Identification of Chelerythrine as an Inhibitor of BclXL Function

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The identification of small molecule inhibitors of antiapoptotic Bcl-2 family members has opened up new therapeutic opportunities, while the vast diversity of chemical structures and biological activities of natural products are yet to be systematically exploited. Here we report the identification of chelerythrine as an inhibitor of BclXL-Bak Bcl-2 homology 3 (BH3) domain binding through a high throughput screening of 107,423 extracts derived from natural products. Chelerythrine inhibited the BclXL-Bak BH3 peptide binding with IC50 of 1.5 μM and displaced Bax, a BH3-containing protein, from BclXL. Mammalian cells treated with chelerythrine underwent apoptosis with characteristic features that suggest involvement of the mitochondrial pathway. While staurosporine, H7, etoposide, and chelerythrine released cytochrome c from mitochondria in intact cells, only chelerythrine released cytochrome c from isolated mitochondria. Furthermore BclXL-overexpressing cells that were completely resistant to apoptotic stimuli used in this study remained sensitive to chelerythrine. Although chelerythrine is widely known as a protein kinase C inhibitor, the mechanism by which it mediates apoptosis remain controversial. Our data suggest that chelerythrine triggers apoptosis through a mechanism that involves direct targeting of Bcl-2 family proteins.

Proteins of the Bcl-2 family are central regulators of apoptosis. While the precise molecular mechanisms by which these proteins confer their biological activities remain to be determined, they are thought to act directly on the mitochondrion (1). Members of the Bcl-2 family can be divided into three subfamilies based on several conserved sequence motifs known as BH1–BH4 domains. The antiapoptotic members (Bcl-2, BclXL, Mcl-1, Bcl-1-w, and Ced-9) share all four BH domains, designated as BH1–4; the proapoptotic members Bax, Bak, Bok, and BclXs contain BH1–3 domains, but other proapoptotic members (Bid, Bad, Bik, and EglI) only have a BH3 domain. Antiapoptotic Bcl-2 family members appear to function, at least in part, by interacting with and antagonizing proapoptotic family members (2). The BH1–3 domains of BclXL form an elongated hydrophobic groove, which is the docking site for the BH3 domains of proapoptotic binding partners (3). It has been demonstrated that BH3 domains from Bak and Bad proteins are required for binding to BclXL and for mediating their proapoptotic effect (4). A synthetic peptide derived from the Bak BH3 domain binds recombinant BclXL protein in vitro with high affinity (3). Furthermore the Bak BH3 peptide alone is able to induce apoptosis when introduced into various cell lines (5), suggesting that the interaction between the BH3 domains from proapoptotic proteins and BclXL is important in mediating the proapoptotic signal. Therefore small molecular weight compounds that inhibit the BclXL-BH3 domain interaction could potentially act as apoptotic modulators. Indeed significant progress has been made in isolating compounds of this nature (6). BH3I-1 and BH3I-2 were discovered by screening a library of 16,320 compounds for ones that disrupted the interaction between BclXL and a Bak BH3 peptide (7), whereas antymycin A was discovered serendipitously (8). A few other compounds have also been discovered in silico, and they are of diverse structures (9–11).

Natural products cover a molecular diversity not available from synthetic libraries with an unrivaled success rate as drug leads (12). We have, therefore, carried out a large scale high throughput screen of natural product extracts to uncover compounds that would disrupt the interaction between BclXL and the Bak BH3 peptide. Here we report the identification of chelerythrine (1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium), which is a natural benzophenanthidine alkaloid and a known protein kinase C inhibitor (13), as an inhibitor of BclXL-Bak BH3 peptide binding. Chelerythrine released cytochrome c (CytC) from isolated mitochondria and induced apoptosis in BclXL-overexpressing cells that were completely resistant to staurosporine or etoposide. Chelerythrine thus represents the first BH3 mimic identified through high throughput screening of natural products.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Human SH-SY5Y and MCF7 cells were maintained as described previously (14). HCT116 cells and FDC-P1 cells were gifts from Bert Vogelstein (Dana Farber Cancer Institute) and David C. Huang (The Walter and Eliza Hall Institute, respectively). LipofectAMINE (Invitrogen) was used for transfections according to the user’s manual, and BclXL stably transfected SH-SY5Y cells were selected with 400 μg/ml hygromycin B (Calbiochem) and maintained in medium containing 100 μg/ml hygromycin B after 2–3 weeks of selection. The peptide protease inhibitor e-VAD-fmk was from Enzyme System Products, Livermore, CA. Staurosporine, H7 (1-(5-isouquinolinesulfonyl)-2-methylpiperazine), and etoposide were from Sigma, and BclXL-1 was from Calbiochem.

