INTRODUCTION

Naturally occurring stilbenes including resveratrol (RSV 1) have attracted a high level of attention due to their biological activities (Inayat-Hussain and Thomas, 2004; Roupe et al., 2006). Stilbenes, characterized by a 1,2-diphenylethylene core structure, exist in some plants in the form of Z or E-isomers (Gorham et al., 1995). The former is represented by the combretastatins (such as combretastatin A 2) which are among the most potent natural antimitotic agents, inducing cell death (Nam, 2003). E-stilbenes, represented by RSV 1, pterostilbene 3 and piceatannol 4 (Fig. 1) have also been extensively investigated but display a different range of biological activities. Apart from antioxidant and cardiovascular protective properties, these compounds have also been found to display significant antitumor properties via the apoptotic death pathway (Surh et al., 1999; Frémont, 2000; Roupe et al., 2006).

Apoptosis pathways are important target for effective cancer therapies (Ghobrial et al., 2005). The extrinsic pathway requires signaling via the death receptor whereas...
the intrinsic pathway requires the release of apoptogenic proteins from the mitochondria (Jin and El-Deiry, 2005). These apoptogenic proteins, specifically cytochrome c, form an apoptosome complex which activates the initiator caspase-9 (Bao and Shi, 2007). The apoptosome mediated caspase-9 activation results in the amplification of downstream executioner caspases including caspases-3 and -7 (Bao and Shi, 2007). To date, several studies on stilbenes especially resveratrol have demonstrated mitochondrial mediated apoptosis (Dong, 2003; Luzi et al., 2004; Gosslau et al., 2008; Reis-Sobreiro et al., 2009). In addition, derivatives of resveratrol including 3,4,4′,5-tetrahydroxystilbene 5, 3,4,4′,5-tetramethoxystilbene 6 and 2,3′,4,4′,5′ pentamethoxystilbene 7 (Fig. 1) induce cytotoxicity primarily via apoptosis where the hydroxy and methoxy moieties are pivotal to the pro-apoptotic activity (Gosslau et al., 2005; Li et al., 2010).

Since resveratrol and resveratrol derivatives show promising anticancer activities in many in vitro and in vivo models (Jang et al., 1997; Aggarwal et al., 2004; Pervaiz, 2004; Roupe et al., 2006), the aim of this study was to characterize the cytotoxic effects of 22 stilbenes with a variety of substitution patterns and functional groups including chloro-, hydroxy-, acetoxy-, benzylloxy-, carboxyl- and methoxy- groups in human K562 chronic myelogenous leukemia cells. The most cytotoxic compound from our stilbene library was a benzylolated stilbene namely 3,5-dibenzyloxy-4′-hydroxystilbene (28). Further investigation of the mechanisms of apoptosis demonstrated that 28 mediated an intrinsic apoptotic pathway with an early activation of the initiator caspase-9. Taken together, this is the first demonstration of a rapid activation of caspase-9 induced by a benzylolated stilbene which may be important for further development of potent chemotherapeutic agents.

**MATERIALS AND METHODS**

**Chemicals**

Cell culture medium RPMI-1640 was purchased from Gibco (Auckland, New Zealand) while fetal bovine serum and penicillin/streptomycin were obtained from PAA (Linz, Austria). Resveratrol, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), ethidium bromide (EtBr), ethylenediaminetetraacetic acid (EDTA), etoposide, bovine serum albumin (BSA), propidium iodide and menadione were purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC was from Pharmingen (San Diego, CA, USA) while dimethyl sulfoxide (DMSO) and hydrogen peroxide (H2O2) were from Merck (Darmstadt, Germany). Rabbit polyclonal anti-caspase-3 antibody, rabbit polyclonal caspase-9 antibody, HRP conjugated goat anti-rabbit antibody and HRP conjugated goat anti-mouse antibody were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Reagents for synthesis were purchased from Sigma Aldrich (St. Louis, MO, USA). Solvents were from Merck or J.T. Baker (Deventer, Netherlands). Each of these compounds was dissolved in DMSO to make a stock solution and kept in -20°C freezer and diluted in cell culture medium to the desired final concentration for treatment purposes.

