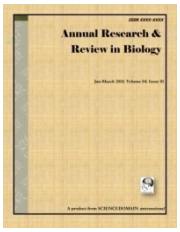

Annual Research & Review in Biology
4(6): 962-977, 2014

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Characterization of the Biochemical Pathway of Apoptosis Induced by D-glucopyranoside Derivatives from *Tulbaghia violacea*

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Author's contribution

This whole work was carried out by the author.

Original Research Article

Received 27th July 2013
Accepted 22nd November 2013
Published 13th December 2013

ABSTRACT

Aim: Chinese hamster ovary (CHO) cells were used to identify the most likely apoptotic biochemical pathway induced by Methyl-α-D-glucopyranoside (MDG), D-fructofuranose-β(2→6)-methyl-α-D-glucopyranoside (DFMDG) and β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside (DFDG) treatments isolated from wild garlic (*Tulbaghia violacea*).
Methodology: Assays, which detect different morphological and biochemical changes in the CHO cells were used to examine the apoptosis induction by individual agents, paying special attention to the mitochondria membrane potential (Δψm) depolarization.
Results: The major events of apoptosis, namely, translocation of phosphatidylserine, mitochondria membrane potential (Δψm) depolarization and activation of caspase-3 in CHO cells were initiated within 2 hours of treatments. The onset of the apoptosis induction was indistinguishable among the MDG, DFMDG and DFDG, and similar to that of staurosporine.
Conclusion: The study shows that CHO cells treated with MDG, DFMDG and DFDG undergo apoptotic death through the mitochondrial (intrinsic) pathway.

Keywords: Apoptosis; mitochondrial pathway; glucopyranosides; mitochondrial permeability transition pore.

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1. INTRODUCTION

Apoptosis, often referred to as cell suicide, is a process in which cells play an active role in their own death. This makes apoptosis distinct from necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems [1]. Apoptosis plays a critical role in many processes such as development, immune responses, elimination of cancerous or infected cells, and eliminating cells that have been damaged by stress e.g. oxidative stress [2]. The apoptotic characteristic changes include the cell shrinkage, nuclear condensation, and collapse of the cell into small intact membrane bound fragments (apoptotic bodies), a form that allows for easy clearance by macrophages [3]. The malfunction of apoptosis is a key hallmark of cancer and is critical for cancer development and tumor-cell survival [2]. Such health implications underscore the potential of using therapeutic strategies to manipulate apoptosis [4].

Apoptosis process involves two major biochemical pathways. The first one is activating the release of cytochrome C from mitochondria through the mitochondrial permeability transition pore (MPTP), also known as, the intrinsic pathway. The other one is activation of cell-surface death receptor known as extrinsic pathway. In the intrinsic pathway, the alterations in mitochondrial structure and function are early events of chemical-induced apoptosis [5, 6]. The MPTP opening leads to the release of pro-apoptotic proteins contained in the mitochondrial intermembrane space. The pro-apoptotic proteins include cytochrome c which activates cascade of the caspases responsible for changes in the cell structure and function that occur in apoptosis, and the caspase independent apoptosis- inducing factor (AIF) [7,8, 9]. In mitochondria, cytochrome c plays an essential role in generating mitochondrial membrane potential ($\Delta\psi m$). This potential is essential in various functions including the production of ATP via oxidative phosphorylation [10]. Therefore, altered $\Delta\psi m$ can cause cytochrome c release and activation of caspases, or, conversely, cytochrome c release may alter mitochondrial function, which in the absence of caspase activity, may lead to the death of the cell.

There are two main pathways leading to apoptosis. One involves the release of cytochrome *c* from mitochondria due to the signals arising within the cell, this pathway is known as the intrinsic or mitochondrial pathway. The second one involves plasma membrane receptors of TNF family triggered by the binding of the death activators, e.g., tumor necrosis factor (TNF), lymphotoxin and Fas ligand (FasL). This pathway is also known as extrinsic pathway [2,4]. Both two mechanisms depend on proteolytic activation of caspases. Caspase independent mechanisms also exist, for instance the mechanism that involves the apoptosis-inducing factor (AIF).

Three -α-D-glucopyranoside derivatives, namely, methyl-α-D-glucopyranoside (MDG), D-fructofuranose-$\beta(2\rightarrow6)$-methyl-α-D-glucopyranoside (DFMDG) and β-D-fructofuranosyl-$(2\rightarrow6)$-α-D-glucopyranoside (DFDG) isolated from the wild garlic (*Tulbaghia violacea*) aqueous extract have been reported to selectively kill Chinese hamster ovary (CHO) cells, estrogen-dependent human breast tumor 'adenocarcinoma' (MCF7) cells and human cervix epitheloid carcinoma (HeLa) cells by inducing apoptosis [11,12]. However, the mechanisms of action through which these glucopyranosides induce the apoptosis are not clearly understood. This paper reports on findings from a study aimed at identifying the biochemical pathway involved in the apoptosis induced by the MDG, DFMDG and DFDG. The findings of this study show that MDG, DFMDG and DFDG activate the intrinsic pathway of apoptosis in CHO cells. CHO cell line was chosen for further studies for possible mechanisms of action

because its apoptotic biochemical network has been thorough extensively studies. Deciphering the rules of the apoptosis induced by MDG, DFMDG and DFDG provide some leads towards improving therapy of cancer and other diseases using *Tulbaghia violacea* extracts.

2. MATERIALS AND METHODS

2.1 α-D-glucopyranosides

Methyl-α-D-glucopyranoside MDG, D-fructofuranose-β(2→6)-methyl-α-D-glucopyranoside (DFMDG) and β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside (DFDG) were isolated from wild garlic (*Tulbaghia violacea*) aqueous extract in earlier studies [11, 12]. The commercial methyl-α-D-glucopyranoside was supplied by Sigma.

