



Loss of mitochondrial transmembrane potential and caspase-9 activation during apoptosis induced by the novel styryl-lactone goniiothalamine in HL-60 leukemia cells

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Accepted 16 April 2003

Abstract

Styryl-lactones such as goniiothalamine represent a new class of compounds with potential anti-cancer properties. In this study, we investigated the mechanisms of goniiothalamine (GTN), a plant styryl-lactone induced apoptosis in human promyelocytic leukemia HL-60 cells. This plant extract resulted in apoptosis in HL-60 cells as assessed by the externalisation of phosphatidylserine. Using the mitochondrial membrane dye (DIOC₆) in conjunction with flow cytometry, we found that GTN treated HL-60 cells demonstrated a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Further immunoblotting on these cells showed activation of initiator caspase-9 and the executioner caspases-3 and -7. Pretreatment with the pharmacological caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (Z-VAD.FMK) abrogated apoptosis as assessed by all of the apoptotic features in this study. In summary, our results demonstrate that goniiothalamine-induced apoptosis occurs via the mitochondrial pathway in a caspase dependent manner.

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Keywords: Apoptosis; Caspase activation; Goniiothalamine; Mitochondrial transmembrane potential; Styryl-lactone

1. Introduction

It has long been known that natural products are important sources of new bioactive low molecular weight structures with promising chemotherapeutic properties (reviewed in Harvey et al., 2000). A number of low molecular weight compounds are currently being investigated for their pharmacological properties in manipulating apoptosis, a cell death program which is pivotal in the pathological process of tumor development (Kinloch et

al., 1999). In this respect, the styryl-lactones found abundantly in the genus *Goniiothalamus* species such as goniiothalamine (GTN, Fig. 1) have been investigated for cytotoxic and antitumor properties (Ali et al., 1997; Hawariah and Stanslas, 1998; Cao et al., 1998; Bermejo et al., 1999).

Apoptotic cell death involves complex yet well regulated biochemical processes leading to the well-characterized features such as condensation of chromatin and internucleosomal DNA cleavage (reviewed in Hengartner, 2000). Current paradigms of apoptosis suggest that the loss of mitochondrial transmembrane potential (MTP or $\Delta\Psi_m$) occurs earlier in the commitment phase of apoptosis which results in the release of mitochondrial apoptogenic proteins including cytochrome-c and the apoptotic inducing factor (Zamzani et al., 1995; Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997).

Abbreviations: GTN, goniiothalamine; MPT or $\Delta\Psi_m$, mitochondrial transmembrane potential; PS, phosphatidylserine; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone.

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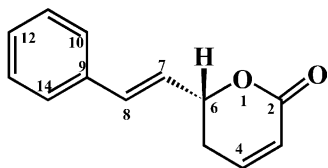


Fig. 1. Chemical structure of goniothalamin.

Cytochrome-c in the presence of dATP or ATP interacts with Apaf-1 resulting in the activation of caspase-9 (Li et al., 1997; Zou et al., 1997). Active caspase-9 subsequently processes the executioner caspases-3 and -7 into their catalytically active subunits (Srinivasula et al., 1998; Nicholson et al., 1995). These executioner caspases are responsible for the degradation of many cellular substrates including the DNA repair enzyme poly (ADP-ribose) polymerase (Nicholson et al., 1995) and gelsolin, a cytoskeletal protein (Kothakota et al., 1997).

In this study, we investigated the upstream mechanisms of GTN-induced apoptosis in human promyelocytic HL-60 leukemia cells. Our data show that GTN-induced apoptosis in HL-60 cells via the loss of $\Delta\Psi_m$ and activation of initiator caspase-9 to its active subunits.

2. Materials and methods

2.1. HL-60 cell culture and reagents

The human promyelocytic HL-60 cells were obtained from ATCC and cultured as described previously (Inayat-Hussain et al., 2000). Media and serum were obtained from GIBCO. The general caspase inhibitor Z-VAD.FMK was obtained from Enzyme Systems. The mitochondrial membrane dye 3,3'-dihexyloxycarbocyanine iodide [DIOC₆(3)] was from Molecular Probes. Procaspase-3 was purchased from Upstate Biotechnology Inc., and caspases-7 and -9 were gifts from Drs. G.M. Cohen and Y. Lazebnik, respectively. Goniothalamin (5-hydroxy-7-phenylhepta-2,6-dienoic acid lactone) was extracted as described previously (Inayat-Hussain et al., 1999). All other reagents were obtained from Sigma Chemical.

