A Dihydrobenzofuran Lignan Induces Cell Death by Modulating Mitochondrial Pathway and G2/M Cell Cycle Arrest

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A dihydrobenzofuran lignan, the dimerization product of caffeic acid methyl ester, has shown pronounced antileishmanial and antiplasmodial activities. The present study showed the effect of this compound on cell cycle and apoptosis. Flow cytometric analysis revealed that the cells were arrested in the G2/M phase. Activation of caspase 3, but not caspase 8, generation of ROS, upstream of caspase-3, release of cytochrome c,increase in Bax level, and decrease in Bcl-2 level suggested the involvement of mitochondrial damage. Loss of mitochondrial transmembrane potential independent of caspase activation further suggested the mode of apoptosis. Dihydrobenzofuran-mediated cell death was absent in *Bcl-xL*-overexpressed cells. Overall, our results justify the role of dihydrobenzofuran lignan as potential antitumor agent, causing G2/M arrest and apoptosis involving the mitochondrial controlled pathway. These findings open promising insights as to how this specific dihydrobenzofuran lignan mediates cytotoxicity and may prove a molecular rationale for future therapeutic interventions in carcinogenesis.

Introduction

Lignans and neolignans are formed in nature by oxidative dimerization of various C_6-C_3 phenols.[1] They are widespread in nature with a wide variety of chemical structures and exhibit a broad range of biological activities.[2] The 3′,4-di-*O*-methylcedrusin belonging to the neolignan class was identified as one of the minor constituents of the red latex called "Dragon's blood" (Sangre de drago) in traditional medicine.[3,4] This latex is obtained by slashing the bark of various South American *Croton* species (Euphorbiaceae) and reported to possess potential antitumor activity by inhibiting cell proliferation.[3] A series of such synthetic dihydrobenzofuran lignan derivatives were explored for their potential antitumor and antiproliferative activities. Synthetic compound methyl(*E*)-3-{2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxy carbonyl-2,3-dihydro-1-benzofuran-5yl}prop-2-enoate,[a] the dimerization product of caffeic acid methyl ester, was found as antileishmanial and antiplasmodial properties[5] and also inhibited cell proliferation.[2] This dimerization product of caffeic acid methyl ester also showed a pronounced antiangiogenic activity in the chorioallantoic membrane (CAM) assay.[6] We looked at the effects of this antitumor agent on the cell cycle progression and the mechanism of inhibiting cell proliferation and found it to be a potent cytotoxic agent causing apoptosis in cancer cell lines.

Anticancer drugs have shown to target diverse cellular functions in mediating cell death in chemosensitive tumors. Cell death can be either necrotic or apoptotic. Necrosis is typically described as a nonspecific form of cell death, and apoptosis is a consequence of a series of precisely regulated events that are frequently altered in tumor cells.[7,8] Apoptosis is further characterized by two pathways, the extrinsic and the intrinsic pathway. The extrinsic pathway is mediated by a group of proteins called death receptors such as Fas and TNF receptors,[9] while the intrinsic pathway involves mitochondria that play an important role in regulation of apoptosis.[10,11] Early events in this process are loss of mitochondrial transmembrane potential $\Psi\Delta_m$ and release of membrane bound cytochrome c into the cytosol.[12] The released cytochrome c induces oligomerization of Apaf-1 (caspase recruitment domain) in the presence of ATP, which then binds to the cytosolic procaspase-9 in a apoptosome complex.[13] Activated procaspase-9 induces the downstream caspase cascade, e.g., caspase-3, which in turn is responsible for the execution of additional features of apoptosis.[14,15]

In this study, we report for the first time that the synthetic dihydrobenzofuran lignan compound (methyl(*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxy carbonyl-2,3-dihydro-1-benzofuran-5yl]prop-2-enoate), designated as dihydrobenzofuran throughout the manuscript, causes G2/M arrest and subsequent apoptosis in Jurkat T cell line. Furthermore, the inhibition of cell cycle progression positively correlates with the decrease in constitutively high phosphotyrosine levels in Jurkat cell line. We investigated the cytotoxic effects of the compound on Jurkat cells, which leads to the generation of ROS and loss of mitochondrial transmembrane potential upstream release of cytochrome c, activation of caspase 3, but not caspase 8, followed by subsequent cleavage of PARP and cell death. In *Bcl-xL*-overexpressed HL-60 cells, the compound did not induce cell death, further confirming the involvement of the mitochondrial pathway. These results suggest that the synthetic dihydrobenzofuran is indeed a candidate for anticancer agent having effect for G2/M arrest and apoptosis following the intrinsic pathway as the mechanism of action.

Results

We examined the effect of the dimerization product of caffeic acid methyl ester (methyl(*E*)-3-[2-(3,4-dihydroxyphenyl)-7-

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[a] Abbreviations: Dihydrobenzofuran lignan, methyl(*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5yl]prop-2-enoate; MDA, malondialdehyde; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PARP, poly-(ADP-ribose) polymerase; PI, propidium iodide; ROI, reactive oxygen intermediate; TBARS, thiobarbituric-acid reactive substances.