**General method for the synthesis of para-hydroxylated stilbenes (15, 25 and 28)**

4-Acetoxyiodobenzene (0.1 g, 1.09 mM) was added into a 2 ml mixture of MeCN/H2O (1:1, v/v). Pd (OAc)2, 48% (0.01 g, 0.045 mM) was added into the solution, followed by Ph3P (0.014 g, 0.05 mM), K2CO3 (0.18 g, 1.3 mM), a phase transfer reagent n-Bu4NCl (0.12 g, 0.43 mM) and a suitably substituted styrene (0.76 mM) (which refers to the use of 3-methoxystyrene and 3,5-dibenzoxystyrene coupling partners in the Heck reaction leading to 15 and 28 respectively). The resulting solution was stirred at room temperature overnight. The reaction was quenched
with water and the mixture was extracted with ethyl acetate. After evaporation, the crude product was purified by preparative thin layer chromatography. The acetyl group was hydrolysed with the help of catalytic amounts of NaOMe in a MeOH/THF mixture at room temperature to yield 12-OH-stilbenes 15 and 28.

**General method for synthesis of fully protected stilbenes (3, 11-14, 16-17, 25-27)**

Protected iodophenols, iodochlorobenzene, or iodo-benzoate derivatives (13.4 mM) were dissolved in 100 ml of DMF. PdCl2 (0.24 g, 1.34 mM) was added to the solutions followed by Ph3P (0.71 g, 2.68 mM), AgNO3 (2.28 g, 13.4 mM), KOAc (1.63 g, 16.60 mM) and appropriate 1,6-Diacetoxy stilbene 13 and 9, respectively. The resulting solutions were stirred at 120°C under nitrogen overnight followed by quenching with water and the mixtures were extracted with ethyl acetate. After evaporation, the crude products were purified by column chromatography to yield 3 and 10-21. 9 and 10 were obtained from thermal decomposition of 19 and 23, respectively. The novel stilbenes synthesized in this study were 13, 20 and 26. The NMR data for the novel compounds are as follow:

**1,6-Diacetoxy stilbene 13**

1H-NMR (CDCl3, 300MHz): δ 2.39 (3H, s, OAc), δ 2.34 (3H, s, OAc), δ 7.10 (2H, s, olefinic CH), δ 7.12 (1H, dd, J = 7.2Hz, 2.1Hz), δ 7.13 (2H, d, J = 8.7Hz, 2.7Hz), δ 7.27 (1H, td, J = 7.2Hz, 1.8Hz), δ 7.32 (1H, td, J = 7.2Hz, 2.1Hz), δ 7.41 (2H, d, J = 8.7Hz, 2.1Hz), δ 7.69 (1H, dd, J = 7.2Hz, 2.1Hz)

**1-Chloro-12- benzyletherstilbene 20**

1H-NMR (CDCl3, 300MHz): δ 6.96 and 7.03 (2H, J = 16.2Hz, olefinic CH), δ 7.00 (2H, d, J = 8.4Hz, 2.1Hz), δ 7.35 (2H, d, J = 8.4Hz), δ 7.28-7.5 (7H, m, overlapped), δ 5.12 (s, CH3)

**3,5-Dimethoxy-12-benzyloxy stilbene 26**

1H-NMR (CDCl3, 300MHz): δ 7.00 and 7.09 (2H, J = 16.2Hz, olefinic CH), δ 6.67 (2H, d, J = 2.4Hz), δ 6.40 (1H, t, J = 2.4Hz, 2.4Hz), δ 7.21 (2H, d, J = 7.8Hz), δ 7.55 (2H, d, J = 7.8Hz), δ 7.63 (1H, tt, J = 7.2Hz, 1.5Hz), δ 7.51 (2H, t, J = 7.2Hz), δ 8.20 (2H, d, J = 6.8Hz), δ 3.82 (3H, s, 2 x OCH3)

**Cell culture**

Human chronic myelogenous leukemia K562 cells were purchased from American Type Tissues Collection (ATCC) and grown in RPMI-1640 medium enriched with 10% fetal bovine serum. These media were supplemented with 1% penicillin-streptomycin and kept in a humidified 5% of CO2 incubator. Cells were subcultured at 1 x 106 cells/ml every 2 to 3 days.