2.2. Assessment of apoptosis

Apoptosis in HL-60 cells was induced by GTN (10 $\mu\text{g}/\text{ml}$, equivalent to 50 μM) for 14 h prior to apoptosis assessment. This concentration has been determined in previous studies and shown to be optimal in inducing apoptosis (Ali et al., 1997, 2000). In some experiments, the general caspase inhibitor, Z-VAD.FMK (100 μM) was added 1 h prior to addition of GTN. The measurement of phosphatidylserine (PS) exposure was carried out using the annexin V assay as described previously (Inayat-Hussain et al., 2000). Briefly, 10 μl of media

binding buffer was added to an aliquot of 0.5×10^6 cells (500 μl) followed by addition of annexin V (1.25 μl of 200 $\mu\text{g}/\text{ml}$). An incubation of 12 min was carried out prior to addition of 10 μl propidium iodide (PI, 30 $\mu\text{g}/\text{ml}$). Flow cytometric analysis using FACScan (Becton Dickinson) was carried out.

The fall in the mitochondrial transmembrane potential ($\Delta\Psi_m$) during apoptosis was detected in the cells using the fluorescent mitochondria membrane dye DIOC₆(3). Briefly, 1 μl of 50 μM DIOC₆(3) was added into an aliquot of 1×10^6 cells (1 ml) and further incubated for 15 min at 37 °C. This was followed by adding 50 μl PI (50 $\mu\text{g}/\text{ml}$) and the cells were incubated further for 2 min prior to analysis by flow cytometer. Flow cytometric data analyses were carried out using the CellQuest software.

2.3. Immunoblotting of caspases-9, -7 and -3

Western blot analysis was performed as described previously (MacFarlane et al., 1997; Rodriguez and Lazebnik, 1999). HL-60 cells (0.5×10^6) were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing condition for both caspases-9, -7 and -3. The gels were then blotted onto a nitrocellulose membrane. Active subunits were detected with either anti-mouse (caspase-9) or anti-rabbit (caspases-3 and -7) horseradish peroxidase-labeled secondary antibodies. Proteins were detected by Enhanced Chemiluminescence staining.

2.4. Statistical analysis

All data were expressed as the mean \pm standard error of mean (S.E.M.). To compare apoptosis between groups in HL-60 cells, an ANOVA test was performed followed by Scheffe *F*-test to determine the statistical significance ($n \geq 3$, $P < 0.05$).

3. Results

3.1. Effects of Z-VAD.FMK on PS exposure and loss of $\Delta\Psi_m$ in GTN-induced apoptosis

In many cell types, the induction of apoptosis is associated with plasma membrane changes where PS is translocated from the inner layer of the plasma membrane to the outer leaflet; a process which is required for phagocytosis by macrophages (Fadok et al., 1992; Savill and Fadok, 2000). The externalization of PS can be assessed by measuring the binding of FITC-conjugated annexin V to cells by flow cytometry. As described earlier (Inayat-Hussain et al., 1999, 2000) apoptotic cells, which expose PS, include both PS+/PI- (early apoptotic) and PS+/PI+ (late apoptotic) cells. In this

study, annexin V was employed to detect HL-60 cells undergoing apoptosis induced by GTN. As shown in Fig. 2a, the background level of apoptosis in HL-60 cells at 14 h was $11.48 \pm 2.8\%$ (CON). After 14 h exposure, GTN significantly induced apoptosis in HL-60 cells ($71.9 \pm 0.7\%$) as assessed by PS exposure. To investigate the role of caspases in GTN induced apoptosis, the effects of the wide spectrum caspase inhibitor Z-VAD.FMK were examined in this study. Pretreatment with Z-VAD.FMK in GTN treated cells resulted in significant inhibition of apoptosis ($17.88 \pm 3.6\%$).