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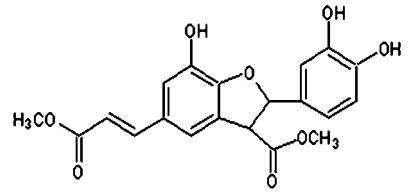

Methyl(E)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5yl] prop-2-enoate

Figure 1. Chemical structure of dihydrobenzofuran lignan compound (methyl(*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5yl]prop-2-enoate). The NMR data, structural formula, designated IUPAC name, and elemental analysis of the compound are shown here. ^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 3.70 (s, 3H, 1 X $-COOCH_3$), 3.74 (s, 3H, 1 X $-COOCH_3$), 4.34 (d, 1H, $J = 6.7$ Hz), 5.84 (d, 1H, $J = 7$ Hz), 6.34 (d, 1H, $J = 15.8$ Hz), 6.6−7.2 (m, 5H, Ar−H), 7.54 (d, 1H, $J = 15.8$ Hz), 9.05 (s, 1H, −OH), 9.06 (s, 1H, −OH), 9.74 (s, 1H, −OH). TOF MS ES: 387 (M + H). Elemental analysis: % of C, H, and O for calculated/found values are 62.174/62.479, 4.695/4.494, and 33.129/33.112, respectively.

hydroxy-3-methoxy carbonyl-2,3-dihydro-1-benzofuran-5yl] prop-2-enoate), designated as dihydrobenzofuran herewith, on the Jurkat E6.1 cell line. The chemical structure as well as the NMR data of this compound is shown in Figure 1. The compound is almost 96% pure, as shown by elemental analysis and HPLC (data not shown). For all experiments, the compound was dissolved in DMSO as 10 mM stock solution and further dilutions were made in the complete medium.

Dihydrobenzofuran Inhibits Cell Viability. The effect of dihydrobenzofuran on cell viability of different tumor cell lines, Jurkat, K562, and MCF-7, was studied by treating cells with varying concentrations for 24 h. The number of viable cells, determined by MTT assay, decreased equally with increasing concentrations of the dihydrobenzofuran in all the tested cells (Figure 2A). When Jurkat cells were treated with varying concentrations of dihydrobenzofuran for 72 h, LDH release into culture supernatant was not observed (Figure 2B). Therefore, this inhibition of cell viability is possibly by apoptosis and not by cytolysis.

Dihydrobenzofuran Arrests Cells at G2/M Phase of Cell Cycle. To understand the mechanism of cell death, dihydrobenzofuran was evaluated for its effect on cell cycle. Jurkat cells were exposed to varying concentrations of the compound for 24 h. The DNA contents of the live population were 66%, 20%, and 15% for untreated cells to 22%, 14%, and 65% for 85 nM dihydrobenzofuran-treated cells for G0/G1, S, and G2/M phase, respectively. Similar effects were also observed for treatments with higher concentrations of dihydrobenzofuran (Figure 2C1). The treatments also lead to significant numbers of apoptotic cells, as shown by an increase in the sub G1 content (Figure 2C2). Together, these results suggest that the compound delayed cell cycle progression by arresting cells in the G2/M phase of the cell cycle, resulting in significant apoptosis.

Dihydrobenzofuran Induces Apoptosis and G2/M Arrest in Jurkat Cells in a Time-Dependent Manner. In the next set of experiments, Jurkat cells were treated with 85 nM dihydrobenzofuran for different times and number of cells was detected in different phases of cell cycle. Dihydrobenzofuran-induced G2/M arrest was detectable between 12 and 24 h (Figure 3A1). A time-dependent increase of apoptosis, measured as sub G1% of the whole cell population, was observed by cell cycle analysis (Figure 3A2).

Dihydrobenzofuran Suppresses Tyrosine Phosphorylation in Jurkat Cells. Phosphorylation on tyrosine residues of key cellular protein substrates is tightly associated with the

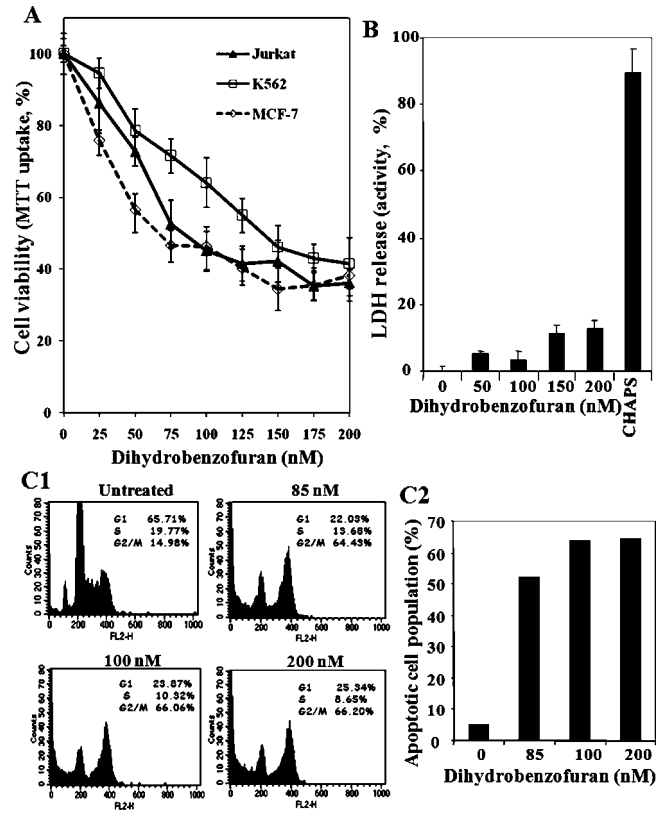

Figure 2. Effect of dihydrobenzofuran on cell death and cell cycle distribution. Jurkat, K562, and MCF-7 cells were treated with different concentrations of dihydrobenzofuran for 24 h in triplicate. Cell viability was determined by MTT assay and indicated in percentage (A). Cytotoxicity was assayed by LDH assay from culture supernatant of Jurkat cells, treated with different concentrations of dihydrobenzofuran for 72 h. Results indicated in % above the value of unstimulated cells. CHAPS (9 mM) extracted cells showed 90% LDH release (B). Jurkat cells (5×10^5) were treated with different concentrations of the dihydrobenzofuran for 24 h, and after staining with PI, cell cycle distribution was analyzed using a flow cytometer. The data indicates the percentage of live cells in each phase of the cell cycle (C1). Data represents the percentage cell death of the total population calculated as the sub G1 population in the cell cycle distribution (C2). All experiments were performed in duplicate and gave similar results.