**Cytotoxicity assay**

Cell viability was evaluated using the MTT assay as described previously (Rajab et al., 2005). Briefly, 5 x 104 cells/ml were treated in 96-well plates (TPP, Switzerland) for 48 hr with all the compounds (0-100 μM). At the end of the incubation time, the cells were incubated with 0.5 mg/ml of MTT for 4 hr.

**Apoptosis detection using Annexin V-FITC/PI dye**

Apoptosis assessment was carried out as described by Inayat-Hussain et al. (2003). Briefly, treated cells (5 x 104 cells/ml) were collected via centrifugation at 220 x g for 5 min and washed using chilled PBS. The resulting pellet was resuspended with 100 μl Annexin-V binding buffer and 2.5 μl Annexin-V FITC was added to the cells suspension before incubation for 15 min at room temperature in the dark. Then, 5 μl of PI (50 μg/ml) was added to the cells and further incubated for 5 min at room temperature. Finally, 400 μl of 1X Annexin V binding buffer was added to the cell suspension. Cells were analyzed immediately using flow cytometry. Data were acquired with CellQuest® software where 10,000 events per sample were collected. The debris was excluded by scattered gating (forward vs. side) and the data were displayed as a two-color dot plot with AnnexinV-FITC (green fluorescence, X axis) versus PI (red fluorescence, Y axis). Viable cells were negative for both Annexin V-FITC and PI as shown in the lower left quadrant; apoptotic cells were derived from Annexin V-FITC positive with low PI (lower right quadrant) and with high PI (upper right quadrant). The latter represents cells that were late in the apoptotic process. Necrotic cells were negative for Annexin V-FITC and positive for PI.

**Mitochondrial membrane potential (ΔΨm) assessment**

The potentiometric dye, tetramethylrhodamine ethyl ester (TMRE), was used to determine ΔΨm (Chan et al., 2010). In this experiment, menadione (50 μM) was used as positive control. Treated cells (5 x 104 cells/ml) were collected via centrifugation at 200 x g for 5 min. The pellets were then suspended with 1 ml medium and 1 μl of 50 μM TMRE was added to the cells. After incubation for 15 min at 37°C, the cells were collected and washed with
lysates were then collected via centrifugation at 10,000 g for 15 min. The resulting crude PBS. The cells were then lysed with RIPA buffer (enriched with complete mini) for 15 min. The resulting crude lysates were then collected via centrifugation at 10,000 g for 15 min at 4°C. Protein concentrations of the cell lysates were determined with Bradford protein assay (Bio-Rad, Hercules, CA, USA) and 20 μg of protein for each sample was loaded onto 12% (resolving gel) SDS-polyacrylamide gels in the Mini-PROTEAN 3 Mini Vertical (Bio-Rad) set. The gels were transferred to PVDF membrane and blotting was performed in Towbin’s buffer for 1½ hr at 100 V constant. Subsequently, membranes were reacted with each primary antibody to caspase-9 or caspase-3 (Cell Signaling Technology) before the membrane was incubated with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase. Finally, immunostaining with the antibody was performed using enhanced chemiluminescence kit (ECL-kit) from Amersham, Piscataway, NJ, USA. X-ray film was exposed to the membrane for the detection of the protein of interest.