In order to study the involvement of mitochondria during GTN induced apoptosis in HL-60 cells, we investigated the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) using flow cytometry. The mitochondrial transmembrane potential was detected using DIOC₆(3), a fluorochrome which has recently been evaluated in HL-60 cells undergoing apoptosis and demonstrated to be comparable to annexin V method (Ozgen et al., 2000). The dot-plot cytogram in Fig. 3 shows a typical results of the loss of $\Delta\Psi_m$ in GTN induced apoptosis in HL-60 cells. In control cells

(CON), the dye loaded population of cells was observed in the lower right quadrant. During apoptosis in GTN treated HL-60 cells (GTN), dissipation of $\Delta\Psi_m$ led to leakage of DIOC₆(3) from the mitochondrial matrix, which can be measured by flow cytometry as a decrease in the fluorescence intensity of the dye (lower left quadrant). Interestingly, the cells in the upper left quadrant were representative of cells losing $\Delta\Psi_m$ and had already included PI, indicative of late apoptotic population. Similar to the annexin V data shown in Fig. 2a, we found a significant loss of $\Delta\Psi_m$ in GTN treated cells over control cells ($77.7 \pm 5.8\%$ and $13.4 \pm 0.9\%$, respectively, Fig. 2b). Z-VAD.FMK significantly inhibited the loss of $\Delta\Psi_m$ ($25.73 \pm 0.5\%$, Fig. 2b) in GTN treated cells with a dot-plot profile essentially similar to control cells (GTN+ZVAD, Fig. 3). The effects of Z-VAD.FMK alone on HL-60 cells as assessed by annexin V and loss of $\Delta\Psi_m$ were essentially similar to control treatment (data not shown).

3.2. Activation of caspases-9, -3, -7 in GTN induced apoptosis

Using the pharmacological caspase inhibitor, the experiments described above (Section 3.1) indicate that caspases are being activated during the apoptotic process. To confirm the processing of caspases into their active subunits during GTN induced apoptosis, immunoblotting of caspases-9, -3 and -7 was carried out as shown in Fig. 4. Intact caspase-9 (46 kDa) was detected in control HL-60 cells (Panel A, CON lane). In addition, some background cleavage of caspase-9 to the p37/p35 fragments was observed indicative of the background level of apoptosis. During apoptosis in GTN treated HL-60 cells, intact caspase-9 was totally cleaved into smaller subunits especially the p35 kDa and another smaller subunit around 30 kDa (Panel a, GTN lane). Activation of caspase-9 into p30 kDa subunit has been previously demonstrated in remoxipride and etoposide induced apoptosis in HL-60 cells respectively (Inayat-Hussain et al., 2000; MacFarlane et al., 1997). In the presence of Z-VAD.FMK, the processing of caspase-9 was inhibited with concomitant accumulation of the intact and p35/p37 kDa subunits in GTN treated cells (Panel a, GTN + ZVAD lane).

As shown in Fig. 4 (panel b), intact caspase-3 (p32 kDa) as detected in control cells (CON lane) was processed into its catalytically active p19 and p17 subunits in GTN treated HL-60 cells (GTN lane). In the presence of the caspase inhibitor Z-VAD.FMK, caspase-3 cleavage to its active p17 kDa subunit was inhibited resulting primarily in the generation of the p20 kDa subunit (GTN + ZVAD). We and others have demonstrated that Z-VAD.FMK inhibition of caspase-3 cleavage to the p20 kDa renders this enzyme to be proteolytically inactive (Inayat-Hussain et al., 2000; Martin et al., 1996).