2.2 Cell Culture and Treatment

Chinese hamster ovary (CHO) cell cultures were grown in nutrient mixture F-12 (Ham) + L-glutamine supplemented with 0.2 % penicillin-streptomycin + 5 % Foetal calf serum medium. Cell counting was performed using a Neubauer haemocytometer cell counting chamber. After 24 – 48 h incubation at 37°C in a 5 % CO_2 humidified incubator, the cells were sub-cultured in 6-well plates at 2 ml of 2.5 x 10^4 cells/ ml/ well, or in 96-well plates at 100 µl of 0.25 x 10^5 cells/ ml/ well. Both sets of culture were incubated under the same conditions for 24 h. Where necessary, one sterilized coverslip was placed in each well before introducing the cell suspension, for microscopic analysis purposes.

2.3 Annexin-V-PE Binding Assay

Cells were treated with 0.1 mg/ml of one of the three glycopyranosides (i.e., MDG, DFMDG and DFDG), and crude aqueous extract prepared in HAM's F-12 medium. 1 µM staurosporine was used as a positive control. Cells were incubated with PE-labelled Annexin-V as described in the Annexin V binding assay manufacturer's manual and analysed by FACScan (BD Biosciences).

2.4 Use of APO P*ercentage*™ dye on FACS to Quantify Apoptosis

Cells were treated with 0.1 mg/ml of one of the three glycopyranosides MDG, DFMDG and DFDG, and crude aqueous extract prepared in HAM's F-12 medium containing the diluted APO Percentage™ dye as described in the manufacturer's manual [13]. A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences).

2.5 Active Caspase-3 Assay

The aim of the active caspase-3 assay was to evaluate the earliest time at which caspase-3 activation occurred in cells treated with MDG, DFMDG and DFDG. It also aimed at comparing these times among the three apoptosis inducers. Cells were treated with 0.1 mg/ml of one of the three glycopyranosides (i.e., MDG, DFMDG and DFDG) at different time intervals ranging between 0 and 12 h. After treatment of the cells, the active caspase-3 was analysed using anti-active caspase-3-PE monoclonal antibody (BD Biosciences). The cells

were analysed using the FL-2 channel on a FACScan flow cytometer (Becton Dickinson). A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences).

2.6 Determination of Mitochondrial Membrane Potential Depolarization($\Delta\psi m$)

$\Delta\psi m$ was measured by lipophilic cation JC-1, which selectively enters mitochondria. JC-1 exists in a monomeric form emitting at 527 nm after excitation at 490 nm. Depending on the $\Delta\psi m$, JC-1 forms J-aggregates which are associated with a large shift in emission (590 nm). Color dye changes reversibly from orange to green as mitochondrial membranes become depolarized. Cells were treated with 0.1 mg of one of the three glycopyranosides (i.e., MDG, DFMDG and DFDG) at different time intervals ranging between 0 and 12 h. The cells were stained with JC-1 according to manufacturer's manual, and, were later on analysed using both the FL-1 (green fluorescence) and FL-2 channels (red fluorescence) on a FACScan (Becton Dickinson). A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences).

2.7 Determination of Fragmentation of Genomic DNA

Internucleosomal DNA fragmentation is a hallmark of apoptosis in mammalian cells [3,4]. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl termini in the DNA. This property was used to identify apoptotic CHO cells by labeling the 3'-hydroxyl ends with directly conjugated fluorescein-deoxyuridine triphosphate nucleotides (FITC-dUTP) catalyzed by deoxynucleotidyl transferase (TdT). Detection of DNA fragmentation in cultured CHO cells, treated with MDG, was achieved using the BioVision's ApoDIRECT TUNEL-based assay kit and was analysed by flow cytometry (Ex/Em = 488/520 nm for FITC) on a FACScan (Becton Dickinson). A minimum of 10,000 cells per sample was acquired and analysed using CELLQuest PRO software (BD Biosciences).

3. RESULTS AND DISCUSSION

MDG, DFMDG and DFDG treatment induced the occurrence of cell shrinkage, translocation of membrane phospholipids phosphatidylserine (PS), loss of mitochondrial membrane potential, caspase-3 activity and genomic DNA fragmentation in CHO cells. The above events are hallmarks of the occurrence of apoptosis [14,3,15,4], which strongly support the use of the wild garlic extract in traditional medicine to treat cancer [16].

Annexin V-PE assay showed that after treatment of CHO cells with MDG, DFMDG and DFDG, wild garlic aqueous crude extract and staurosporine, M1 peak was displaced to the right indicating the presence of Annexin V-PE positive (Figure 1). Annexin V-PE serves as a sensitive probe for flow cytometric analysis (in FL-2 channel). There is a direct relationship between Annexin V-PE fluorescence and the number of the apoptotic cells. In Figure 1, the percentages of positive cells are in majority of cases, around one half, of the positive control, and higher than the crude extract. The observed difference between positive control (staurosporine) and the three D-glucopyranosides may be attributed to their differences in molecular structures leading to differences in how they interact with their target biomolecules in the CHO cells.