upregulation of cell cycle progression.[16] Total amount of tyrosine phosphorylation in Jurkat cells was determined by flow cytometry with FITC-labeled monoclonal antibody against phosphotyrosine. As shown in Figure 3B1, the number of cells with high phosphotyrosine concentration was significantly reduced with increasing time of dihydrobenzofuran treatment. The numbers of phosphotyrosine positive cells were indicated in Figure 3B2. These data suggest dihydrobenzofuran treatment reduced the concentration of cellular phosphotyrosine.

Dihydrobenzofuran Activates Caspase 3, but Not Caspase 8, and Releases Cytochrome c. Cell death is related to cellular and molecular events in the cells and occur via two independent processes, i.e., necrosis and apoptosis.[17] Necrotic cell death, an accidental cell death, did not occur on dihydrobenzofuran treatment described in the previous section. Apoptosis, however, occurs through activation of caspases, the key proteases. In this study, we found the activity of caspase 3 increased without altering activity of caspase 8 in dihydrobenzofuran treated cells (Figure 4A). As shown in Figure 4B, significant amounts of PARP cleavage was found in cell population treated with 85 nM of dihydrobenzofuran in a time-dependent manner. Dihydroben-

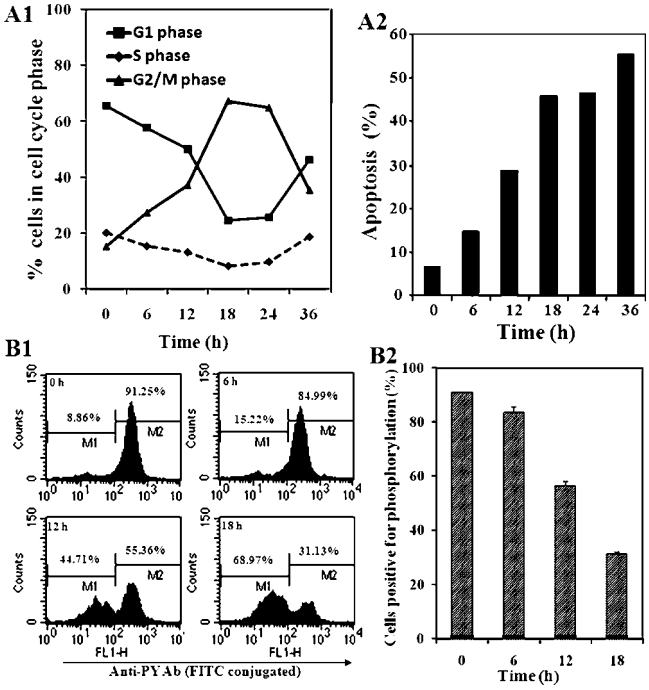

Figure 3. (A) Effect of dihydrobenzofuran in cell cycle distribution kinetics in Jurkat cells. Jurkat cells treated with 85 nM of the compound for different times and stained with PI and cell cycle analysis was performed using flow cytometer. Data represented in percentage of live cells in each phase of the cell cycle (G1, S, and G2/M) (A1). A time-dependent increase in the percentage cell death of the total population was expressed (A2). Representative results of two independent experiments are shown. (B) Effect of the dihydrobenzofuran on the amounts of phosphotyrosine in Jurkat cells. Jurkat cells (1×10^6) were treated with 85 nM dihydrobenzofuran for indicated times. Cells were fixed and permeabilized as described in the Experimental Section, and the extent of tyrosine phosphorylation in the cells was determined by measuring the increase in fluorescence produced by the FITC-labeled monoclonal antibody compared to the FITC-labeled isotype control antibody (B1). Number of cells positive for tyrosine phosphorylation is represented as the M2 gated population (B2).

zofuran-treated cells showed increased concentrations cytochrome c in the cytoplasm in a time-dependent manner (Figure 4C), in conformity with the known feature of release of cytochrome c, activating caspase 3.

Dihydrobenzofuran Alters Mitochondrial Transmembrane Potential ($\Psi\Delta_m$). The mitochondrial transmembrane potential alteration was determined using mitochondria-specific fluorescence dye, $DiOC_6(3)$. The number of fluorescent-positive cells increased with increasing time of dihydrobenzofuran treatment (Figure 4D). Almost equal number of fluorescent-positive cells was found in z-VAD-fmk, Pan caspase inhibitor-pretreated Jurkat cells. These results suggest that dihydrobenzofuran induces mitochondrial transmembrane potential change in addition to release of cytochrome c into cytoplasm.

