

Altholactone, a novel styryl-lactone induces apoptosis via oxidative stress in human HL-60 leukemia cells

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Abstract

Plant styryl-lactone derivatives isolated from *Goniothalamus* sp. are potential compounds for cancer chemotherapy. In this study, we have examined the mechanisms of apoptosis induced by altholactone, a styryl-lactone isolated from the Malaysian plant *G. malayanus* on human HL-60 promyelocytic leukemia cells. Flow cytometric analysis of the externalization of phosphatidylserine (PS) using the annexin V/PI method on altholactone treated HL-60 cells showed a concentration-dependent increase of apoptosis from concentrations ranging from 10.8 (2.5 µg/ml) to 172.4 µM (40 µg/ml). Pre-treatment with the antioxidant *N*-acetylcysteine (1 mM) completely abrogated apoptosis induced by altholactone, suggesting for the involvement of oxidative stress. Further flow cytometric assessment of the level of intracellular peroxides using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) confirmed that altholactone induced an increase in cellular oxidative stress in HL-60 cells which was suppressed by *N*-acetylcysteine. In summary, our results demonstrate for the first time that altholactone induced apoptosis in HL-60 cells occurs via oxidative stress. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Apoptosis; Altholactone; Styryl-lactone; Oxidative stress; *N*-acetylcysteine

1. Introduction

Low molecular weight compounds especially derived from natural sources such as plants are

currently being investigated for their pharmacological properties in regulating apoptosis, a cell death program which is pivotal in the pathological process of tumor development (Kinloch et al., 1999). In this respect, the styryl-pyrone derivatives found abundantly in the genus *Goniothalamus* species have also been investigated for cytotoxic and antitumor properties (Ali et al., 1997; Cao et al., 1998; Hawariah and Stanslas, 1998; Bermejo et al., 1999).

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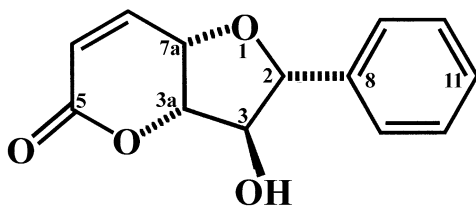


Fig. 1. Structure of altholactone (2-phenyl-3-hydroxy-6,7-dihydro-furano-pyrone).

Recent studies have demonstrated that goniothalamin, a plant styryl-lactone isolated from *Goniothalamus malayanus* is a potential cytotoxic compound especially by inducing apoptosis in a variety of tumor cell lines (Ali et al., 1997; Hawariah and Stanslas, 1998; Inayat-Hussain et al., 1999). In addition, altholactone which is also a styryl-lactone (Fig. 1) isolated from the *Goniothalamus* sp. has been shown to possess some cytotoxicity properties (Bermejo et al., 1999). These earlier observations allude to the potential of styryl-lactones to be developed as anti-cancer agents.

A flurry of recent work has clearly demonstrated the importance of apoptosis as a mechanism of cell death in the treatment of cancer (Jaffrezou et al., 1998; Debatin, 2000). It is currently known that chemotherapeutic agents can induce apoptosis via multiple mechanisms including DNA topoisomerase inhibition, intercalation into DNA, cell membrane damage or generation of reactive oxygen species (ROS) (Barry et al., 1990; Chandra et al., 2000). Although the roles of ROS in apoptosis are controversial, many cytotoxic compounds such as etoposide, adriamycin and methotrexate require the involvement of ROS in signaling apoptotic cell death (Verhaegen et al., 1995; McClain et al., 1995; Davis et al., 2001). In this study, we have investigated the mode of cell death induced by altholactone and the possible role of ROS in altholactone induced cytotoxicity in human promyelocytic HL-60 leukemia cells. Our results show for the first time that the mode of death induced by altholactone is apoptosis, which occurs via oxidative stress.

2. Materials and methods

2.1. Reagents and cells

The human promyelocytic HL-60 cells were obtained from ATCC (Rockville, MD) and cultured as described previously (Inayat-Hussain et al., 2000). Annexin V/FITC was purchased from R&D Laboratories and 2',7'-dichlorofluorescein diacetate (DCFH-DA) from Molecular Probes. Altholactone (MW. 232) was extracted from *G. malayanus* as described previously (Zakaria et al., 1998). All other reagents were from Sigma Chemical (St. Louis, MO).