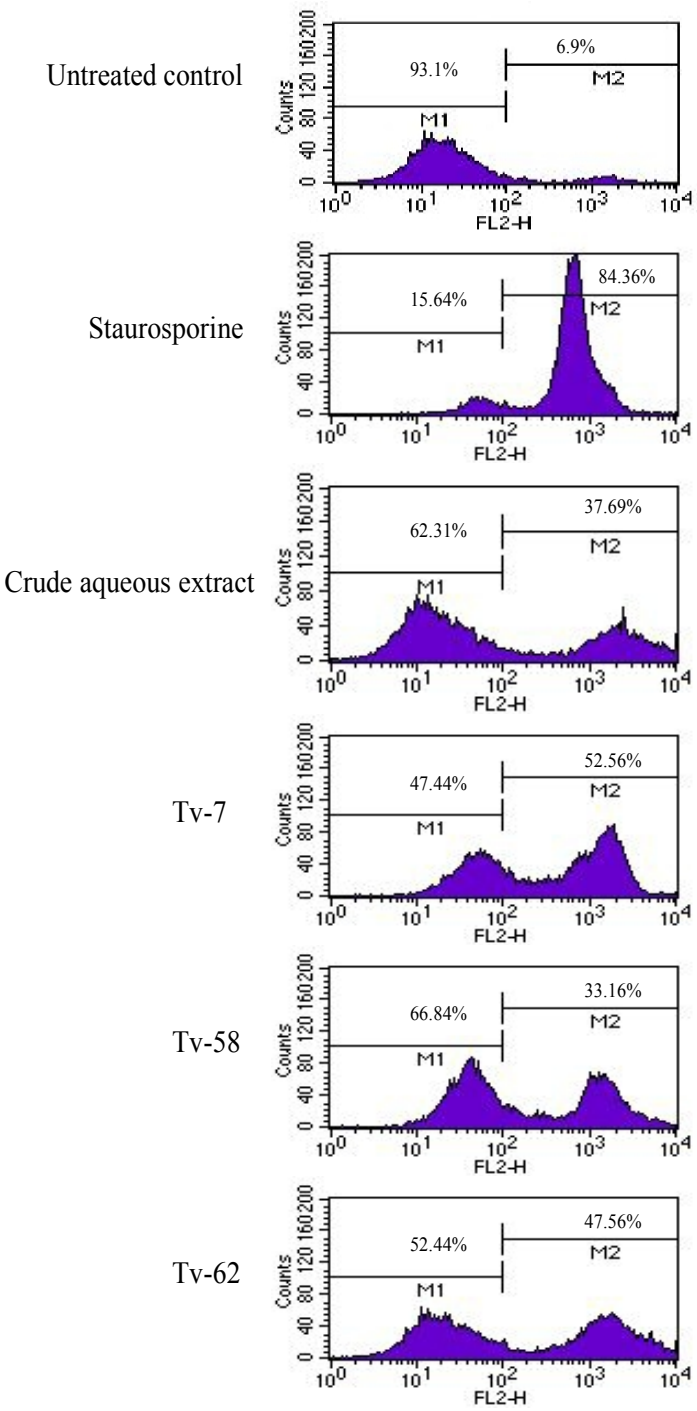

Fig. 1. Annexin V-PE binding assay on CHO cells treated with MDG (Tv-7), DFMDG (Tv-58), DFDG (Tv-62), crude aqueous extract from *T. violacea* and staurosporine. M1 is the Annexin V-PE negative and M2 is the Annexin V-PE positive populations.

The results of the APO Percentage™ apoptosis assay on CHO cells treated with MDG, DFMDG, DFDG and then compared to the positive control 1 µM staurosporine. The FACScan analysis showed that cells in the samples treated with MDG, DFMDG, DFDG crude aqueous extract were APO Percentage™ dye positive (Fig. 2). It seems, from Figure 2, crude aqueous extract showed highest apoptotic activity compared to positive control as well as MDG, DFMDG and DFDG. There might be a possibility that some other (unidentified) compound is also contributing to apoptotic effect.

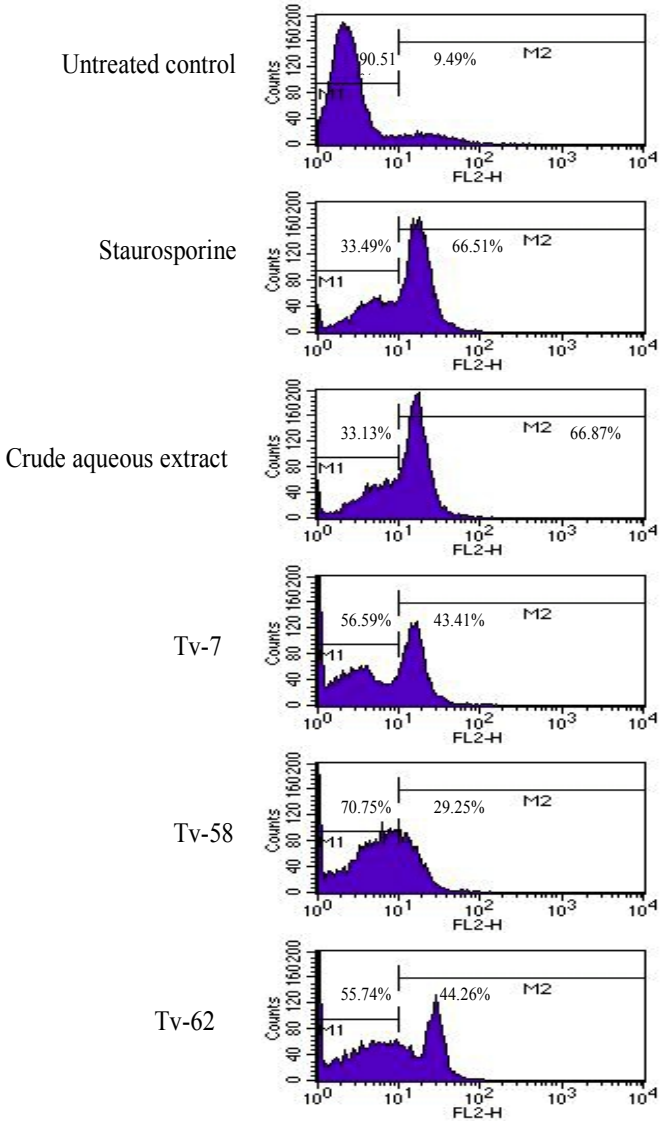

Fig. 2. APO Percentage™ apoptosis assay on CHO cells treated with MDG (Tv-7), DFMDG (Tv-58), DFDG (Tv-62) and staurosporine. M1 indicates viable cells and M2 apoptotic cells.

