Induction of Apoptosis and Down-Regulation of Bcl-XL in Cancer Cells by a Novel Small Molecule, 2[[3-(2,3-Dichlorophenoxy)propyl]amino]ethanol

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ABSTRACT

In a search for new anticancer agents, we identified that 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol (2,3-DCPE) induced apoptosis more effectively in various cancer cells than in normal human fibroblasts. We further evaluated the cell-killing effects of this compound in vitro in several human cancer cell lines and normal human fibroblasts. A cell viability assay showed that IC50s for human colon cancer cell lines LoVo and DLD-1, for human lung cancer cell lines H1299 and A549, and for normal human fibroblasts were 0.89, 1.95, 2.24, 2.69, and 12.6 μM, respectively. Subsequent studies revealed that 2,3-DCPE could cause cleavage of caspase-8, caspase-3, caspase-9, and poly(ADP-ribose) polymerase and release of cytochrome c in cancer cells but not in normal human fibroblasts. Our data also showed that 2,3-DCPE attenuated the protein level of Bcl-XL and that apoptosis induction by 2,3-DCPE could be blocked by enforced overexpression of Bcl-XL. Our results suggest that 2,3-DCPE might be a potential new anticancer agent.

INTRODUCTION

Resistance to treatment is one of the major causes of failure or discontinuation of anticancer therapy. Consequently, despite the use of many therapeutic strategies and new therapeutic agents, most cancers remain incurable. With over 10 million people diagnosed with cancer and over 6 million deaths attributable to cancer in the world every year, cancer has become a global burden (1). Cancer is the second leading cause of death in the United States and is expected to become the leading cause within a few years (2). Thus, there is a need to develop new therapeutic agents for successful treatment of cancer.

A study with 60 cell lines from the National Cancer Institute’s in vitro anticancer drug screen and a panel of 122 standard chemother-apy agents showed that basal Bcl-XL levels were strongly negatively correlated with sensitivity to drugs in all of the mechanistic classes except one class of antimitabolites (3). Bcl-XL, a mitochondrial membrane protein, promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria in response to a variety of stimuli (4, 5). Overexpression of Bcl-XL is reported to confer a multidrug resistance phenotype (6, 7). Moreover, inhibition of Bcl-XL expression by treatment with the chemopreventive agent sulindac or other nonsteroidal anti-inflammatory drugs resulted in an altered ratio of BAX to Bcl-XL and subsequent mitochondria-mediated cell death (8). Thus, Bcl-XL may serve as a molecular target of anticancer therapy, and agents that can down-regulate Bcl-XL expression may be useful for cancer treatment or prevention.

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In searching for new potential anticancer agents, we recently screened a chemical library obtained from ChemBridge Corporation (San Diego, CA) for agents that can modulate expression of genes required for tumorigenesis and tumor maintenance. We found that 2,3-DCPE was more potent in killing human cancer cells than in killing normal human fibroblasts (NHBs). Subsequent studies showed that 2,3-DCPE elicited apoptosis and down-regulated Bcl-XL expression in cancer cells. Our findings indicated that further investigation of the antitumor activities of 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol (2,3-DCPE) and its analogs was warranted. In the study described here, we evaluate the cell-killing effects of these compounds in vitro in six human cell lines and NHBs.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The following cell lines were used: the human lung cancer cell line A549, H1299, and H460, the human breast cancer cell line MDA-MB-231, the human colon adenocarcinoma cell lines LoVo, DLD-1, and DLD-1 stably transfected with Bcl-XL (DLD-1/Bcl-XL), and NHBs. The DLD-1/Bcl-XL cells were derived from parental DLD-1 cells as described previously (9). NHBs were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, and 1% antibiotics and cultured at 37°C in a humidified incubator containing 5% CO2.