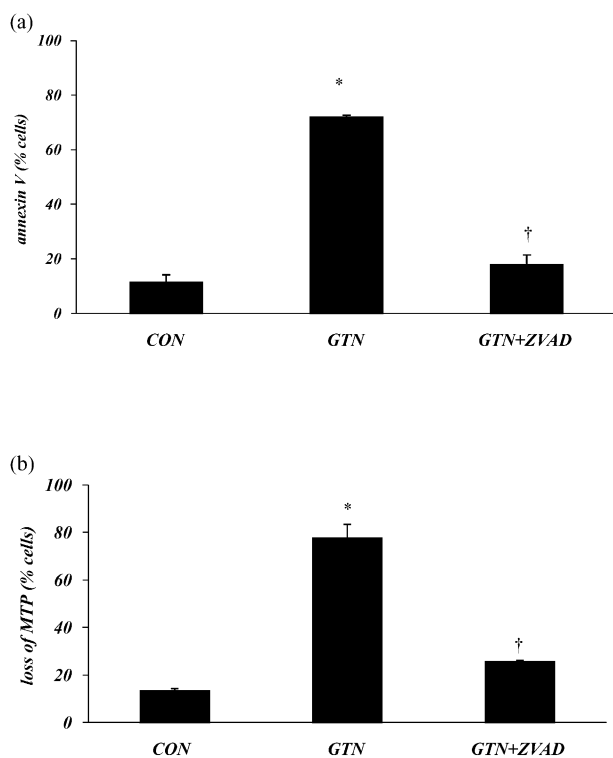


Fig. 2. Effects of Z-VAD.FMK on GTN induced apoptosis in HL-60 cells as assessed by (a) Annexin V/PI assay and (b) loss of mitochondrial transmembrane potential (MTP). Cells (1×10^6) were pre-incubated for 1 h with $100 \mu\text{M}$ Z-VAD.FMK (ZVAD) before adding $10 \mu\text{g/ml}$ GTN in HL-60 cells. These cells were further incubated for 14 h prior to flow cytometric analysis as described in Materials and methods. CON represents control cells. Asterisk (*) indicates significant difference from control and dagger (†) indicates significant difference from GTN treatment ($P < 0.05$) by ANOVA and Scheffe *F*-test.

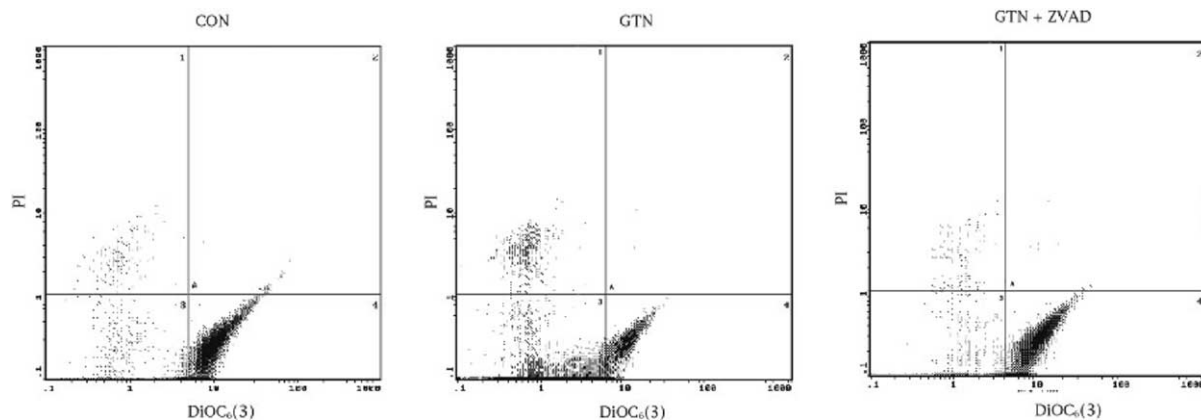


Fig. 3. Flow cytometric profile of the effects of Z-VAD.FMK on GTN induced loss of mitochondrial transmembrane potential in HL-60 cells. Cells were treated as described in Fig. 2. The x-axis represents the fluorescence of DIOC₆(3) and the y-axis represents PI fluorescence.

In addition to caspase-3, we investigated if caspase-7, another member of the executioner caspases was activated during GTN induced apoptosis in HL-60 cells (Fig. 4, panel c). Similar to caspase-3, intact caspase-7 (p35 kDa, CON lane) was also processed to its active p19 kDa subunit during apoptosis (GTN lane). The cleavage of caspase-7 to its active subunit in GTN induced apoptosis was blocked in the presence of Z-VAD.FMK (GTN + ZVAD lane). PARP, a substrate for these executioner caspases, was also cleaved in GTN treated cells in Z-VAD.FMK inhibitable manner (data not shown), further confirming the activation of caspases-9, -3 and -7.

4. Discussion

The styryl-lactones found abundantly in *Goniothalamus* species (Annonaceae) have been investigated for its pharmacological activities especially its cytotoxic properties (Ali et al., 1997; Cao et al., 1998; Blasquez et al., 1999). Although many investigators have shown that these compounds are cytotoxic (Tsukubi et al., 1999), the mechanism of action of styrylpyrone derivative is not fully understood.