The APO Percentage^{TM} dye staining of the MDG, DFMDG and DFDG that were treated CHO cells, confirms the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (also known as 'flip-flop mechanism') which has been linked to the onset of the execution phase of apoptosis [13]. The results of APO Percentage^{TM} assay agree with the Annexin V-binding assay. This confirms that the observed effect from the treatment of CHO cells with fractions of MDG, DFMDG and DFDG, is indeed apoptosis and not necrosis. In the APO percentage apoptosis assay results (Fig. 2), the crude extract activity is equal to that of the positive control, and activity of all the three purified D-glucopyranosides is lower. Staurosporine is an optimized positive control for apoptosis induction, so, it will always show optimum results, whereas, the experimental agents may need further optimization to achieve their optimal apoptosis induction potentials. In addition, the crude extract is a mixture of many compounds whose activity maybe complex to explain. The results confirmed that the three α-D-glucopyranoside derivatives induce apoptotic activity.

Fig. 3 shows that MDG, DFMDG and DFDG induce caspase-3 activity, meanwhile, the time course flow cytometric analysis showed that the untreated cells were negative for active caspase-3, whereas, at least 40 % of treated cells were positive for active caspase-3 staining as early as 2 h after the treatments (Fig. 3). The data in Fig. 3 represents the average of 4 experiments. There was no difference in the time of activation among the MDG, DFMDG and DFDG samples.

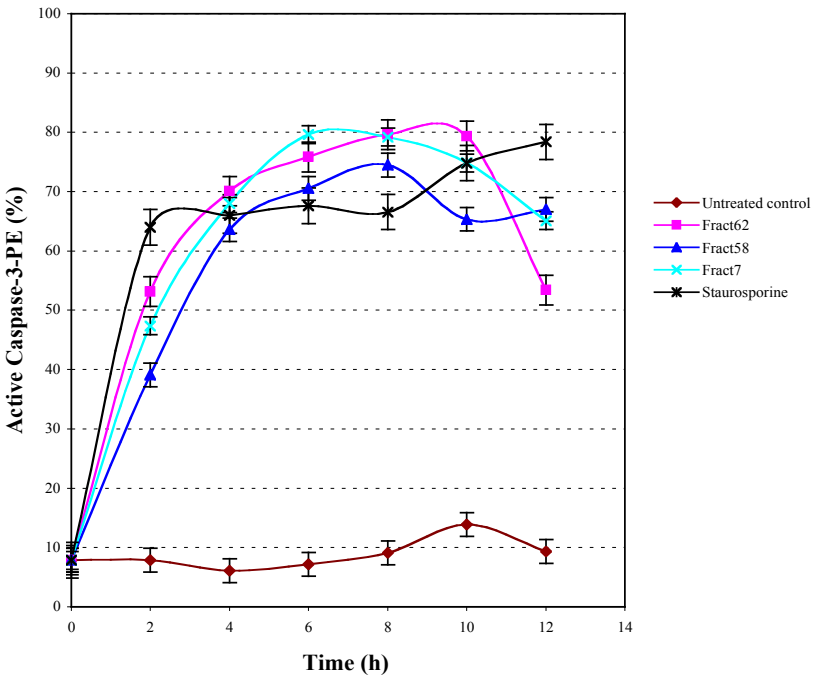

Fig. 3. Time course flow cytometric analysis of populations of untreated control cells and cells induced with MDG (Fract7), DFMDG (Fract58), DFDG (Fract62) and staurosporine using anti-active caspase-3 antibody. The graphic shows median of three experiments at each tested point and also shows a standard deviation.

It has been widely documented that cascade of members of the cysteine protease family caspases, is indispensable for apoptosis to occur [17,18,19]. Caspase-3 is the most downstream in the apoptotic protease cascade, and, the inhibition of caspase-3 by synthetic peptide inhibitors often prevents apoptosis induced by various stimuli [7]. This study supports these hypotheses: the active caspase-3 staining data indicate the same induction time of caspase-3 activity, suggesting that MDG, DFMDG and DFDG activate the same apoptotic pathway.

As shown in Fig. 4, the decrease in the red fluorescence emitted by JC-1 was observed in the CHO cells treated with staurosporine, MDG, DFMDG and DFDG. This indicates that staurosporine, MDG, DFMDG and DFDG induce mitochondrial depolarization. For example, DFDG induces 52% reduction of $\Delta\psi m$ compared to untreated cells death of 6% at the 4th h of the experiment. The graphs of the time-course apoptosis induction profiles (Fig. 5) were indistinguishable from the three agents.