**Western blot analysis**

Treated cells were collected and washed with chilled PBS. The cells were then lysed with RIPA buffer (enriched with complete mini) for 15 min. The resulting crude lysates were then collected via centrifugation at 10,000 g for 15 min at 4°C. Protein concentrations of the cell lysates were determined with Bradford protein assay (Bio-Rad, Hercules, CA, USA) and 20 μg of protein for each sample was loaded onto 12% (resolving gel) SDS-polyacrylamide gels in the Mini-PROTEAN 3 Mini Vertical (Bio-Rad) set. The gels were transferred to PVDF membrane and blotting was performed in Towbin’s buffer for 1½ hr at 100 V constant. Subsequently, membranes were reacted with each primary antibody to caspase-9 or caspase-3 (Cell Signaling Technology) before the membrane was incubated with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase. Finally, immunostaining with the antibody was performed using enhanced chemiluminescence kit (ECL-kit) from Amersham, Piscataway, NJ, USA. X-ray film was exposed to the membrane for the detection of the protein of interest.

**Structure-activity relationship**

The Leadscope® software provides capabilities for looking at the relationships between families of structures that share a common structural feature and any biological response data (Roberts et al., 2000; Johnson et al., 2001; Blower et al., 2002; Cross et al., 2003; Yang et al., 2004). The software uses a dictionary of over 27,000 pre-defined structural features, such as common functional groups and heterocycles, which are used to classify the dataset (Roberts et al., 2000). In addition, new features were generated by combining different combinations of features (Cross et al., 2003). Statistics are calculated to determine whether there is a significant association between the chemical family and the biological response (Roberts et al., 2000). The software has been used and validated by many organizations, including the US FDA, to analyze the relationships between chemical structural features and toxicity data (Matthews et al., 2008).

The chemical structures and associated cytotoxic activity data were loaded into the Leadscope software. All these features were analyzed to determine those structural motifs that differentiate the active from inactive compounds. Compounds 3, 15 and 28 contain the phenolic structural motif; however no differentiating structural features distinguished 19 from the non-active compounds, despite a series of similar compounds in the dataset.

**Statistical analysis**

Data were presented as mean ± S.E.M. for at least three independently performed experiments. Statistical significance (p < 0.05) was assessed by unpaired t-test for cytotoxicity result. Meanwhile, ANOVA was used in the statistical determination of the mode of cell death (apoptosis assay) and the assessment of mitochondrial membrane potential.

**RESULTS AND DISCUSSION**

There is increasing evidence demonstrating that resveratrol and its derivatives are potential anticancer drugs (Athar et al., 2009). In this study, a series of stilbene derivatives based on resveratrol were synthesized. These compounds have been constructed with various substituent patterns and functional groups such as hydroxy-, chloro-, acetoxy-, benzylxyox-, carboxyl- and methoxy- groups (Fig. 2). All compounds were investigated for their cytotoxicities in human K562 chronic myelogenous leukemia cells.

The viability of K562 cells treated with stilbenes for 48 hours was measured using the MTT assay for all the 22 compounds. However, only four stilbenes namely, 3 (pterostilbene), 15, 19 and 22 demonstrated significant cytotoxicity in K562 leukemic cells with IC50s of 78 μM, 38 μM, 67 μM and 19.5 μM, respectively (Fig. 2). All other compounds including resveratrol, the positive control in this study, showed no IC50. Interestingly, resveratrol was not cytotoxic in K562 cells even at 200 μM where it induced a decrease in the cell viability to only 64 ± 6% (data not shown). In agreement, Jeong et al. (2009) have demonstrated that K562 cells are resistant to resveratrol cytotoxicity where 80% of cells were still viable at 100 μM of treatment. In contrast, Tolomeo et al. (2005) have shown that resveratrol kills K562 cells with an IC50 of 28 ± 6 μM. However, this group demonstrated that pterostilbene is cytotoxic in K562 cells with an IC50 of 10 ± 3μM that is again dissimilar to our findings (Tolomeo et al., 2005). The discrepancy between these results may be due to the different methods employed to assess cytotoxicity (Kim et al., 2009). In this study, MTT assay was employed where the viability was measured based on the intact mitochondrial activity, whereas Tolomeo et al. (2005) used trypan blue, in which the viability was assessed based on the uptake of the dye in dead cells (Mosmann, 1983). It is also possible that the resistance of K562 cells observed in work by Jeong et al. (2009) study
may be due to the difference in BCR-Abl protein expression (McGohan et al., 1994).