Dihydrobenzofuran Induces ROI Generation and Lipid Peroxidation in Jurkat Cells. ROI generation is an intermediate step in apoptosis induced by different agents. Dihydrobenzofuran treated cells showed increase in ROI generation. This dihydrobenzofuran-mediated increase in ROI generation was inhibited on pretreating cells with 1.5 mM ascorbic acid (Figure 5A). As lipid peroxidation is another marker of apoptosis,[18] we also examined the effect of this compound on lipid peroxidation. As shown in Figure 5B, MDA production increased with increasing concentration of dihydrobenzofuran in treated Jurkat cells. Treatment of cells with ascorbic acid showed 80%

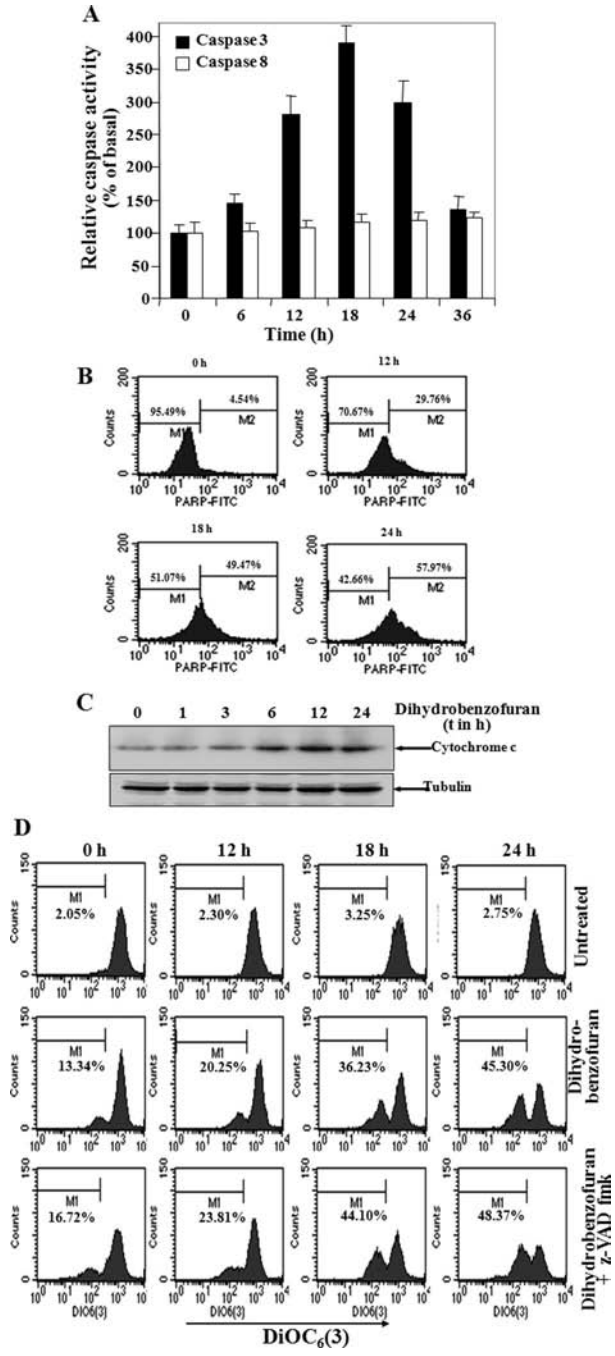

Figure 4. Effect of the dihydrobenzofuran on activation of caspases, cleavage of PARP, release of cytochrome c, and mitochondrial transmembrane potential ($\Psi\Delta_m$). Jurkat cells were treated with 85 nM dihydrobenzofuran for different times and cell extracts were prepared. Caspase 3 and 8 activities were measured by incubating the cell extracts with Ac-DVED-pNA and Ac-ITED-pNA, respectively, as described in the Experimental Section, and the absorbance was recorded at 405 nm. Caspases activities were indicated in % considering unstimulated cells as 100% (A). Jurkat cells were treated with 85 nM dihydrobenzofuran for indicated times, and PARP cleavage was determined using FACS analysis as described in the Experimental Section. Percentage apoptotic population is represented as the M2 gated population (B). Jurkat cells were treated with dihydrobenzofuran (85 nM) for indicated times, and cytosolic fractions (100 μg proteins) were used to detect cytochrome c by Western blot (C). The same blot was reprobed against tubulin. Jurkat cells, pretreated with z-VAD-fmk (3 μM) for 2 h and treated with 85 nM of the dihydrobenzofuran for different times were stained with $DiOC_6(3)$, and the percentage of cells having loss in mitochondrial transmembrane potential ($\Psi\Delta_m$) was determined by FACS analysis. The M1 gated population showing low $DiOC_6(3)$ fluorescence represents the percentage of cells with loss of mitochondrial transmembrane potential (D).