Chemicals. 2,3-DCPE, 2[[3-(3,4-dichlorophenoxy)propyl]amino] ethanol (3,4-DCPE), and 2[[2-(2,6-dichlorophenoxy)ethyl]amino]ethanol (2,6-DCEE) were purchased from ChemBridge Corporation. DMSO (Sigma-Aldrich Co., St. Louis, MO) was used to dissolve 2,3-DCPE, 3,4-DCPE, and 2,6-DCEE.

Cell Viability Analysis. Cell viability was determined by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay as described previously (9, 10). Briefly, 1 day after 3000 cells/well in 100 μL of medium were cultured in 96-well plates, the cells were treated with 2,3-DCPE, 3,4-DCPE, and 2,6-DCEE at concentrations ranging from 0.1 to 100 μM. DMSO alone was used as a control. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Four days after the treatment, medium was removed. The cells were washed once with PBS. Cell viability was measured by XTT assay using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. Each experiment was performed in quadruplicate and repeated at least twice.

Flow Cytometry Assay. Cells (1 × 106) were plated into 10-cm tissue culture dishes 1 day before the treatment. Cells were then treated with 2,3-DCPE at various concentrations. Cells treated with DMSO alone were used as a control. Four days after the treatment, floating and attached cells were harvested, washed with PBS, and fixed in 70% ethanol overnight at 4°C. Cells were then stained with propidium iodide for analysis of DNA content. Apoptotic cells were quantified by flow cytometry performed in the Flow Cytometry Core Laboratory at our institution as described previously (9, 10).

Western Blot Analysis. For Western blot analysis, cells were washed with cold PBS and subjected to lysis in Laemmli’s lysis buffer. Equal amounts of protein were separated by SDS-PAGE and electroblotted onto nitrocellulose. The membranes were blocked with 5% nonfat dry milk and incubated with primary antibodies that were diluted 1:200 or 1:1000 with PBS. Membranes were washed with 0.05% Tween 20, and incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoblot results were visualized by enhanced chemiluminescence. The bands were scanned and quantitated using software (Molecular Dynamics, Sunnyvale, CA).
kit (ECL kit; Amersham Bioscience, Buckinghamshire, United Kingdom). Rabbit anti-human Bax, Bcl-XL, caspase-3, and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human caspase-8 and mouse anti-human poly(ADP-ribose) polymerase (PARP), cytochrome c, and Apaf-1 were obtained from BD PharMingen (San Diego, CA). An ApoAlert Cell Fractionation Kit from Clontech (Palo Alto, CA) was used for mitochondria preparation. β-actin was used as a loading control.

**Statistical Analysis.** Differences among the treatment groups were assessed by ANOVA using StatSoft statistical software (Tulsa, OK). *P* < 0.05 was regarded as significant.

### RESULTS

**Growth-inhibiting Effect of 2,3-DCPE on Human Cancer Cell Lines and NHFBs.** 2,3-DCPE is a synthetic compound with a molecular weight of 264. Chemically, it is a phenoxyl compound with chlorine substituent groups (Fig. 1). 2,3-DCPE is soluble in both water and DMSO. Because 2,3-DCPE was dissolved in DMSO in the original chemical library obtained from ChemBridge Corporation, we used DMSO as a solvent in all of the subsequent studies.

In a primary screening test, we found that 2,3-DCPE was able to kill human lung cancer H1299 and A549 cells at a concentration at which most of the other chemicals in the library did not kill these cells. Thus, the cell killing effects of this compound were further evaluated *in vitro* in six human cancer lines and NHFBs. Cells were treated with 2,3-DCPE at concentrations ranging from 0.1 to 100 μM. DMSO alone was used as a control. Cell viability was determined 4 days after the treatment. Whereas treatment with the same concentration of DMSO did not result in much difference from those treated with PBS, treatment with 2,3-DCPE at various concentrations resulted in loss of cell viability (Fig. 2). The IC₅₀s for LoVo, H1299, and DLD-1 cells were 0.89, 2.24, and 1.95 μM, respectively. The IC₅₀ for NHFBs was 12.6 μM (Table 1).