In order to elucidate the molecular mechanisms of the styryl-lactone, we studied the effects of GTN isolated from *Goniothalamus andersonii* on human promyelocytic HL-60 leukemia cells. In agreement with our previous studies (Ali et al., 1997; Inayat-Hussain et al., 1999), we found that GTN caused cell death in HL-60 cells and apoptosis was the mode of death observed in these cells. Flow cytometric analysis of the treated cells showed externalization of PS as assessed by annexin V binding (Fig. 2a). PS is normally restricted to the inner-membrane leaflet, however dying cells expose this phospholipid as it is one of the key signals for phagocyte recognition (Savill and Fadok, 2000).

The molecular machinery of chemotherapeutic drugs-induced apoptosis has been shown to involve the release

of apoptogenic factors from the mitochondria as a result of the loss of $\Delta\Psi_m$ which subsequently leads to the activation of the initiator caspase-9 (Susin et al., 1997; Constantini et al., 2000; Desagher and Martinou, 2000). By using the mitochondrial membrane dye DIOC₆(3) in conjunction with flow cytometry, we demonstrated that GTN treated HL-60 cells resulted in the loss of $\Delta\Psi_m$ in a Z-VAD.FMK inhibitable manner (Fig. 2a). It has recently been demonstrated by Susin et al. (1999) that caspase-9 is found in the intermembrane space of mitochondria from five different organs (liver, kidney, heart, brain, and spleen), as well as in several cell lines (including different lymphoid and neuroblastoma cell lines). This apical/initiator caspase is released when there is a loss of $\Delta\Psi_m$ in isolated mitochondria and upon induction of apoptosis in cells. Our data is consistent with the loss of $\Delta\Psi_m$ and activation of caspase-9 into its active subunits (35/37 kDa and the smaller fragment corresponding to around 30 kDa, Fig. 4) (Inayat-Hussain et al., 1999; Susin et al., 1999; Mesner et al., 1999). In the presence of Z-VAD.FMK, the generation of smaller fragment (i.e. ~30 kDa) of caspase-9 during apoptosis was inhibited with concomitant accumulation of the p35/37 kDa subunits. In agreement with the Z-VAD.FMK inhibition of the loss of $\Delta\Psi_m$ and caspase-9 activation, we found that the processing of the executioner caspases-3 and -7 in GTN treated cells was also inhibited (Fig. 4). Similar concentration of Z-VAD.FMK (100 μ M) blocked the activation of caspases-9, -3 and -7 in polyphenolic metabolites of remoxipride induced apoptosis in HL-60 cells (Inayat-Hussain et al., 2000).

The caspase inhibitor Z-VAD.FMK has recently been demonstrated to inhibit all of the first 10 caspases (Garcia-Calvo et al., 1998). This further emphasizes its use as a general caspase inhibitor in many studies. However, it is interesting to note that our recent studies showed that in hydroquinone induced apoptosis in HL-60 cells, the PS exposure occurred via