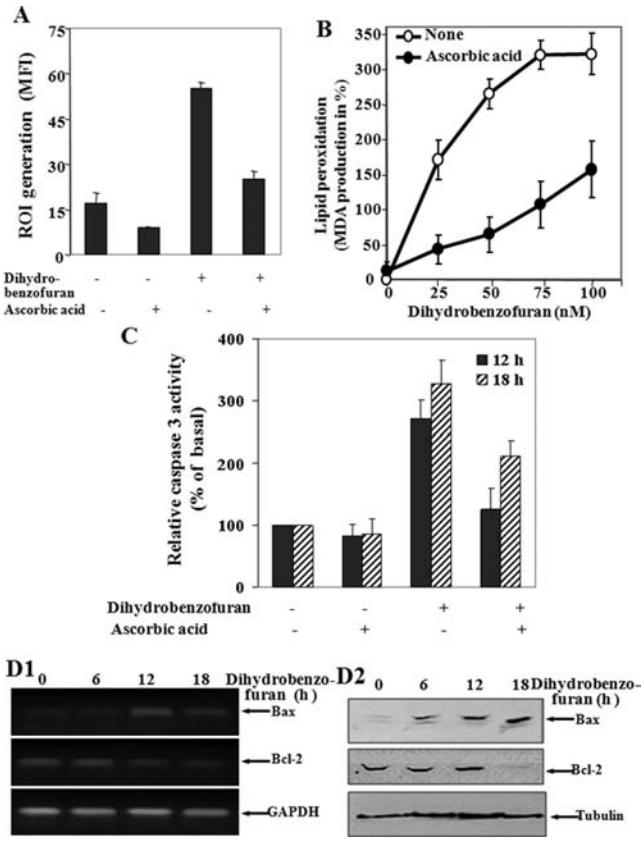

Figure 5. Effect of dihydrobenzofuran on lipid peroxidation, ROI generation, and *Bax* and *Bcl-2* expression. Jurkat cells, pretreated with ascorbic acid (1.5 mM) for 3 h were treated with 85 nM dihydrobenzofuran for 12 h. Generation of ROI was measured by using dihydrorhodamine 123. The increase in the rhodamine 123 fluorescence intensity was measured using a flow cytometer (A). Jurkat cells, pretreated with ascorbic acid (AA), were treated with different concentrations of dihydrobenzofuran for 18 h. Cell extracts prepared by freeze−thaw method were assayed for lipid peroxidation, as described in the Experimental Section. Results indicated as lipid peroxidation in % above untreated cells (B). Untreated cells showed 0.549 ± 0.09 nmol of TBA-reactive substances/mg protein. Jurkat cells, pretreated with ascorbic acid (1.5 mM) for 3 h, were treated with dihydrobenzofuran (85 nM) for 12 and 18 h. The caspase-3 activation was measured from whole cell extracts and activity is indicated in %, considering untreated cells as 100% (C). Jurkat cells were treated with 85 nM dihydrobenzofuran for different times and the amounts of *bax* and *bcl-2* expression was detected by RT-PCR method from total RNA (D1) and by Western blot from whole cell extracts (D2).

inhibition of this MDA production. These results suggest that dihydrobenzofuran induces ROI generation and lipid peroxidation, and these changes are prevented by ascorbic acid, a ROI-scavenger.

Ascorbic Acid Partially Protects Dihydrobenzofuran-Mediated Caspase 3 Activation. Ascorbic acid-pretreated Jurkat cells showed significant reduction of caspase 3 activity induced by dihydrobenzofuran treatment initial phase of 12 h (Figure 5C). The inhibition caused by ascorbic acid is less effective after 18 h of exposure of dihydrobenzofuran, possibly because ascorbic acid may be oxidized then. These data strongly suggest that the ROI play an important role in regulating pathway of dihydrobenzofuran-induced apoptosis and is upstream of caspase-3 activation. The amount mRNA for *Bax* was increased and for *Bcl-2* was decreased with the treatment of dihydrobenzofuran (Figure 5D1). Similar data were obtained for the amounts of Bax and Bcl-2 proteins (Figure 4D2). These

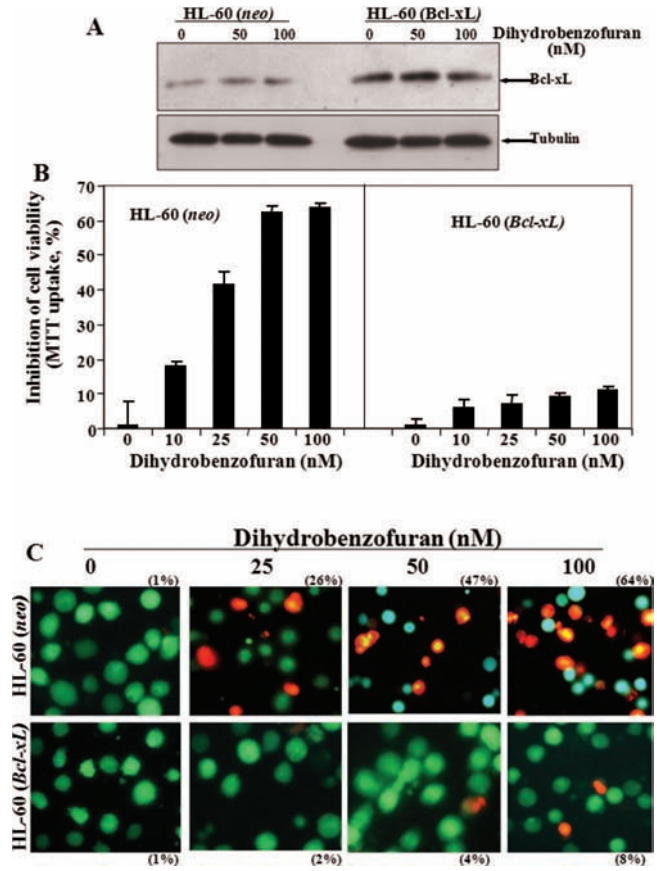

Figure 6. Effect of dihydrobenzofuran on cell death in Bcl-xL-overexpressed HL-60 cells. HL-60 (*neo*) and HL-60 (*Bcl-xL*) cells were treated with different concentrations of dihydrobenzofuran for 72 h. Amounts of Bcl-xL was detected from whole cell extracts by Western blot (A). Cell viability was detected by MTT assay as indicated in inhibition of cell viability in % (B) and number of apoptotic cells (red in color) was counted in fluorescence microscope as detected by Live and Dead assay kit and indicated in % in the bracket (C).

data show that dihydrobenzofuran treatment is able to alter the *bcl-2*/*bax* ratio, which in turn could favor apoptosis in Jurkat cells.