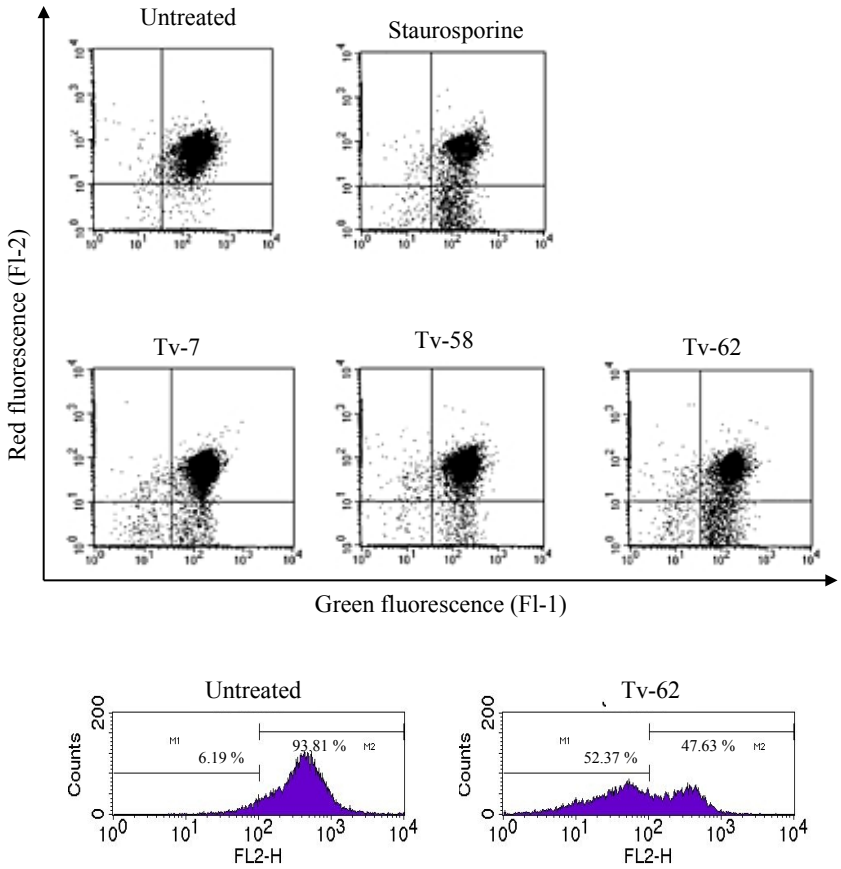

Fig. 4. Fluorescence pattern of CHO cells stained with JC-1 after a 4 h incubation with 1 µM of staurosporine, and 0.1 mg/ml MDG (Tv-7), DFMDG (Tv-58), DFDG (Tv-62). Green fluorescence (FL-1) represents mitochondrial mass whereas red fluorescence (FL-2) represents mitochondrial transmembrane potential ($\Delta\psi m$). M1 and M2 indicate the cell populations with low and high $\Delta\psi m$ respectively.

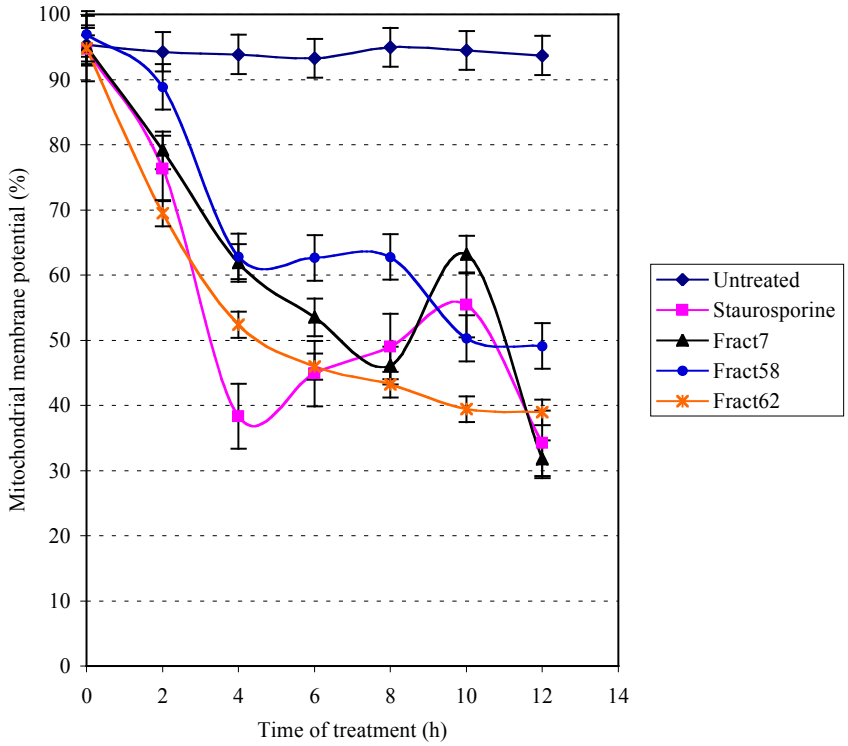

Fig. 5. Time course analysis of mitochondrial membrane potential (Δψm) during apoptosis on CHO cells treated with 1 µM of staurosporine, and 0.1 mg/ml of MDG (Fract7), DFMDG (Fract58), DFDG (Fract62). The graphic shows median of three experiments at each tested point and also shows a standard deviation.

The drop in the mitochondrial membrane potential (Δψm) in all the CHO cell cultures treated with MDG, DFMDG and DFDG within 2 h coincides with the time course of the caspase-3 activation. The profiles of time-dependent induction of drop in Δψm (apoptosis) were indistinguishable in all the three agents. These results indicate that mitochondrial dysfunction and subsequent cell death occur in CHO cells treated with MDG, DFMDG and DFDG and suggest that Δψm is a marker of the observed cell death. These data support the hypotheses that disruption of mitochondrial membrane has been reported to be an early and irreversible event leading to apoptosis, along with the opening of permeability transition pores in the mitochondrial inner membrane and the subsequent release of mitochondrial intermembrane proteins (cytochrome c, apoptosis-inducing factor) into the cytosol and activation of endogenous caspases [20].

Commercial methyl-α-D-glucopyranoside (MDG) induced occurrence of a concentration-dependent PS flip flop mechanism (Fig. 6) in CHO cells. At 2 mM, 21% of the CHO cell culture was apoptotic (Annexin V PE positive).