2.2. Flow cytometric assessment of apoptosis using annexin V/PI assay

HL-60 cells were treated with altholactone (10.8–172.4 μM) for 14 h prior to apoptosis assessment. In some experiments, the antioxidant *N*-acetylcysteine (1 mM) was added 1 h prior to addition of altholactone. The measurement of phosphatidylserine (PS) exposure was carried out using the annexin V assay as described previously (Inayat-Hussain et al., 2000). Briefly, 1×10^6 cells were collected and resuspended in 1 ml annexin V buffer containing 1.5 μl annexin V and incubated for 8 min. Propidium iodide (2.5 $\mu\text{g/ml}$) was then added and followed by flow cytometric analysis using FACS Calibur (Becton Dickenson Laboratory).

2.3. Flow cytometric analysis of oxidative stress using DCFH-DA

Oxidative stress was analyzed using the fluorescent dye DCFH-DA as described previously (Kayanoki et al., 1996). For these experiments, altholactone (86.2 μM) was added to HL-60 cells (1×10^6) and further incubated for 3 h. In experiments employing NAC (1 mM), this antioxidant was added 1 h prior to addition of altholactone. This was followed by centrifuging the cells at 1000 rpm per 5 min and resuspending it in PBS. About 1 μl of DCFH-DA (5 mM in DMSO) was then added to the cell suspension and incubated in a waterbath (37 $^\circ\text{C}$)

for 15 min. Subsequently, the cells were kept on ice and flow cytometry was immediately carried out on the samples.

3. Results and discussion

Apoptosis has been an intensive research area, which involves the study of compounds that trigger or inhibit this mode of death. Being an important process involved in many pathological diseases including cancer, a number of low molecular weight compounds have been used to inhibit or trigger this fundamental cellular process making apoptosis amenable to pharmacological intervention (Kinloch et al., 1999). In this study, we report the potential apoptogenic activity of a styryl-lactone compound, altholactone isolated from *G. malayanus* plant found abundantly in Malaysia, on human promyelocytic HL-60 leukemia cells. Apoptosis induced by altholactone in HL-60 cells was determined using the annexin V/PI assay in conjunction with flow cytometry. Annexin V is used to detect the externalization of PS on the outer leaflet of plasma membrane during the apoptotic process (Fadok et al., 1992).

Cells were treated with a range of concentrations from 10.8 to 172.4 μM for 14 h. As shown in the histogram (Fig. 2A), altholactone induced apoptosis in a concentration dependent manner. At 86.2 μM altholactone, $55.5 \pm 7.3\%$ apoptosis was observed in HL-60 cells and the highest concentration used in this study (172.4 μM) clearly showed that this compound was able to induce apoptosis culminating to $89.5 \pm 3.1\%$ (Fig. 2B). Although previous studies (Cao et al., 1998; Bermejo et al., 1999) have demonstrated that altholactone is cytotoxic to a variety of human tumor cell lines, no studies to date have elucidated the mode of cell death induced by this compound. It is important to note that apoptosis has always been a preferred mode of cell death in the treatment of cancer as this death process unlike necrosis does not result in inflammatory reactions (Fadok et al., 1992).

Recently, Peris et al. (2000) have reported that styryl-lactone compounds such as altholactone, 3-acetylaltholactone and 5-acetoxyisogoniothala-

min oxide inhibit the mitochondrial respiratory chain by studying the NADH oxidase activity of beef heart submitochondrial particles. In addition, inhibition of mitochondrial respiratory chain complex I could affect the electron flow through other complexes leading to release of cytochrome c in an antioxidant sensitive pathway during apoptosis (Higuchi et al., 1998). From these observations, it may be possible that styryl-lactones disrupt the function of mitochondria, which may lead to homeostasis imbalance leading to oxidative stress. Current models of apoptosis favor that the loss of mitochondrial transmembrane potential occurs earlier during apoptotic death which results in the release of cytochrome c and the apoptotic inducing factors (Zamzami et al., 1995; Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997). In agreement, goniiothalamine, another styryl-lactone induces the loss of mitochondrial transmembrane potential during apoptosis in HL-60 cells further supporting the involvement of mitochondria in this cell death process (data not shown).