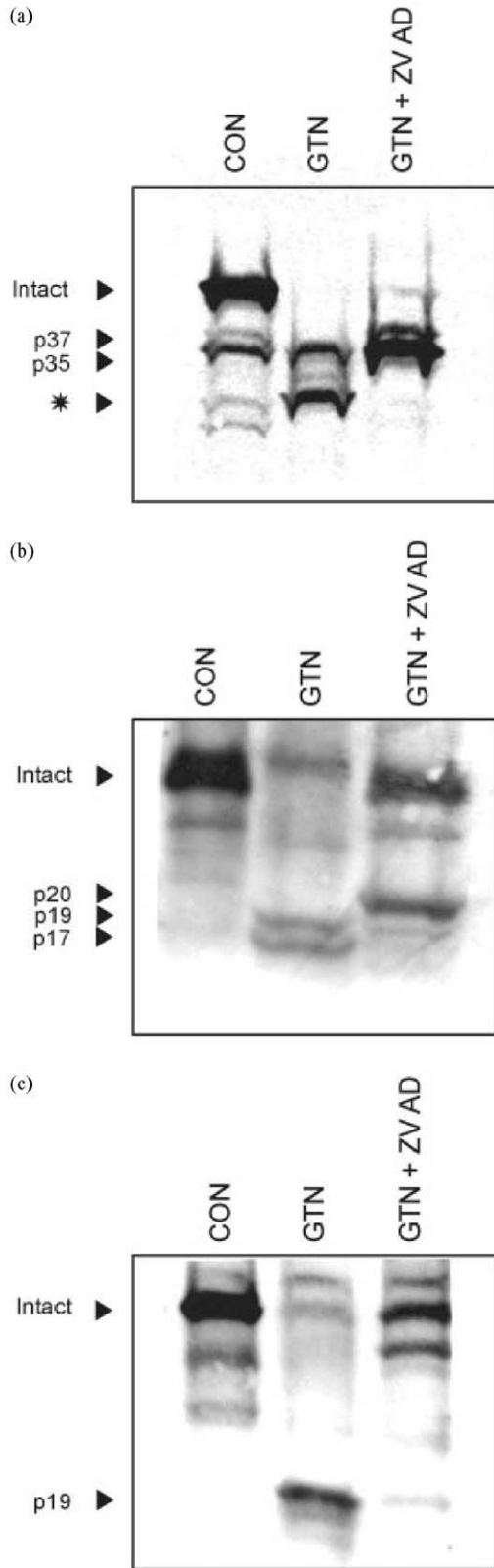


Fig. 4. Effects of Z-VAD.FMK on the activation of caspase-9, -3, -7 in GTN induced apoptosis. Cells were treated as described in Fig. 2 and the whole cell lysates were immunoblotted with antibodies to caspase-9 (panel a), caspase-3 (panel b) and caspase-7 (panel c). The intact proteins for caspases-9, -3 and -7 are 46 kDa, 32 kDa and 35 kDa, respectively. *Corresponds to small fragment of around 30 kDa.

a ZVAD-insensitive mechanism (Inayat-Hussain et al., 2001). In addition, Z-VAD.FMK did not completely abrogate the externalization of PS in HL-60 cells treated with the hydroquinone and catechol metabolites of the antipsychotic remoxipride (Inayat-Hussain et al., 2000). Similarly, the loss of $\Delta\Psi_m$ in these treatments (hydroquinone and the remoxipride metabolites) was not inhibited by Z-VAD.FMK suggesting the existence of a caspase-independent pathway(s) in the mitochondria (Inayat-Hussain et al., 2001). These findings show that different chemical stimuli may result in different involvement of caspases during the apoptotic cell death.

Previous study by Bossy-Wetzel and Green (1999) demonstrated the ability of caspase-8, another initiator caspase, to signal through mitochondria by cleaving p22 Bid yielding a truncated Bid. The latter is responsible to target mitochondria and stimulate the release of cytochrome c. It is therefore tempting to speculate the involvement of caspase-8 in GTN-induced apoptosis where its activation leads to the loss of $\Delta\Psi_m$ in a ZVAD-FMK inhibitable manner. In this respect, an upregulation of Fas mediated apoptosis system is possible after GTN treatment. Interestingly, more recent studies using human cell lines also raise the importance of caspase activation leading to the release of AIF from the mitochondria (reviewed in Penninger and Kroemer, 2003). Future studies need to be carried out to identify the upstream caspase(s) if any, leading to the loss of $\Delta\Psi_m$ in ZVAD.FMK inhibitable manner (Fig. 5).

Collectively, results presented in this study demonstrate that apoptosis induced by GTN in HL-60 cells is associated with the loss of $\Delta\Psi_m$, PS exposure and activation of caspases-9, -3, -7. Furthermore, the Z-VAD.FMK inhibition data on the loss of $\Delta\Psi_m$, PS exposure and activation of caspases suggest that GTN

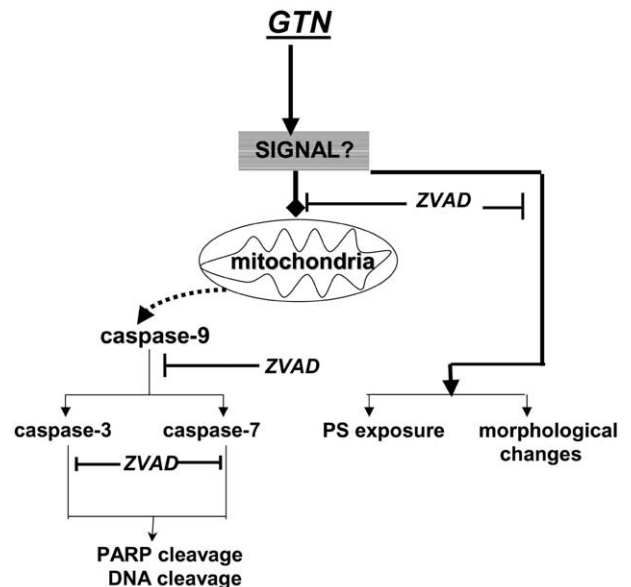


Fig. 5. A proposed model of Goniiothalamin-induced apoptosis.