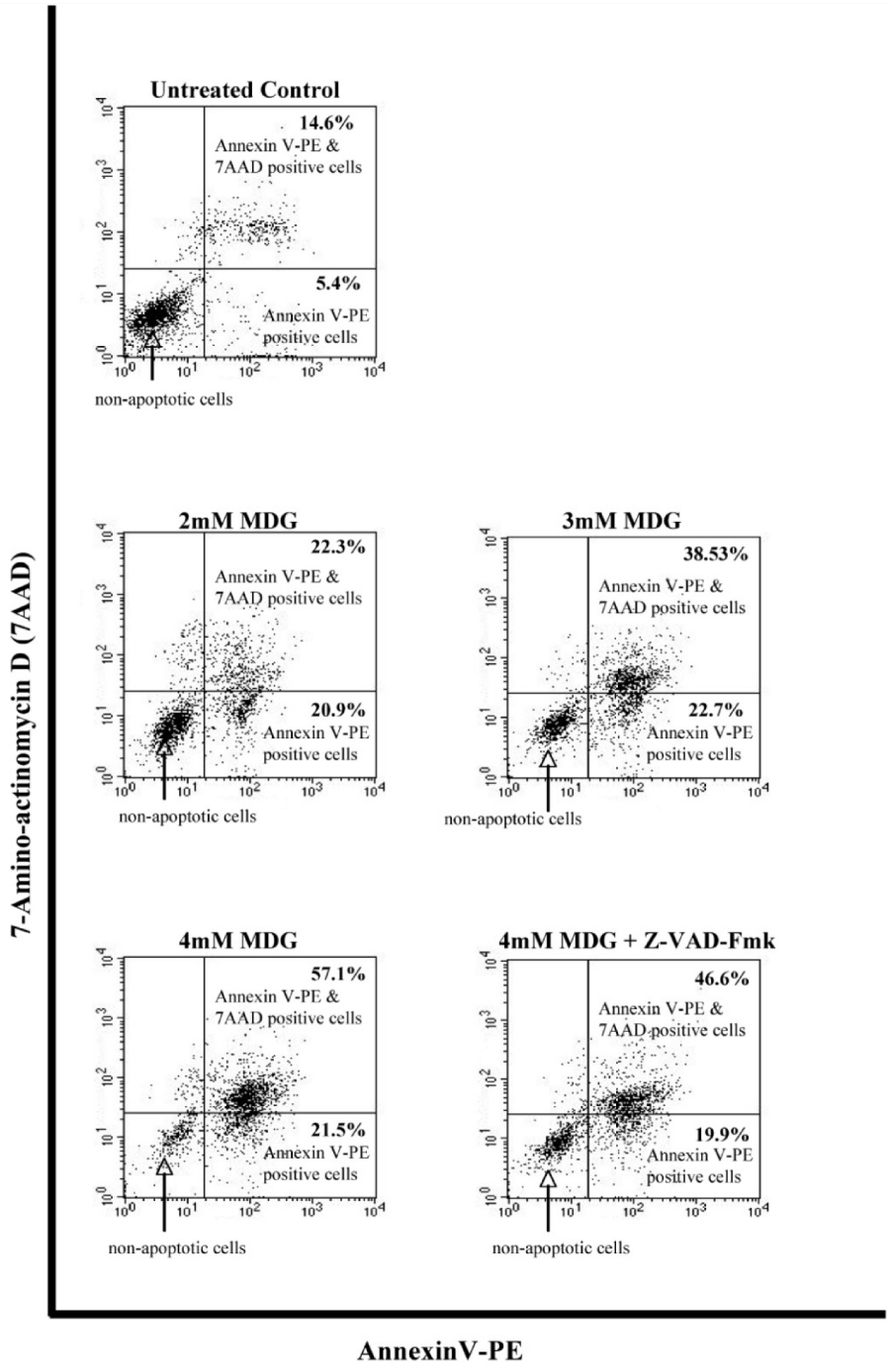

Fig. 6. Occurrence of the concentration-dependent phosphotidylserine flips mechanism induced in CHO cells treated with commercial methyl-α-D-glucopyranoside (commercial MDG). Annexin V-PE indicates apoptotic cells with membrane intact and 7AAD indicates membrane-damaged cells.

One of the most easily measured features of apoptotic cells is the break-up of genomic DNA by cellular nucleases. The commercial MDG also induced occurrence of a concentration-dependent DNA fragmentation (Fig. 7) in CHO cells. At 4mM about 90% cells had their genomic DNA fragmented. However, at higher concentrations, more cells stained 7-Amino-actinomycin D (7AAD) positive because when the concentration of inducer is too high cells undergo necrotic death instead of apoptosis.