To compare the cell growth inhibition effect of 2,3-DCPE with some existing chemotherapeutic agents, we tested the effects of 5-fluorouracil, cisplatin, and paclitaxel in the same cell lines as we had tested for 2,3-DCPE (Table 1). The IC₅₀ for 5-fluorouracil and cisplatin were in the micromole range, similar to that of 2,3-DCPE. However, the IC₅₀ for paclitaxel was in the nanomole range for most cell lines.

![Fig. 1. Chemical structure of 2[(3-(2,3-dichlorophenoxy)propyl]amino] ethanol (2,3-DCPE), 2[(3-(3,4-dichlorophenoxy)propyl]amino] ethanol (3,4-DCPE), and 2[(2-(2,6-dichlorophenoxy)ethyl]amino] ethanol (2,6-DCEE).](image)

![Fig. 2. Effects of 2,3-DCPE (A) and 3,4-DCPE (B) on cell viability. Cells were treated with the indicated agent for 4 days, and then cell viability was measured by XTT assay. In each treatment group, cells treated with DMSO were the control, and their viability was set as 100%. Each experiment was performed in quadruplicate and repeated at least twice. Values are means for quadruplicate assays; bars, SD.](image)

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<th>Cisplatin (μM)</th>
<th>Paclitaxel (μM)</th>
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![Fig. 3. Induction of apoptosis by 2,3-DCPE. A, proportion of cells that were apoptotic according to fluorescence-activated cell sorter analysis at 4 days after treatment with 2,3-DCPE at 3.3 μM or DMSO. Data shown represent one of two experiments with similar results. B, Western blot analysis of cleavage of caspase-8, caspase-3, and caspase-9, and PARP in NHFBs, DLD-1, and A549 cells but not in NHFB. C, distribution of cytochrome c in DLD-1 cells. Cells were treated with DMSO or 2,3-DCPE at 20 μM for 24 h and then harvested. Cytochrome c (cyto) and mitochondria (mito) were separated with an ApoAlert cell fractionation kit from Clontech according to the manufacturer’s protocol. COX-4 was used as a marker of mitochondrial proteins.](image)
cells tested, suggesting that paclitaxel is more potent in killing cells than other agents tested. Nevertheless, all of the three existing chemotherapeutic agents did not have a selective killing effect for cancer cells versus normal fibroblasts.

We also tested the cell-killing effects of 3,4-DCPE and 2,6-DCE (Fig. 1), which have chemical structures similar to that of 2,3-DCPE. We found that 3,4-DCPE killed cells with less potency than 2,3-DCPE and without obvious selectivity (Fig. 2B), whereas 2,6-DCE did not kill any cells at the highest concentration tested (100 μM; data not shown). Thus, we decided to focus on 2,3-DCPE in our subsequent experiments.

**Induction of Apoptosis by 2,3-DCPE in Various Cancer Cell Lines.** Many antitumor agents function by inducing apoptosis in tumor cells (11). To test whether the cell-killing effects observed with 2,3-DCPE were caused by induction of apoptosis, we used fluorescence-activated cell sorter analysis to quantify apoptotic cells in 2,3-DCPE-treated cells. Cells were treated with 2,3-DCPE at 3.3 μM or DMSO for 4 days and then cells with less DNA than G1 cells (designated sub-G1 cells) were counted after propidium iodide staining as we have described previously (10, 12). The results showed that treatment with 2,3-DCPE but not with DMSO alone resulted in a dramatic increase in the proportion of apoptotic cells in the cancer cell lines LoVo, A549, and H1299 (Fig. 3A). In contrast, only a minimal increase in the proportion of apoptotic cells was observed in NHFBs. These results suggested that 2,3-DCPE induces apoptosis in cancer cells but not normal cells.