induced apoptosis occurs in a caspase-dependent pathway (Fig. 5). We observed that inhibition of these caspases caused the abrogation of PARP and DNA cleavage (data not shown). To the best of our knowledge, however, this is the first report demonstrating that a styryl-lactone induces apoptosis via a mitochondrial pathway leading to the activation of the apical caspase-9. These data have relevance for defining mechanisms underlying the mechanism of cytotoxicity of goniothalamin and may also be of importance to the observed chemotherapeutic actions of this natural product.

Acknowledgements

We would like to thank the University of Colorado Health Sciences Center Flow Cytometry Core Facility for assistance in the flow cytometric analysis. This work was partially supported by Malaysian IRPA grant No. 06-02-04-03474-PR0014/06-05.

References

- Ali, A.M., Mackeen, Hamid, M., Aun, Q.B., Zauyah, Y., Azimahtol, H.L., Kawazu, K., 1997. Cytotoxicity and electron microscopy of cell death induced by goniothalamin. *Planta Medica* 63, 81–83.
- Ali, A.M., Umar-Tsafe, N., Mohamad, S.M., Oo, K.T., Yusoff, K., Dhaliwal, J., Abdullah, N., Din, L.B., Inayat-Hussain, S.H., 2000. Cell cycle and apoptosis induction by goniothalamin in CEM-SS T-lymphoblastic leukemia cell line. *Journal of Biochemistry, Molecular Biology and Biophysics* 5, 1–9.
- Bermejo, A., Leonce, S., Cabedo, N., Andreu, I., Caignard, D.H., Atassi, G., Cortes, D., 1999. Semisynthesis and cytotoxicity of styryl-lactone derivatives. *Journal of Natural Products* 62, 1106–1109.
- Blasquez, M.A., Bermejo, A., Zafra-Polo, M.C., Cortes, D., 1999. Styryl-lactones from *Goniothalamus* species—a review. *Phytochemical Analyses* 10, 161–170.
- Bossy-Wetzel, E., Green, D.R., 1999. Caspases induce cytochrome c release from mitochondria by activating cytosolic factors. *Journal of Biological Chemistry* 274, 17484–17490.
- Cao, S.G., Wu, X.H., Sim, K.Y., Tan, B.K.H., Pereira, J.T., Goh, S.H., 1998. Styryl-lactone derivatives and alkaloids from *Goniothalamus borneensis* (Annonaceae). *Tetrahedron* 54, 2143–2148.
- Costantini, P., Jacotot, E., Decaudin, D., Kroemer, G., 2000. Mitochondrion as a novel target of anticancer chemotherapy. *Journal of the National Cancer Institute* 92, 1042–1053.
- Desagher, S., Martinou, J.C., 2000. Mitochondria as the central control point of apoptosis. *Trends in Cell Biology* 10, 369–377.
- Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., Henson, P.M., 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of Immunology* 148, 2207–2216.
- Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W., Thornberry, N.A., 1998. Inhibition of human caspases by peptide-based and macromolecular inhibitors. *Journal of Biological Chemistry* 273, 32608–32613.
- Harvey, A., 2000. Strategies for discovering drugs from previously unexplored natural products. *Drug Discovery Today* 5, 294–300.
- Hawariah, A.L., Stanslas, J., 1998. In vitro response of human breast cancer cell lines to the growth-inhibitory effects of styrylpyrone derivative (SPD) and assessment of its antiestrogenicity. *Anticancer Research* 18, 4383–4386.
- Hengartner, M.O., 2000. The biochemistry of apoptosis. *Nature* 12, 770–776.
- Inayat-Hussain, S.H., Annuar, B.O., Din, L.B., Ali, A.M., Snowden, R.T., MacFarlane, M., Cain, K., 1999. Caspases-3 and -7 are activated in goniothalamin-induced apoptosis in human Jurkat T-cells. *Federation of European Biochemical Society Letters* 456, 379–383.
- Inayat-Hussain, S.H., McGuinness, S.M., Johansson, R., Lundstrom, J., Ross, D., 2000. Caspase-dependent and -independent mechanisms in apoptosis induced by hydroquinone and catechol metabolites of remoxipride in HL-60 cells. *Chemical and Biological Interactions* 128, 51–63.