In order to investigate the possible role of oxidative stress in altholactone induced apoptosis, we pretreated the cells with the antioxidant NAC as shown in Fig. 3. The cytogram in Fig. 3A shows a bivariate annexin V/PI analysis of HL-60 cells. Viable cells were negative for both PI and annexin V (lower left quadrant), apoptotic cells were positive for annexin V and negative for PI (early apoptosis, lower right quadrant) whereas late apoptotic cells displayed both high annexin V and PI labeling (upper right quadrant). Non-viable cells which underwent necrosis were positive for PI and negative for annexin V (upper left quadrant). Apoptosis induced by altholactone (AL, 57.2%) was inhibited back to control levels by pretreatment of the cells with NAC (AL + NAC, 6.2%, Fig. 3A). It is clear from this cytogram that altholactone induced massive apoptosis and most of the cells were already at late apoptotic stage at 14 h. Nevertheless, pretreatment with NAC was effective in abrogating apoptosis induced by altholactone resulting in a cytogram with similar profile to control cells. NAC alone did not induce any increase of apoptosis and essentially similar to control cells (Fig. 3B). These results are in agreement with previous

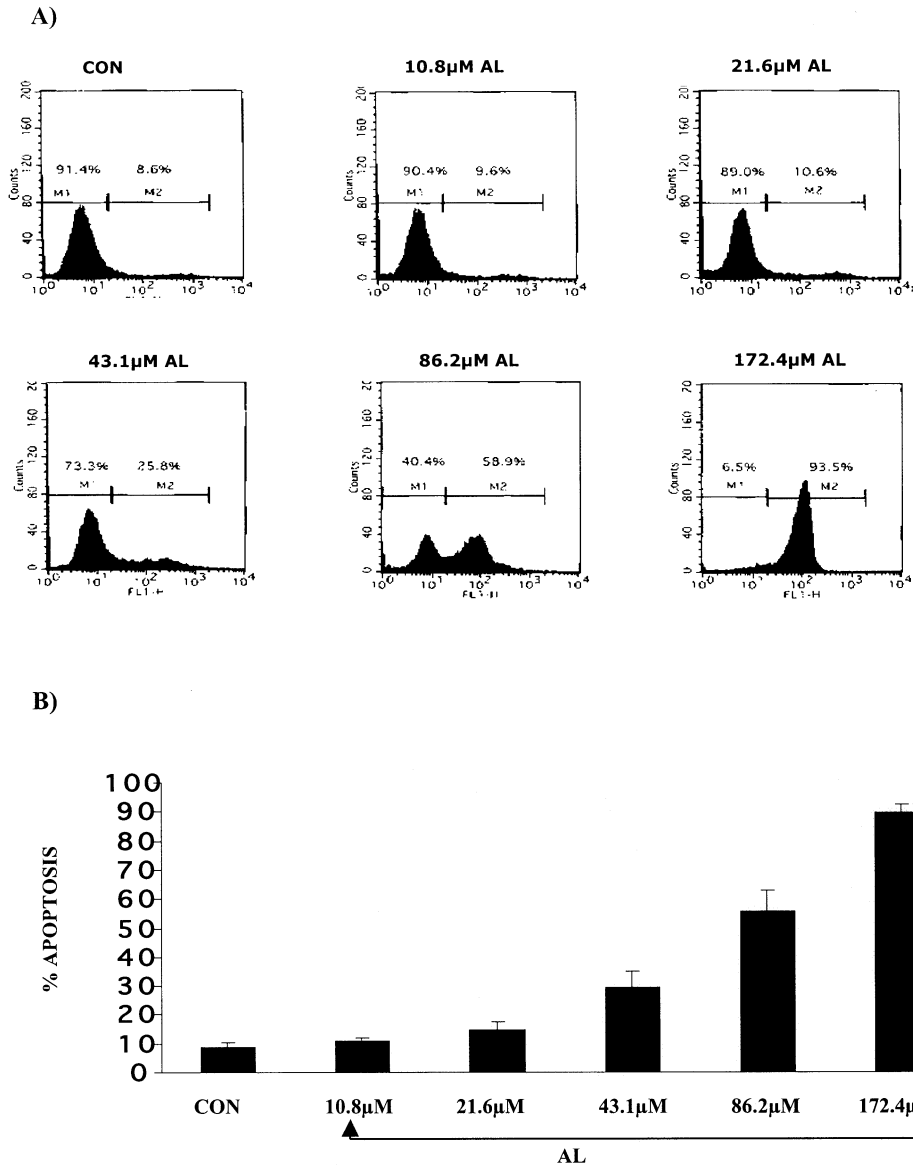


Fig. 2. Concentration dependent increase in altholactone induced apoptosis in HL-60 cells. (A) Cells were incubated with either alone (CON) or in the presence of various concentrations of altholactone (AL). The percentage of apoptosis was determined by annexin V-FITC as shown in the histogram using flow cytometry. The M1 region represents normal viable cells and M2 region represents apoptotic cells, which stained positive for annexin V. (B) Cumulative data represent the mean \pm S.E.M. of at least three separate experiments.

studies where NAC inhibits oxidative stress and apoptosis in HL-60 cells induced by certain compounds including actinomycin D, gallic acid and β -lapachone (Chau et al., 1998; Ikeda et al., 1999; Inoue et al., 2000).

The results with NAC (Fig. 3) strongly suggest that oxidative stress is a key feature of apoptosis induced by altholactone. In order to confirm that oxidative stress occurred in altholactone treated cells, flow cytometric analysis was carried out to

determine if DCFH-DA was oxidized to DCF, an indicator for oxidative stress in cells (Burow and Valet, 1987). In this experiment, the cells were treated with altholactone for only 3 h before addition of the dye. As shown in Fig. 4, there was

a marked increase in the fluorescence of DCFH-DA in altholactone treated cells (AL) as compared with control cells (CON), further confirming the presence of ROS. This marked increase in the fluorescence of DCFH-DA in al-