Bcl-xL-Overexpressed Cells Protect from Dihydrobenzofuran-Mediated Cell Death. As dihydrobenzofuran is inducing change in mitochondrial transmembrane potential, we are interested to find its effect in *Bcl-xL*-overexpressed HL-60 [HL-60 (*Bcl-xL*)] cells. These cells were treated with different concentrations of dihydrobenzofuran and cell viability was determined by MTT and "Live and Dead" assays. Dihydrobenzofuran induced cell death in HL-60 (*neo*), but not in HL-60 (*Bcl-xL*) cells, as shown by MTT assay (Figure 6B) and "Live and Dead" assay (Figure 6C). The expression of *Bcl-xL* was shown in Figure 6A. These results suggest that dihydrobenzofuran induces cell death through *Bcl-2* family proteins and mitochondrial pathways.

Discussion

We studied the effects of the promising chemotherapeutic compound methyl(*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5yl]prop-2-enoate, also known as dihydrobenzofuran lignan derivative (designated as dihydrobenzofuran) on Jurkat T cells. Flow cytometric analysis reveals that dihydrobenzofuran blocks cells in the G2/M phase and an increase in the sub G1 cell population suggesting apoptosis. These effects are not restricted in one cell

types but showed in multiple cell types including human T-cell carcinoma, myeloid leukemia, breast carcinoma, and promyelocytic lymphoma. The dihydrobenzofuran is very much pure as analyzed by HPLC and also C, H, and O elemental analysis.

Phosphorylation on tyrosine residues of key cellular protein substrates is tightly associated with the regulation of cell activation and proliferation.[19,20] Protein tyrosine phosphorylation is tightly regulated in normal cells. But these may acquire transforming functions due to mutations or overexpression of certain protein tyrosine kinases, resulting in abnormal cell growth and malignancy.[16,21] In contrast to normal cells, tumor cells, especially the leukemic and breast cancer cells, are known to display a higher constitutive level of tyrosine phosphorylation.[19,22] This increase in tyrosine phosphorylation is known to control cell cycle regulatory proteins, positively correlating with enhanced progression in the cell cycle. The simultaneous analysis of total phosphotyrosine levels in cells by the PY-FACS method[17,19] and the study of cell cycle phases has become an interesting approach to the study of signal transduction events, leading to cell activation and proliferation.[19,23] We observed a significant decrease in the total tyrosine phosphorylation in dihydrobenzofuran-treated Jurkat cells, in complete agreement with the above-discussed results stating inhibition of cell proliferation by arresting the cells in the G2/M phase. As tyrosine phosphorylation is the result of opposite influences from protein tyrosine kinase (PTK) and protein tyrosine phosphatases (PTPases),[24] whether dihydrobenzofuran could possibly inhibit some class of tyrosine kinases or activate tyrosine phosphatases is a subject of interest for further studies. The arrest in the G2/M phase could be due to modulation of proteins involved as check points in the cell cycle and/or due to its interaction with microtubule function causing mitotic arrest.

Induction of apoptosis is considered as a possible mechanism of most of the chemotherapeutic agents,[25−27] and targeting the apoptosis signaling system is becoming a promising strategy for the development of novel chemotherapeutic molecules.[28,29] As caspases play an important role in many forms of apoptosis and both the intrinsic and extrinsic apoptotic pathways are known to trigger caspase activation,[30] the caspase activation effect of dihydrobenzofuran found in this investigation is of significance. Surprisingly, dihydrobenzofuran activates caspase-3 without activating caspase-8. Alternately, cytochrome c, released into cytoplasm from mitochondria, binds with Apaf-1 and activates caspase-9 and subsequently caspase-3.[13,31] The activation of caspase-3 also resulted in the cleavage of it is substrate PARP (poly(ADP-ribose) polymerase) indicating that almost 60% of the cell population underwent apoptosis within 24 h after dihydrobenzofuran treatment. Mitochondrial alterations coordinate events in apoptosis signaling.[32] One such alteration involves disruption of the inner membrane transmembrane potential ($\Psi\Delta_m$) through the opening of mitochondrial permeability transition pores. After the pore opening, the normally impermeable inner membrane becomes permeable to ions and solutes and the negatively charged environment of the matrix is lost. In this study, intermembrane permeabilization was assessed indirectly by determining a reduction in the mitochondrial transmembrane potential ($\Psi\Delta_m$). Furthermore, pretreatment of cells with the pan-caspase inhibitor did not seem to alter the percentage of cells with decreased mitochondrial transmembrane potential, clearly suggesting that the changes occur earlier or is upstream of caspase-3 and independent of caspases activation. Thus, induction of apoptosis by this compound in Jurkat cells follows the steps commonly observed for the intrinsic pathway involving mitochondrial damage.

Stress factors, e.g., reactive oxygen species, are known to induce apoptosis involving the mitochondrial pathway.[32−34] Our results show that the compound was able induce lipid peroxidation and ROI generation, further indicating that the cells were following the apoptotic pathway. Bcl-2 family proteins are critical for cell survival and Bax levels can shift the process in favor of apoptosis.[35] In *Bcl-xL*-overexpressed cells dihydrobenzofuran unable to induce cell death further, suggesting that this mitochondrial pathway is targeted by this compound.

Experimental Section

Cell Lines. The cell lines used in this study were as follows: Jurkat E6.1 TIB-152 (human T cell leukemia), MCF-7 (human breast cancer cell line), and K562 (human myeloid leukemia) were obtained from American Type Culture Collection (Manassas, VA). Human promyelocytic lymphoma HL-60 cells stably transfected with vector and *Bcl-xL* constructs [designated as HL-60 (*neo*) and HL-60 (*Bcl-xL*)] were obtained from Prof. Bharat B. Aggarwal, MD Anderson Cancer Center, Houston, TX. Jurkat and HL-60 cells were cultured in RPMI 1640, K562 cells in Iscove's modified Dulbecco's medium, and MCF-7 cells in DMEM, supplemented with 10% FBS, penicillin (1000 U/ml), and streptomycin (100 μg/mL). All cells were free from mycoplasma contamination.