To further analyze the effect of 2,3-DCPE, we tested activation of caspase-8, caspase-3, and caspase-9 and cleavage of PARP after treatment with DMSO or 2,3-DCPE. DLD-1 and A549 cells and NHFBs were harvested at 24 h after treatment with DMSO or 2,3-DCPE at 20 μM. Activation of caspase-3, caspase-8, and caspase-9 was analyzed by Western blot assay. Treatment with 2,3-DCPE resulted in apparent cleavage of capase-3, capase-8, and caspase-9 in A549 and DLD-1 cancer cells but not in NHFB cells (Fig. 3B). PARP was also cleaved in A549 and DLD-1 cells but not in NHFB cells after treatment with 2,3-DCPE. Moreover, Western blot analysis of cytochrome c distribution showed release of cytochrome c to the cytosol in DLD-1 cells after treatment with 2,3-DCPE (Fig. 3C). Together, these results provided further evidence that 2,3-DCPE induces apoptosis in cancer cells.

**Down-Regulation of Bcl-XL by 2,3-DCPE.** To analyze possible mechanisms underlying the 2,3-DCPE-induced apoptosis, we tested expression of Bax, Bcl-XL, and Apaf-1 in cells after treatment with 2,3-DCPE. Western blot analysis showed no apparent change in levels of Bax and Apaf-1 after treatment with 2,3-DCPE at 20 μM for 24 h. However, the level of Bcl-XL was decreased after treatment with 2,3-DCPE (Fig. 4A). A time course study showed that decrease of Bcl-XL started at 8–16 h after treatment and became more obvious at 24 h after treatment (Fig. 4B). We also tested levels of Bcl-XL in DLD-1 cells 24 h after treatment with various concentrations of 2,3-DCPE. A reduction of Bcl-XL was observed when cells were treated with 5 μM of 2,3-DCPE (Fig. 4C).

To test whether down-regulation of Bcl-XL can also be induced by existing chemotherapeutic agents, we analyzed Bcl-XL levels in DLD-1 cells after treatment with 200 μM 5-fluorouracil for 3 days or 100 nM paclitaxel for 24 h. The concentrations and time points for 5-fluorouracil and paclitaxel were selected because peak levels of apoptosis were detected at those conditions (data not shown). Nevertheless, we did not detect a change of Bcl-XL levels in DLD-1 cells after treatment of 5-fluorouracil and paclitaxel (Fig. 4D).

**Blockage of 2,3-DCPE-Induced Apoptosis by Enforced Overexpression of Bcl-XL.** To further test the role of Bcl-XL expression in 2,3-DCPE-induced apoptosis, we evaluated the effects of 2,3-DCPE in Bcl-XL stable-transfected DLD-1 cells (DLD-1/Bcl-XL) (9). DLD-1 and DLD-1/Bcl-XL cells were treated with 2,3-DCPE at 20 μM. Thirty-two h after treatment, cells were harvested, and apoptotic cells were quantified by fluorescence-activated cell sorter analysis as described in “Materials and Methods”. About 16% of parental DLD-1 cells underwent apoptosis after 2,3-DCPE treatment, whereas only about 4% of DLD-1/Bcl-XL cells underwent apoptosis (Fig. 5A), suggesting that overexpression of Bcl-XL could block 2,3-DCPE-induced apoptosis. Western blot analysis also showed that cleavage of caspase-8, caspase-3, and caspase-9 could not be detected in DLD-1/Bcl-XL cells, even at 72 h after treatment (Fig. 5B).

**DISCUSSION**

Here we showed that 2,3-DCPE induced apoptosis in various cancer cells more effectively than in NHFBs. 2,3-DCPE also reduced
Bcl-XL blocked 2,3-DCPE-induced apoptosis significantly (P < 0.05). B. Western blot analysis of cleavage of caspases in DLD-1/Bcl-XL cells treated with 2,3-DCPE at 20 μM and harvested at the indicated times. Parental DLD-1 cells treated with 2,3-DCPE and harvested at 24 h were used as a control. Cleavage of caspase-8, caspase-3, and caspase-9 was observed in DsLD-1 cells but not in DLD-1/Bcl-XL cells.

