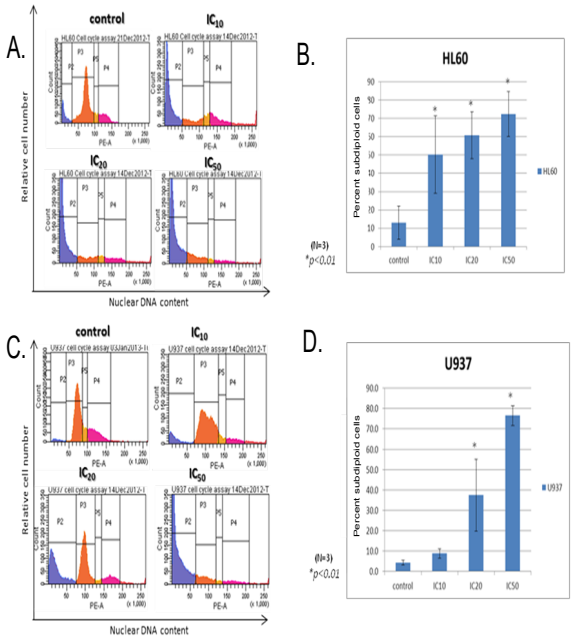

Figure 3. Histograms and Analysis of Percent Cells in Subdiploid Areas of Goniothalamin-treated Human Leukemic Cells. HL-60 (A, B) and U937 (C, D) cells were treated with GTN for 24 h, stained with propidium iodide (PI) and processed by flow cytometry. Representative histograms and data are shown from three independent experiments. *p <0.01, compared to control

difference between control and treated group was determined by one-way ANOVA (Kruskal Wallis analysis) at limit of p<0.05 from 3 independent experiments conducted in triplicate. For comparison between two groups, data were analyzed using Student's t-test.

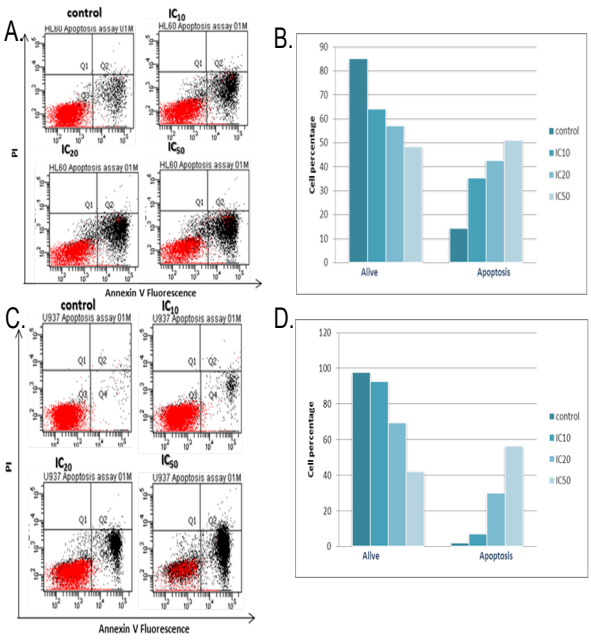

Figure 4. Dot Plots and Percent Analysis of Goniothalamin-treated Human Leukemic Cells for Phosphatidylserine Externalization of the Apoptotic Cells. HL-60 (A, B) and U937 (C, D) cells were treated with GTN for 24 h and stained with annexin V-FITC and PI and processed by flow cytometer as described in Materials and Methods. Representative dot plots and data are shown from three independent experiments. *p<0.05, compared to control

Results

Cytotoxicity of goniothalamin

GTN inhibited human HL-60 and U937 leukemic cell growth in a dose response manner. HL-60 cells were sensitive to GTN more than U937 cells with lower IC_{50} (5.67 μM and 11.5 μM, respectively) as shown in Figure 2 and Table 1. Goniothalamin at IC_{10}, IC_{20} and IC_{50} levels of each cell line were calculated and selected for further experiments.

Cell cycle distribution

The cells were induced to die with the characteristic DNA content as subdiploid (subG1) or hypodiploid which is less than 2n (Figure 3A and 3C), shown as cells under P2 area. The percentage of cells with hypodiploid DNA content increased in a dose-dependent manner both in HL-60 and U937 cells (p<0.01) as shown in Figure 3B and 3D. The subG1 is a characteristic hallmark of apoptotic cells.