In our investigation, we found that 28 was the most cytotoxic compound in K562 cells. It is interesting to note that in this compound, positions 3′ and 5′ were substituted with benzyloxy moieties. Although 28 has been synthesized by Orsini et al. (2004), its biological activities have not been investigated. Significantly, 28 showed the greatest cytotoxic effect in K562 cells. Compound 19 was also cytotoxic in K562 cells. This compound contains a halogen (chlorine) at position 4 and an acetoxy group at position 4′. Previous studies by Lee et al. (2004) on trans- and cis-stilbenoids containing bromo groups demonstrated their superior cytotoxic effects compared to resveratrol and combrestatin in human lung cancer cells. This suggests that halogen substitution may enhance the cytotoxic effects of stilbenoids in cancer cells.

We also found that the presence of methoxy groups in these stilbenes improved the induction of cytotoxicity in K562 cells since 3 and 15 were more potent than resveratrol. This is in agreement with a previous study where methoxy stilbenes have been shown to induce cell death more effectively than resveratrol (Hooberman et al., 1994). Moreover, pterostilbene (3) was more cytotoxic in K562 cells than resveratrol, which is essentially similar to the findings of Tolomeo et al. (2005).

To study cell death triggered by 28 in K562 cells, flow cytometric analysis using Annexin-V/PI was employed (Vermes et al., 2000). Based on the flow cytogram, compound 28 clearly induced apoptosis following 24 hr of treatment (Fig. 3; Panel A). A concentration-dependent increase was observed in 28-treated cells (Fig. 3; Panel B). A significant increase in the percentage of apoptotic cells most of which were late apoptotic cells was observed with 75 μM and 100 μM of 28. In this assay, etoposide was used as a positive control. As expected, etoposide at 100 μM also induced apoptosis in human chronic myelogenous (K562) cells. Our results are in agreement with
previous studies by Fukumi et al. (2000) where etoposide induces apoptosis in K562 cells. Interestingly, the percentage of apoptotic cells in K562 treated etoposide is much lower than that for K562 treated 28 at the same concentration used. Since 3,5-dibenzyloxy stilbene (28) induced a greater percentage of apoptotic cells compared to etoposide, further mechanistic studies were carried out.

The involvement of mitochondrial membrane potential (ΔΨm) in K562 cells treated with 28 was investigated. As presented in Fig. 3 (Panel C), the mean fluorescent unit (mfu) which represents the ΔΨm decreased gradually in a concentration-dependent manner. Following 24 hr of treatment of 28, ΔΨm was completely abolished at concentrations of 75 μM and 100 μM. These results were almost identical to menadione which served as the positive control. The dissipation of ΔΨm induced by 28 is

Fig. 3. Flow cytometric analysis of K562 cells following treatment with 28 for 24 hr. The apoptotic and necrotic cells as assessed by Annexin V-FITC (x-axis) and PI (y-axis) staining is shown in cytogram (Panel A) and the cumulative result in Panel B. (Panel B). The mean fluorescent units (mfu) for ΔΨm as assessed by TMRE staining is shown in Panel C. Etoposide (100 μM) and menadione (50 μM) were positive controls for Panel B and C respectively. Values are expressed as mean ± S.E.M. of three separate experiments. *p < 0.05 against vehicle control. (VC – vehicle control; PC – positive control).

Fig. 4. Activation of caspases-9 and -3 in K562 cells incubated with compound 28. Panels A and B are the immunoblots for caspase-9 and caspase-3, respectively.
suggestive of the activation of apical caspase-9 cascade in the intrinsic apoptotic pathway.