Materials. Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, propidium iodide (PI), caspase-3 substrate (Ac-DVED-pNA), caspase-8 substrate (Ac-ITED-pNA), ascorbic acid, thiobarbituric acid, antitubulin Ab, and antiphosphotyrosine FITC conjugate were purchased from Sigma Aldrich Chemicals (St Louis, MO), 3,3′-Dihexyloxycarbocyanine iodide and dihydrorhodamine 123 (DHR 123) were obtained from Molecular Probes (Inc., Eugene, OR). Antibodies against cytochrome c, Bax, Bcl-2, and Bcl-xL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TRIzol, penicillin, streptomycin, neomycin, RPMI 1640, DMEM, Iscove's Dulbecco's medium, fetal bovine serum (FBS), and FITC-phalloidin were obtained from Life Technologies (Grand Island, NY). Anti PARP-FITC conjugate was purchased from Novus Biologicals (Littleton, CO). One-Step Access RT-PCR kit was purchased from Promega (Madison, WI).

Chemical Synthesis and Characterization of the Compound. Methyl(*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5yl]prop-2-enoate was prepared according to the method described previously.[2] Briefly, methyl caffeate was dimerized using silver oxide in the presence of anhydrous benzene and anhydrous acetone and the product was purified by silica gel column chromatography with ethyl acetate-*n*-heptane as the eluent. After evaporation, a white foam was obtained (32%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ_{ppm} 3.70 (s, 3H, 1 X $-COOCH_3$), 3.74 (s, 3H, 1 X $-COOCH_3$), 4.34 (d, 1H, J = 6.7 Hz), 5.84 (d, 1H, J = 7 Hz), 6.34 (d, 1H, J = 15.8 Hz), 6.6−7.2 (m, 5H, Ar−H), 7.54 (d, 1H, J = 15.8 Hz), 9.05 (s, 1H, −OH), 9.06 (s, 1H, −OH), 9.74 (s, 1H, −OH). TOF MS ES: 387 (M + H). Molecular formula: $C_{20}H_{18}O_8$. The compound ($C_{20}H_{18}O_8$) was also characterized by high performance liquid chromatography, and purity was found to be 96%. Further purity of the compound was carried out by elemental analysis for C, H, and O, which suggested the standard error was ±0.5% (the calculated/found values for C, H, and O were 62.174/62.479, 4.695/4.484, and 33.129/33.112 in percentage). For each biological experiment, the compound was freshly dissolved in DMSO as 10 mM stock solution and further dilutions were made in the complete medium.

Cytotoxicity Assay. Cytotoxicity was assayed by the modified tetrazolium salt (MTT) assay.[36] Briefly, 5 × 10^4 cells/well of a 96-well plate were treated various concentrations of the dihydrobenzofuran in triplicate and the cell viability was determined by incubating the cells with the 25 μL MTT dye (5 mg/mL in PBS) for 4 h at 37 °C. The cells were lysed in lysis buffer (20% SDS in 50% dimethylformamide) by overnight incubation at 37 °C. The plate was read at 570 nm. The mean absorbance was calculated and value for untreated cells was conserved as 100% cell viability.

Cell Cycle Analysis. Cells (log phase culture) were treated with various concentrations of the compound for indicated time. Untreated cells were also included in this experiment for comparison. After treatments, the cells were harvested and washed with cold EDTA/PBS (5 mM) and then resuspended in cold EDTA/PBS (300 μL) and 100% chilled ethanol (700 μL), vortexed, and incubated at room temperature for 1 h. Samples were centrifuged at 500*g* for 5 min, and the supernatant was removed. A solution containing propidium iodide (100 μg/mL) and RNase A (1 μg/mL) was added to the samples and incubated for 1 h at room temperature. Samples were then transferred to 5 mL tubes and analyzed on a flow cytometer. Flow cytometery analyses were done on FACS Calibur (Becton Dickinson, San Jose, CA) after gating (FL2-A/FL2-W) the single nuclei population.

Tyrosine Phosphorylation Assay. Tyrosine phosphorylation assay was performed by the method described earlier.[17] Briefly, cells (10^6) were washed with ice-cold phosphate buffered saline (PBS) and exposed to 1% paraformaldehyde for 30 min at 4 °C. Cells were washed and incubated with 5 mL of chilled 70% ethanol. The cells were washed with PBS and permeabilized with saponin (0.05% in PBS) for 10 min at room temperature. Nonspecific binding was blocked by incubating the cells with BSA (0.1%) and 0.1% Tween 20 (v/v) in PBS for 30 min. Thereafter the cells were stained with 20 μg/mL of FITC-conjugated antiphosphotyrosine antibody for 30 min. Extent of tyrosine phosphorylation in the cells was determined by measuring the increase in fluorescence produced by the FITC-labeled monoclonal antibody compared to the FITC-labeled isotype control antibody. Fluorescence events for 10000 cells were collected and analyzed by flow cytometry (FACS Calibur cytometer with Cell Quest software, Becton Dickinson, San Jose, CA).