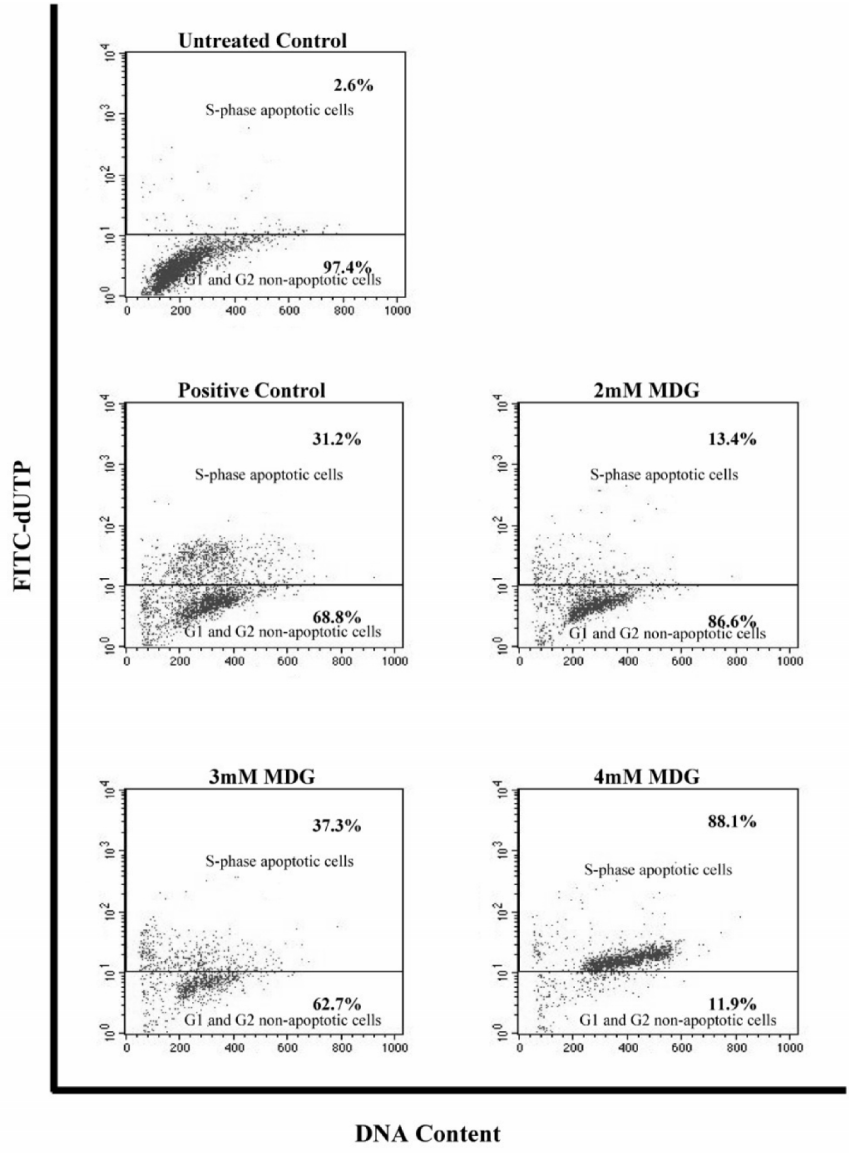

Fig. 7. Occurrence of the concentration-dependent genomic DNA fragmentation induced in CHO cells treated with commercial methyl-α-D-glucopyranoside (MDG). Staurosporine was used as a positive control. Terminal FITC-dUTP nick end labeling (TUNEL), a gold-standard assay for detecting DNA fragmentation by labeling the terminal end of nucleic acids was used.

These data strongly suggest that the apoptotic activity of MDG, DFMDG and DFDG samples was a glycoside-induced apoptotic cell death. Elevated sugar metabolism generates reactive oxygen species (ROS), reactive nitrogen species and oxidative products of glycation, and such products may lead to apoptosis [21,22]. Glycoside has high affinity to the endogenous glucose transporters (GLUT1-5) as well as the endogenous Na^+/glucose co-transporter proteins, which form channels in the membrane through which they are transported. High cellular concentrations of glucose or fructose have been reported to reduce cell proliferation and induce apoptosis through oxidative stress, drop of mitochondrial membrane potential ($\Delta\psi_m$), release of cytochrome *c,* and proteolytic processing of caspases-3 [22,23]. The observation of the early drop of $\Delta\psi_m$ in the cells treated with MDG, DFMDG and DFDG is in agreement with these reports.

ROSs are known to induce apoptosis involving Bax [24]. ROS may also trigger an apoptotic death program through an oxidative stress-involving JNK signaling pathway [25]. Also, an increase in generation of ROS may lead to activation of NF-κB, and induction of apoptosis by a glucose-specific and NO synthase-dependent mechanism [26]. Increased generation of ROS, activation of NF-κB, and induction of apoptosis has also been observed in cells incubated with 3-O-methyl-D-glucose [26]. These evidences supports the suggestion that there was ROS generation in the MDG, DFMDG and DFDG -treated cells due to the metabolism of the chemical compounds present in the samples, and led to the ROS-induced apoptotic cell death.

Glucose- or fructose-induced apoptosis requires oxidative phosphorylation [27]. The oxidative phosphorylation step involves mitochondrial surface hexokinases, which can phosphorylate reducing sugar moieties such as glucose and fructose [28]. Mammalian cells express four hexokinase (HK) isoforms; HKI, HKII HKIII and HKIV [29]. Hexokinases I-III are each about 100 kD in size, and have relatively high affinities for several different hexose substrates [29]. Hexokinase IV, more commonly known as glucokinase (GK) is about 50 kD [30]. HKI and HKII are associated with the cytoplasmic face of the outer mitochondrial membrane. An amino-terminal hydrophobic domain found only in HKI and HKII mediates this interaction [31]. HKI and HKII bind to the voltage-dependent anion channel (VDAC) [32]. Increased expression of hexokinase has been associated with protection from apoptosis while loss of mitochondrial hexokinase provides binding sites for Bax and this has been associated with apoptosis [33]. For instance in glucose phosphorylation, hexokinase catalyses the transfer of a γ-phosphoryl group from ATP to the C-6 hydroxyl of glucose to generate glucose-6-phosphate. The mechanism involves the attack of C-6 hydroxyl oxygen of glucose on the γ-phosphorate of $MgATP^{2-}$ displacing MgADP. Bax competes with the HKs for binding and accumulation at the outer mitochondria membrane. The elevated glucose levels increases glucose metabolism (the glycolysis); thus more hexokinase dissociates from the mitochondrial sites, allowing the Bax to bind, leading to the opening of the MPTP and apoptosis.