We next investigated the activation of apical caspase-9 and its substrate caspase-3 in 28-induced apoptosis in K562 cells. As shown in Fig. 4A, the activation of caspase-9 occurred in a concentration- and time-dependent manner as demonstrated by the loss of intact zymogen and its active subunits namely p37 and p35. To the best of our knowledge, this is the first demonstration of a rapid activation of caspase-9 as early as 2 hr induced by a

Fig. 5. Quantitative structure-activity relationship (QSAR) studies on selected compounds. Panel A shows the structure comparison among three cytotoxic compounds (3, 15 and 28) where each of them has a hydroxyl group. Panel B shows the possible metabolic transformation of 28. Abstraction of an electron from the benzylic CH of I via oxygen (or the corresponding diradical) gives rise to the benzylic radical (2). This can be converted to the peroxyradical (4) via (3). This radical (4) is set up to attack the para position of ring B-assisted by conversion of the ring A phenolic OH to the phenoxy radical (4) → (5) to produce the highly conjugated quinone (7) via (6).
stilbene. Caspase-9 activation in 28-induced apoptosis resulted in the processing of caspase-3 to its active subunits (p19 and p17). The pattern of caspase-3 activation mirrored caspase-9 activation (Fig. 4B). Although stilbenes such as resveratrol, pterostilbene and piceatannol have been demonstrated to induce apoptosis, the caspases-3 and -9 activation occur only in longer treatment exposure (Dörrie et al., 2001; Wieder et al., 2001; Alosi et al., 2009). In all concentrations used, the intact and activated forms of caspase-9 and caspase-3 almost disappeared at 12 and 24 hr of treatment. This may be due to the progression of apoptotic cells to late apoptosis or secondary necrosis (Wolbers et al., 2004). During the late stages of apoptosis, organelles in the apoptotic bodies will lose their membrane integrity and most protein including caspases will be degraded. This was further supported with our flow cytometric analysis of apoptosis where most of the cells were shifted to the upper right quadrant (late apoptosis) at 24 hr treatment with 28. In addition, the complete loss of mitochondrial membrane potential is most likely due to the mitochondriotoxic effect of stilbenes. Interestingly, stilbenes such as resveratrol have been shown to target the mitochondria to trigger the apoptosis process (Fulda et al., 2010).

Structural examination of 3, 15 and 28 using Leadscope® software suggests 28 to be capable of benzylic oxidation (Fig. 5A, position of the asterisks) leading to, according to our hypotheses, the ester hydrolysis via the benzoate (structure 7 in Fig. 5B) or alternative oxidation at the para position of the aromatic ring of the benzyl group (note arrow in Fig. 5A). This reaction sequence may generate the reactive intermediate benzyl radical (structure 2 in Fig. 5B) and formation of quinone methide (structure 7 in Fig. 5B) via intermediates (structure 2 to 7 in Fig. 5B). It has been proposed previously that stilbene derivatives such as tamoxifen causes toxicity via the formation of quinone methide (Bolton, 2002). This electrophilic reactive intermediate has the affinity to bind nucleophilic macromolecules such as proteins which can result in dysfunction of organelles (Thompson et al., 1993). Therefore, the formation of quinone methide may play an important role in 28-induced mitochondrial toxicity leading to the loss of ΔΨm and early activation of caspase-9. As an alternative mechanism, it is important to point out that oxidation of the free OH can also lead to quinone methide oxidation. Although we have demonstrated the intrinsic pathway of 28-induced apoptosis, the fact that this compound has a marked ability to overcome resistance of K562 cells by BCR/Ab1 protein warrants further investigation.

In conclusion, the study on the 22 synthesized stilbenes shows that only four of them were cytotoxic against K562 cells with a significant decrease in the percentage of viable cells. The most cytotoxic compound was 28 whereby it induced cell death in K562 cells via apoptosis. In addition, this study provides evidence that this dibenzyloxy stilbene (28) induced apoptosis via a mitochondrial pathway.

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3,5-dibenzyloxy-4′-hydroxystilbene induces mitochondria-mediated apoptosis


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