Phosphatidylserine externalization and apoptotic induction

When HL-60 and U937 cells were treated with GTN at IC_{10}, IC_{20} and IC_{50} levels for 24 h, and the flip-flop of phosphatidylserine (PS) was determined by using annexin V fluos staining kit. As shown in Figure 4A and 4B, the early apoptotic cell population (right lower

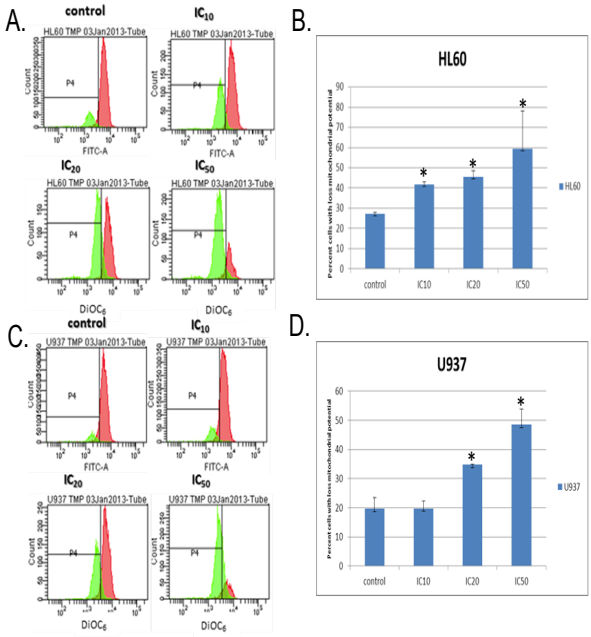

Figure 5. Reduction of Mitochondrial Transmembrane Potential in Goniothalamin-treated HL-60 and U937 Cells. Histograms and data analysis of HL-60 (A, B) and U937 (C, D) cells represents the percentage of cells with loss of mitochondrial transmembrane potential (MTP) which are under P4 area. Representative histograms and data are shown from three independent experiments. *p<0.05, compared to control

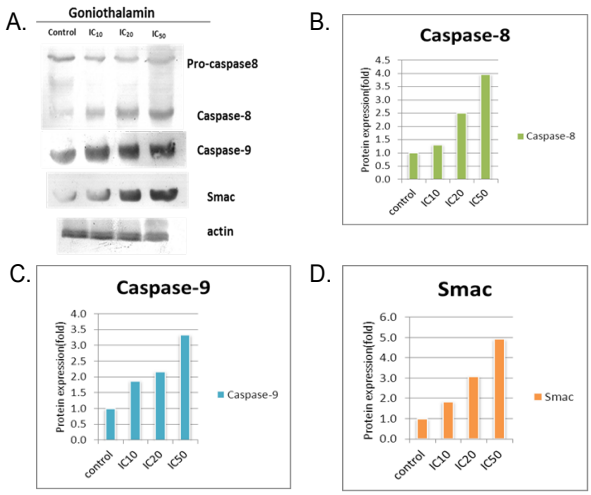

Figure 6. Effect of Goniothalamin on Initiation Caspase-8 and -9 and Apoptosis-related Smac/Diablo Protein Expressions in Human HL-60 Cells. Immunoblots of caspase-8 and -9 and Smac/Diablo were determined in GTN-treated cells at various concentraitons, viz. IC_{10}, IC_{20} and IC_{50} levels. Representative bands (A) and band intensity analysis of caspase-8 (B), caspase-9 (C) and Smac/Diablo (D) are obtained from three independent experiments

quadrant) increased in a dose response manner in HL-60 cell lines. U937 cells were also induced to die via apoptosis with PS flip-flop out to the outer layers (Figure 4C and 4D). Both HL-60 and U937 cells responded to GTN in a concentration-dependent manner significantly at doses of IC_{10}, IC_{20} and IC_{50} levels for HL-60 cells ($p<0.01$), whereas for U937 cells at doses of IC_{20} and IC_{50} levels ($p<0.01$) (Figure 4B and 4D).