3.1 The Proposed Model of Mechanism of the Apoptosis Induction

The proposed model of mechanism used by the MDG in Fig. 8 shows that (A) under normal physiological glucose level in a cell, hexokinase binds to the voltage-dependent anion channel (VDAC) on the outer surface of the mitochondrial outer membrane, thereby inhibiting Bax-induced MPTP [24,32]. VDAC acquires capacity for hexokinase binding by interacting with adenine nucleotide trans-locator (ANT), the most abundant protein of the inner mitochondrial membrane. The complex formed by hexokinase, VDAC and ANT allows

for the efficient utilization of mitochondrially generated ATP, enabling the first step of glycolysis to occur (called aerobic glycolysis). This is a vital step towards sugar-induced apoptosis. ANT catalyzes the specific exchange (antiport) of ATP and ADP on the inner membrane, while VDAC allows for the passive diffusion of solutes including ATP and ADP. VDAC plays an important role in apoptosis by participating in the release of intermembrane space proteins e.g. cytochrome *c* [34]. VDAC adopts a different structure when it forms a complex with the ANT. This new VDAC structure has a higher capacity for binding of hexokinase. Cytochrome *c* is associated to the VDAC-ANT complexes [35], supporting the role played by the complex to release cytochrome *c* to the cytosol.

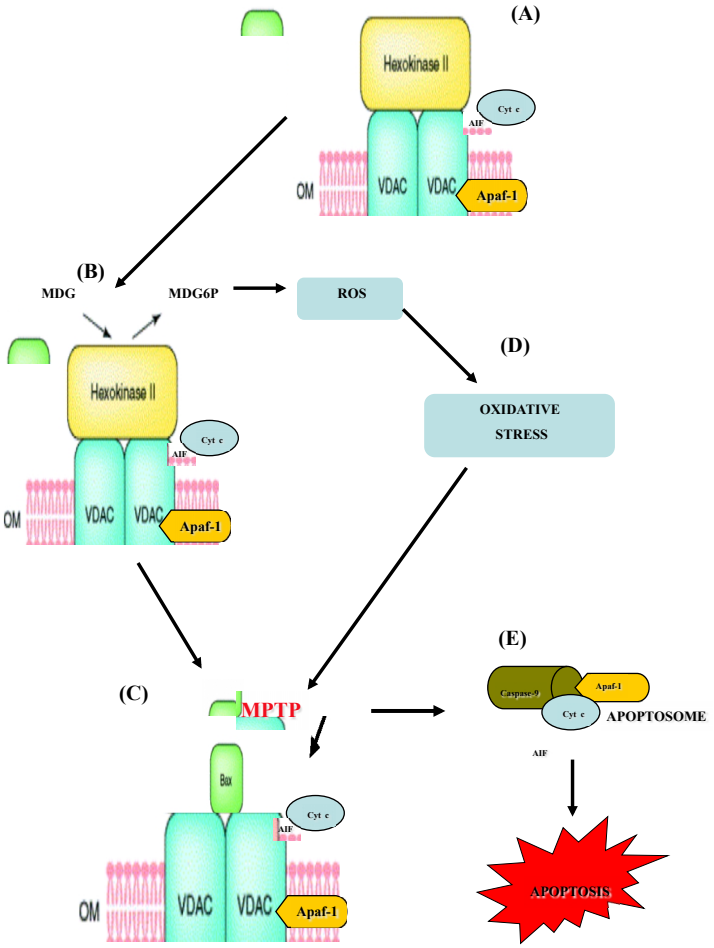

Fig. 8. Hexokinase-VDAC-ANT complex (A). Hexokinase-dependent phosphorylation of MDG (B) leads to the dissociation of the Hexokinase from its ANT-VDAC complex and the production of ROS (D). The exposed VDAC is accessible by Bax (C) and might form a composite channel with Bax or facilitate the insertion of Bax into the outer membrane (OM) of the mitochondrion, thereby allowing for Bax-mediated MPTP formation (C), release of pro-apoptotic proteins from the mitochondria and formation of the APOPTOSOME complex (E), and subsequent apoptosis.

MDG is taken up into the cells by glucose transporters and is oxidative phosphorylated by hexokinase into methyl-α-D-glucopyranoside-phosphate (MDG-P) as shown in Fig. 8 (B). This MDG metabolism may lead the cell to apoptotic death through several ways. The dissociation of the mitochondrial hexokinases from the mitochondrial surface, and participate in the MDG phosphorylation, exposes Bax-binding sites of VDAC, and allows the Bax-induced opening of MPTP (C), and the release of the proapoptotic proteins such as cytochrome c, Apaf-1 and AIF from the mitochondria [36]. As a result, the collapse of $\Delta\Psi m$, the activation of caspase-3, the fragmentation of the genomic DNA and cell death occur. The critical event may be the actual decrease of hexokinase over time indicating open sites for Bax binding rather than the absolute concentration of hexokinase because the relative number of hexokinase binding sites is unknown [24]. As in glucose, the products of the oxidative phosphorylation of MDG and production of the MDG-P may lead to increased production of ROSs, oxidative stress (D), also leading to the opening of MPTP, release of cytochrome c, Apaf-1 and AIF, formation of the apoptosome (a complex of cytochrome c, Apaf-1 and caspase-9) in the cytosol, cascade of caspases and subsequent induction of apoptosis.

Thus, the observation of early externalization of the phosphatidylserine in the cells treated by MDG, DFMDG and DFDG supports the occurrence of the MPTP, most probably due to the oxidative stress from ROSs and Bax-binding to the VDAC. This supports the hypothesis that MDG, DFMDG and DFDG activate the intrinsic (mitochondrial) apoptosis pathway.

CONCLUSION

Methyl-α-D-glucopyranoside (MDG), D-fructofuranose-β(2→6)-methyl-α-D-glucopyranoside (DFMDG) and β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside (DFDG) isolated from wild garlic (*Tulbaghia violacea*) aqueous extract kill Chinese hamster ovary (CHO) cells through the intrinsic mitochondrial apoptosis pathway. Deciphering the rules of the apoptosis induced by MDG, DFMDG and DFDG may contribute to the drug discovery efforts towards improving cancer therapy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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