Assay of Caspase-3 and -8 Activities. To evaluate caspase-3 and -8 activities, cell lysates were prepared after their respective treatments with the dihydrobenzofuran. Then 50 μg of the cell lysate proteins were incubated with 200 μM caspase-3 substrate (Ac-DVED-pNA) or caspase-8 substrate (Ac-ITED-pNA) in 100 μL of reaction buffer (1% NP-40, 20 μM Tris-HCl, pH7.5, 137 mM NaCl, and 10% glycerol) and incubated for 2 h at 37 °C. The release of chromophore *para*-nitroaniline (pNA) was monitored spectrophotometrically at 405 nm.[37]

Lactate Dehydrogenase (LDH) Release Assay. Necrosis of cells was measured by assaying LDH, the cytosolic marker, from dihydrobenzofuran-treated cells culture supernatant. Culture supernatants were incubated with the substrate solution (230 mM sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5) and absorbance was taken at 340 nm.

PARP Cleavage Assay Using Flow Cytometry. Extent of PARP cleavage was determined using FITC conjugated antibody specifically recognizing the 85 kDa fragment of cleaved PARP (NSB 699 Novus Biologicals) and was used as a marker for detecting apoptotic cells. Treated cells were fixed with 70% chilled ethanol and permeabilized for 30 min at RT (PBS + 0.5% BSA + 0.02% NaN_3 + 0.5% saponin) and stained with anti PARP-FITC (10 μL/10^6 cells) for 1 h at RT. Cells were washed twice with wash buffer (PBS + 1% heat inactivated FBS) and analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson).

Determination of Thiobarbituric Acid-Reactive Substances (TBARS). Lipid peroxidation was assessed by the TBARS assay, which detects mainly malondialdehyde (MDA), a product of the peroxidation of polyunsaturated fatty acids and related esters. TBARS were measured by the method described previously.[38] Jurkat cells (6×10^6/2 mL), after different treatments were washed and undergone three cycles of freeze−thawing in 200 μL of water. A 20 μL aliquot was subsequently removed for protein determination, and 800 μL of assay mix (0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS, 9.4% (v/v) acetic acid, pH 3.5) was added to the remaining sample. Samples were incubated for 1 h at 95 °C, cooled at room temperature, and the absorbance of the supernatants was read at 532 nm against a standard curve prepared using the MDA standard (10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCL, pH 7.4). Results were calculated as nmol of MDA equiv/mg protein and expressed in percentage of matched control values.

Measurement of Reactive Oxygen Intermediates (ROI). The generated ROI in cells were determined by flow cytometer as described previously.[39] Briefly Jurkat (5×10^5) cells, after different treatments, were exposed to dihydrorhodamine 123 (5 mM stock) at a final concentration of 1 μM for 1 h at 37 °C with moderate shaking (100 rpm) and then washed with PBS three times and resuspended in 1 mL of PBS. Rhodamine 123 fluorescence intensity was measured by FACS Calibur (Becton-Dickinson) with excitation at 488 nm and was detected between 515 and 550 nm. Data was analyzed using Cell Quest software (Becton-Dickinson).

Analysis of Mitochondrial Transmembrane Potential ($\Psi\Delta_m$). To evaluate $\Psi\Delta_m$, the cationic lipophilic fluorochrome 3,3′-dihexyloxycarbocyanine iodide [$DiOC_6(3)$] was used. Five $\times$ 10^5 cells were washed with cold PBS and the cell pellets were mixed gently with 20 nM $DiOC_6(3)$ and incubated for 30 min, at 37 °C in dark. Cells were washed and analyzed by flow cytometry with an excitation wavelength of 488 nm (emission 529 nm) (BD FACS Calibur). $\Psi\Delta_m$ low cells were those displaying [$DiOC_6(3)$] fluorescence less than the fluorescence of control cells not treated with the apoptotic stimulus.

Reverse Transcriptase (RT)-PCR. Total RNA was isolated using the standard TRIzol method (Gibco BRL). First, 1 μg of total RNA (quantified by spectrophotometer at 260 nm) was used to reverse transcribe into cDNA by One-Step Access RT-PCR kit (Promega, Madison, WI), followed by the amplification of the gene of interest using gene specific primers. PCR was performed after AMV RT inactivation and RNA/cDNA/primer denaturation for 4 min at 94 °C, and repeating the cycles at 94, 55, and 72 °C. Amplified products were separated by agarose gel electrophoresis (1.8%) and visualized by ethidium bromide staining. The primer sequence and product size are as follows: *bcl-2*: *127bp* {forward}5′-CTGTGGATGACTGAGTACCT-3′, {reverse}5′GAGACAGCC-AGGAGAAA TCA-3′; *bax*: 489bp{forward}5′-GTTTCATCCAG GATCGAGCA-3′, {reverse}5′-CCATCTTCTTCCAGATGGTG-3′; *GAPDH: 239bp* {forward} 5′-TGATGACATCA AGAAG-GTGGTGAA-3′, {reverse}5′-TCCTTGGAGGCCATGTGGGCC AT-3′.

Cytochrome c Release Assay. Cells (5×10^7) were treated with dihydrobenzofuran. Cytoplasm and mitochondria were prepared by differential centrifugation, as described previously.[37] Cells were pelleted, washed once with PBS, resuspended in 1 mL of ice-cold buffer A (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM dithiothreitol, 170 μg/mL PMSF (phenylmethylsulfonyl fluoride), 16 μg/mL aprotinin, and 4 μg/mL leupeptin), and incubated for 10 min on ice. Cells were lysed in a Down's homogenizer. Unbroken cells and nuclei were pelleted at 120*g* for 10 min at 4 °C. The supernatant was spun at 10000*g* for 25 min at 4 °C. The supernatant was collected as cytoplasm removing the pellet (mitochondrial fraction). Then 100 μg of cytosolic proteins were loaded on a 12% SDS-PAGE and subjected to Western blotting for cytochrome c.

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