Fluorescence Polarization (FP) Assay—The Bak BH3 peptide labeled

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CH$_2$Cl$_2$ fraction. The active fraction was fractionated using gradient modified Kupchan solvent partition method (15) to give an active assay buffer (50 mM Tris, pH 8, 150 mM NaCl, and 0.1% bovine serum albumin). 5

Mitochondria were isolated from SH-SY5Y cells. Cells were suspended in isolation buffer (320 mM sucrose, 1 mM EDTA, 50 mM HEPES (pH 7.5), 1 mM EGTA, and 10 mM sodium succinate, 2 mM Mg$^2+$, and 5 mM KH$_2$PO$_4$) and disrupted by 10 expulsions through a 27-gauge needle. Disrupted cells were centrifuged at 1000 g for 10 min and disrupted by 10 expulsions through a 27-gauge needle. Disrupted cells were centrifuged at 3000 g for 5 min to remove cell debris and nucleus. The supernatants were centrifuged at 3000 g to pellet the mitochondria. The mitochondria pellets were resuspended in assay buffer (250 mM sucrose, 2 mM KH$_2$PO$_4$, 5 mM sodium succinate, 2 mM EGTA, and 10 mM HEPES (pH 7.5)) at 0.5 mg/ml and treated at room temperature with the indicated compounds for 15 min followed by centrifugation. CytC released into the supernatant was subjected to fractionation by 10% SDS-PAGE followed by Western blotting analysis. Flow Cytometry—For detection of sub-G$_1$ DNA, cells were washed once, resuspended in 200 μl of phosphate-buffered saline, and fixed in a 50-fold excess of ice-cold 70% ethanol. Cells were recovered by centrifugation at 1000 × g for 5 min at 4 °C, washed, stained with 50 mg/ml propidium iodide for 30 min at room temperature, and analyzed in a FACScan flow cytometer (BD Biosciences). Mitochondrial potential change as measured by JC-1 staining was performed in accordance with the manufacturer’s instructions (Molecular Probes). A minimum of 10,000 cells/sample were analyzed.

RESULTS AND DISCUSSION

Identification of Chelerythrine as an Inhibitor of BclXL and Bcl-2 Peptide Interaction—A high throughput screen based on FP (7) was devised to identify compounds that disrupt the interaction between BclXL and the BH3 domain of Bak. A total of 107,423 extracts prepared from plants, actinomycetes, fungi, marine invertebrates, and marine bacteria were screened. Twelve extracts were chosen for isolation of active compounds, and the active principle of four extracts from plants was found to be chelerythrine (Fig. 1A). Chelerythrine displaced the fluorescently labeled BH3 domain peptide from a recombinant GST-BclXL fusion protein with IC$_{50}$ of 1.5 μM (Fig. 1B). Similar concentrations of chelerythrine with GST protein and the labeled peptide produced no significant change in polarization (Fig. 1B).