Bcl-XL protein levels in cancer cells and enforced overexpression of Bcl-XL-blocked 2,3-DCPE-mediated apoptosis. The exact mechanisms of 2,3-DCPE-mediated apoptosis and down-regulation of Bcl-XL remain to be characterized. Interestingly, real-time PCR analysis showed that treatment with 2,3-DCPE did not decrease the Bcl-XL mRNA level (data not shown), suggesting that down-regulation of Bcl-XL is not caused by suppressed transcription. Down-regulation of Bcl-XL was, however, not observed in apoptosis induced by 5-fluorouracil and paclitaxel, suggesting that apoptosis itself may not result in down-regulation of Bcl-XL. We also found that knockdown of Bcl-XL by small interfering RNA can directly induce apoptosis in DLD-1 cells (data not shown), suggesting that down-regulation of Bcl-XL itself may contribute to 2,3-DCPE-induced apoptosis. Nevertheless, it is possible that other cellular molecules may also be involved in 2,3-DCPE-induced apoptosis. Although we found that enforced overexpression of Bcl-XL blocked 2,3-DCPE-induced apoptosis, overexpression of Bcl-XL may block apoptosis signals induced by 2,3-DCPE other than its effect on Bcl-XL levels because the Bcl-XL level negatively correlates with sensitivity to signals induced by 2,3-DCPE other than its effect on Bcl-XL levels. Therefore, knockdown of Bcl-XL by small interfering RNA can directly induce apoptosis in DLD-1 cells (data not shown), suggesting that down-regulation of Bcl-XL itself may contribute to 2,3-DCPE-induced apoptosis. Nevertheless, it is possible that other cellular molecules may also be involved in 2,3-DCPE-induced apoptosis. Although we found that enforced overexpression of Bcl-XL blocked 2,3-DCPE-induced apoptosis, overexpression of Bcl-XL may block apoptosis signals induced by 2,3-DCPE other than its effect on Bcl-XL levels because the Bcl-XL level negatively correlates with sensitivity to most anticancer drugs of various action mechanisms.

A search of the online Chemical Abstracts Service database revealed an entry for 2,3-DCPE but not a report of its biological function. Several chemicals with structures similar to that of 2,3-DCPE were found in the Chemical Abstracts Service database. The biological functions of these chemicals also were not reported. We tested two of these chemicals, 3,4-DCPE and 2,6-DPEE. Although 2,6-DPEE did not kill any cells at a concentration up to 100 μM, 3,4-DCPE killed all cells at a concentration range of 10–100 μM without obvious selectivity. These findings indicate that 2,3-DCPE kills cells through a cellular target that may not be recognized by its analogs 3,4-DCPE and 2,6-DCE. Whether other analogs of 2,3-DCPE can selectively kill cancer cells is not yet clear.

The selective cell-killing effect of 2,3-DCPE suggested that this compound or its analogs might be potential new anticancer agents. However, the mechanisms underlying this selectivity are not yet clear. It is possible that differences in uptake of the agent, metabolism, or cellular levels of proapoptotic/antiapoptotic proteins may account for the selective cell killings observed. Interestingly, cell-killing effects by 5-fluorouracil, cisplatin, and paclitaxel also varied greatly among the cell lines tested, although no selective advantage for fibroblasts was observed. It is noteworthy that inducing induction of apoptosis of 2,3-DCPE in cancer cells is only the first step in our research on potential new anticancer agents. It will be necessary to test various analogs of 2,3-DCPE in vitro to identify chemicals with better efficacy and selectivity. It may also be necessary to screen for analogs with acceptable in vivo pharmacokinetic properties, including absorption, distribution, metabolism, and excretion (13, 14). It is noteworthy that in ~40% of the cases in which the development of drug is halted, the reason is inappropriate pharmacokinetics in humans (15). Thus, much work remains to be done to determine whether 2,3-DCPE or its analogs can be used for treatment of cancers.

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REFERENCES

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