Reduction of mitochondrial transmembrane potential (MTP)

The loss of mitochondrial transmembrane potential was found in both HL-60 and U937 cells. The percentage of cells with reduction or loss of mitochondrial transmembrane potential (cells under P4 area) increased in a dose-dependent manner ($p<0.05$) as shown in Figure 5A, 5B for HL60 and Figure 5C, 5D for U937 cells.

The expression of Smac/Diablo, caspase-8 and -9

The human leukemic HL-60 and U937 cells were incubated with GTN at various concentrations for 24 h and the immunoblots of Smac/Diablo, caspase-8 and -9 were performed. The expression of Smac/Diablo, cleaved caspase-8 and cleaved caspase-9 increased in a concentration-dependent manner as shown in Figure 6A-6D. The pro-caspase-8 band intensity decreased and the cleaved form increased dose-dependently (Figure 6A, 6B), whereas the band intensity of cleaved caspase-9 (Figure 6A, 6C) and Smac/Diable (Figure 6A, 6D) also increased in a concentration-dependent manner.

Discussion

Goniothalamin is cytotoxic towards both cancerous (HGC-27, MCF-7, PANC-1, HeLa) and non-cancerous (3T3) cells but these cells die via necrotic cell death (Ali et al., 1997). However, many cancer cells, such as leukemic HL-60 cells, Jurkat T cells, colon HT29 cells were induced to undergo apoptosis by GTN as well (Alabsi et al., 2012; Inayat-Hussain et al., 2003; Inayat-Hussain et al., 2010; Inayat-Hussain et al., 1999). Even though there are reports of mechanisms of cancer cell apoptosis induced by GTN to be mitochondrial pathway, the cross-link between the extrinsic and intrinsic pathways remains elusive. The present study compared the characteristic of HL-60 and U937 apoptotic cell death, which underwent the same mechanism. IC_{50} levels of GTN towards HL-60 and U937 cells were 5.67±2 and 11.5±1.1 μM, respectively. It has been shown that U937 cells were less sensitive to goniothalamin than HL-60 cells.

The apoptosis of HL-60 and U937 cells was confirmed by the cell cycle profile demonstrating of the increase of subG1 population of both cells in concentration-dependent manner. The flip-flop of PS to the outer layer of cell membrane also occurred in HL-60 and U937 cells induced by GTN. The loss of mitochondrial transmembrane potential indicated the involvement of the mitochondrial pathway of apoptosis in both cell lines. The release of Smac/Diablo, a protein in the intermembranous space of mitochondria, into the cytosol increased as shown by immunoblot. Smac/Diablo acts as a negative regulator or an antagonist of inhibitors of apoptotic protein (IAPs), so it acts as a stimulator of apoptosis and is proposed to be of use in anti-cancer

treatment (de Almagro and Vucic, 2012).

The expression of caspase-9 and Smac/Diablo together with the reduction of MTP indicated the mitochondrial pathway of apoptotic cell death in HL-60 cells. However, the increase of cleaved caspase-8 expression was related to the extrinsic pathway. This suggests the cross-talk between the intrinsic and extrinsic pathways of apoptosis induced by goniothalamin in human leukemic cell lines. The combined treatment of GTN and conventional chemotherapeutic drugs may be helpful in reducing the adverse effects of the chemotherapy and increase the apoptosis induction effect of such combination. The differences of the cell surface carbohydrate residues between HL-60 and U937 cells can be attributed to their differentiation within the myelomonocytic cell lineage (Schumacher et al., 1996). There are also differences in protein expression of angiopoietins and kindlins in human leukemic cells (Wu et al., 2012), HtrA2 and WT1 in acute myeloid leukemia (Li et al., 2012). Nevertheless, the mode of cell death induced by goniothalamin in both cell lines showed a similar mode and mechanism of cell death as proven in the recent study but the extensiveness of apoptosis induction may be different according to the characteristics and sensitivity of each cell line. In conclusion, goniothalamin induced human leukemic cells to die via apoptosis involving mitochondrial (caspase-9 and Smac) and death receptor (caspase-8) pathways.

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