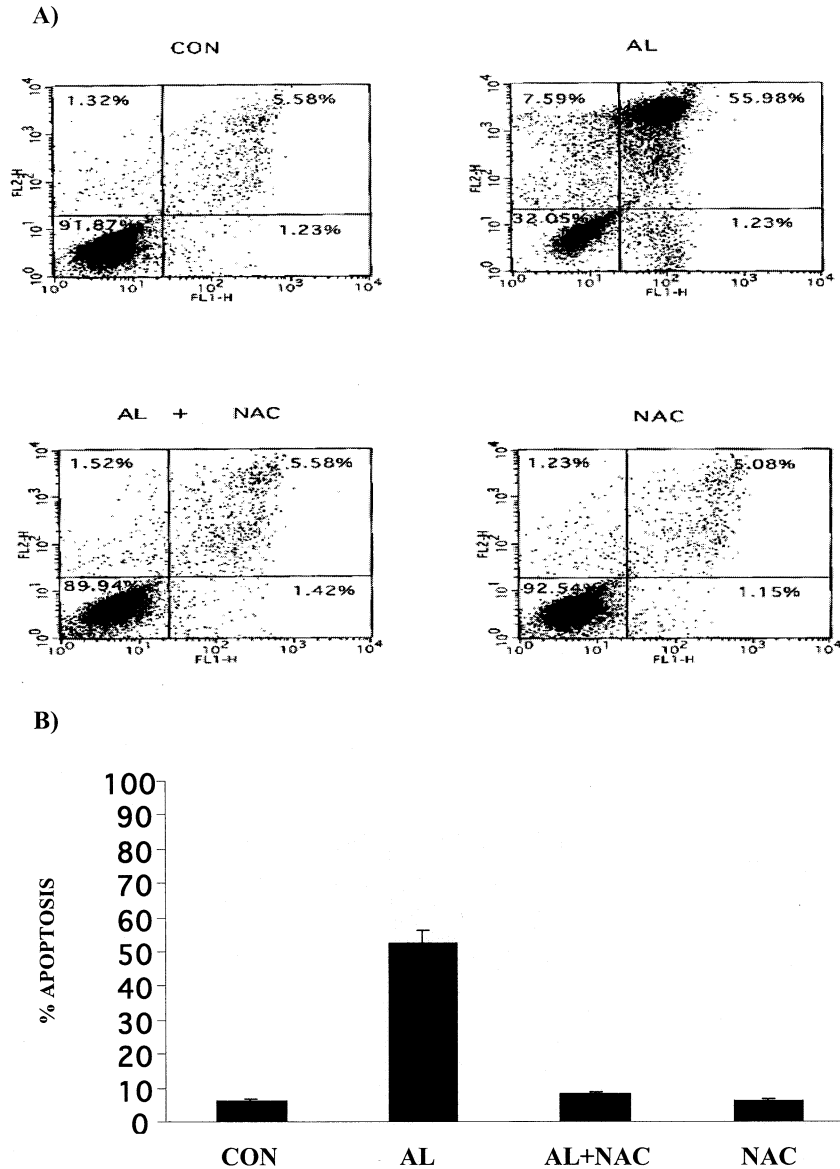


Fig. 3. Flow cytometry of N-acetylcysteine (NAC) inhibition of altholactone (AL) induced apoptosis. (A) cells treated as shown in Fig. 2 were analyzed using annexin V/PI method. NAC (1 mM) was preincubated for 1 h prior to addition of AL (86.2 μ m). The x-axis FL1-H represents annexin V labeling while the y-axis (FL2-H) represents the PI staining. (B) The cumulative data obtained from the cytogram which represents the means (\pm) S.E.M. of three separate experiments.

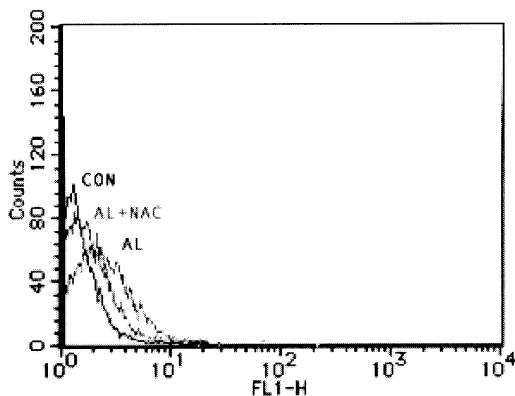


Fig. 4. Effects of *N*-acetylcysteine (NAC) on Altholactone (AL) treated HL-60 cells as assessed by changes in DCFH fluorescence using flow cytometry. Cells were treated either alone (CON), with Al (86.2 μ M) or Al in the presence of NAC (AL + NAC) for 3 h, followed by flow cytometric analysis as described in the Section 2.

tholactone treated cells was reduced in the presence of NAC (Fig. 4, AL + NAC) resulting in a similar pattern with the control treatment. Annexin V assay on altholactone treated cells for 3 h showed that the treatment did not induce any increase of apoptosis over control untreated cells (data not shown). Therefore, any increase in fluorescence seen in this study was not contributed by oxidative stress generated as a result of cell death.

The redox status of a cell is influenced by the balance between the levels of ROS and endogenous thiols such as glutathione (Davis et al., 2001). Recently, Chen et al. (2001) have demonstrated that NAC can reverse the depletion of GSH which occurs during caffeic acid phenylethyl ester induced apoptosis in HL-60 cells. In agreement, Anuradha et al. (2001) have shown that sodium fluoride induces apoptosis in HL-60 cells via an oxidative stress dependent pathway resulting in the loss of mitochondrial transmembrane potential which can be blocked by the antioxidants NAC and GSH. It is tempting to speculate that in our study, altholactone treatment may result to a reduction of cellular GSH and subsequently leading to elevation of ROS. Such elevation as shown in Fig. 4, alters the redox status of the cells, causing a sustained activation of signal-

ing that will initiate the apoptotic cell death execution program (Davis et al., 2001).

In summary, our results have demonstrated that altholactone induces apoptosis in HL-60 cells via the generation of oxidative stress. Future studies will be required to further understand the molecular mechanisms of this potential anticancer compound.

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