- Inayat-Hussain, S.H., Winski, S.L., Ross, D., 2001. Differential involvement of caspases in hydroquinone-induced apoptosis in human leukemic HL-60 and Jurkat cells. *Toxicology and Applied Pharmacology* 175, 95–103.
- Kinloch, R.A., Treherne, J.M., Furness, L.M., Hajimohamadreza, I., 1999. The pharmacology of apoptosis. *Trends in Pharmacological Sciences* 20, 35–42.
- Kluck, R.M., Bossy-Wetzel, E., Green, D.R., Newmeyer, D.D., 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275, 1132–1136.
- Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J., Williams, L.T., 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278, 294–298.
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, I.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91, 479–489.
- Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 12, 147–157.
- MacFarlane, M., Cain, K., Sun, X.M., Alnemri, E.S., Cohen, G.M., 1997. Processing/activation of at least four interleukin-1beta converting enzyme-like proteases occurs during the execution phase of apoptosis in human monocytic tumor cells. *Journal of Cellular Biology* 137, 469–479.
- Martin, S.J., Amarante-Mendez, G.P., Shi, L., Chuang, T.H., Casiano, C.A., O'Brien, G.A., Fitzgerald, P., Tan, E.M., Bokoch, G.M., Greenberg, A.H., Green, D.R., 1996. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO Journal* 15, 2407–2416.
- Mesner Jr, P.W., Bible, K.C., Martins, L.M., Kottke, T.J., Srinivasula, S.M., Svingen, P.A., Chilcote, T.J., Basi, G.S., Tung, J.S., Krajewski, S., Reed, J.C., Alnemri, E.S., Earnshaw, W.C., Kaufmann, S.H., 1999. Characterization of caspase processing and activation in HL-60 cell cytosol under cell-free conditions. Nucleotide requirement and inhibitor profile. *Journal of Biological Chemistry* 274, 22635–22645.
- Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T.-T., Yu, V.L., Miller, D.K., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376, 37–43.
- Ozgen, U., Savasan, S., Buck, S., Ravindranath, Y., 2000. Comparison of DiOC(6)(3) uptake and annexin V labeling for quantification of apoptosis in leukemia cells and non-malignant T lymphocytes from children. *Cytometry* 42, 74–78.
- Penninger, J.M., Kroemer, G., 2003. Mitochondria, AIF and caspases—rivaling for cell death execution. *Nature Cell Biology* 5, 97–99.
- Rodriguez, J., Lazebnik, Y., 1999. Caspase-9 and APAF-1 form an active holoenzyme. *Genes Development* 13, 3179–3184.
- Savill, J., Fadok, V., 2000. Corpse clearance defines the meaning of cell death. *Nature* 407, 784–788.

- Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., Alnemri, E.S., 1998. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Molecular Cell* 1, 949–957.
- Susin, S.A., Zamzami, N., Castedo, M., Daugas, E., Wang, H.G., Geley, S., Fassy, F., Reed, J.C., Kroemer, G., 1997. The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. *Journal of Experimental Medicine* 186, 25–37.
- Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M.C., Alzari, P.M., Kroemer, G., 1999. Mitochondrial release of caspase-2 and -9 during the apoptotic process. *Journal of Experimental Medicine* 189, 381–394.
- Tsukubi, M., Kanai, K., Nagase, H., Honda, T., 1999. Stereocontrolled syntheses of novel styryl lactones (+)-goniodiol, (+)-goniotriol, (+)-8-acetylgoniotriol, (+)-goniofufurone, (+)-9-deoxygoniopyrone, (+)-goniopyrone and (+)-altholactone from common intermediates and cytotoxicity of their congeners. *Tetrahedron* 55, 2493–2514.
- Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275, 1129–1132.
- Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S., Petit, P.X., Mignotte, B., Kroemer, G., 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *Journal of Experimental Medicine* 182, 367–377.
- Zou, H., Henzel, W.J., Liu, X., Lutschg, A., Wang, X., 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90, 405–413.