Chelerythrine Disrupts the Interaction between BclXL and Bax—The ability of chelerythrine to displace the Bak BH3 peptide in the FP assay suggests that it may be able to displace BH3-containing proteins from BclXL. In vitro translated $[^{35}$S]Bax bound specifically to GST-BclXL immobilized on glutathione beads, and the addition of chelerythrine resulted in a dose-dependent decrease in Bax binding (Fig. 1C and D). Chelerythrine was, however, unable to disrupt interaction between the Caenorhabditis elegans sex determination proteins $[^{35}$S]FEM3 and GST-FEM2 (18) immobilized on glutathione beads (data not shown), suggesting that the action of chelerythrine on Bax and BclXL was specific. The solution structure of Bel-2 has been solved (19), and the data suggest that Bel-2 and BclXL have highly similar three-dimensional structures, including the hydrophobic groove. Interestingly we found that
the binding of Bax to Bcl-2 was disrupted by chelerythrine in a dose-dependent manner (data not shown).

Chelerythrine-mediated Apoptosis Exhibits Characteristic Features Similar to Cell Death Induced by Proapoptotic Members of the Bcl-2 Family—Since the mitochondria play a key role in the control of apoptosis and it is the main site where BclXL and Bcl-2 exert their function, we evaluated mitochondrial function in response to chelerythrine with the fluorescent dye JC-1 that allows the analysis of mitochondrial potential ($\Delta \psi_m$). Treatment of human neuroblastoma SH-SY5Y cells (20) with chelerythrine at 2.5 and 5 $\mu$M for 16 h induced a substantial decrease in mitochondrial potential as indicated by an increase in JC-1 green fluorescence (Fig. 2, A and B). Chelerythrine-induced mitochondrial potential changes were partially inhibited by the broad spectrum caspase inhibitor ZVAD (Fig. 2, A and B), similar to reports showing that Bax-induced mitochondrial potential change was partially sensitive to caspase inhibition (21). Treatment of SH-SY5Y cells with chelerythrine also induced the appearance sub-G1 DNA that is indicative of apoptosis (Fig. 2, C and D). The appearance of sub-G1 DNA is totally blocked by the addition of ZVAD (Fig. 2, C and D), which is consistent with the notion that DNA fragmentation is dependent on caspase activation (22). The ZVAD-treated cells without sub-G1 DNA, however, were not viable since they were unable to grow upon replating on fresh tissue culture plates (data not shown). Similar to apoptosis mediated by proapoptotic members of the Bcl-2 family, inhibition of caspases only slows down but does not abrogate the cell death process (23, 24). The change in mitochondrial potential and the appearance sub-G1 DNA upon chelerythrine treatments were observed in two other cell lines, HCT116, a colon carcinoma cell line, and MCF7, a breast cancer cell line (data not shown), suggesting that the effect is not limited to SH-SY5Y cells.

Chelerythrine Triggers CytC Release from Isolated Mitochondria—Many death stimuli trigger apoptosis through the release of CytC from the mitochondrial intermembrane space to activate Apaf-1, thus coupling this organelle to caspase activation. Treatment of SH-SY5Y cells with etoposide, staurosporine, and H7 (20) as well as chelerythrine induced mitochondrial potential change, CytC release from the mitochondria (Fig. 3, A and B), and the appearance of sub-G1 DNA (data not shown), which are hallmarks of apoptosis. However, if the action of chelerythrine is on BclXL or Bcl-2 on the mitochondria, it should be able to trigger CytC release directly from isolated mitochondria as observed with proapoptotic Bcl-2 family members (25, 26). To investigate this, mitochondria were isolated from healthy SH-SY5Y cells and subjected to treatment with various death stimuli. Chelerythrine released CytC from isolated mitochondria in a dose-dependent manner (Fig. 3, C and D). Etoposide and other protein kinase C inhibitors such as H7 and staurosporine were unable to do so (Fig. 3, C and D) even at concentrations exceeding the required amount to induce apoptosis in intact cells (Fig. 3, B–D, and data not shown). The interactions of Bax or Bak with BclXL in the mitochondrial preparation, if any, appeared to be very weak (data not shown).
It was therefore technically difficult to determine whether there was a reduction in heterodimerization between the proteins upon chelerythrine treatment. It is possible that the CytC release represents a direct antagonistic effect of chelerythrine on BclXL/Bcl-2 function.

Chelerythrine Induces Apoptosis in BclXL-overexpressing SH-SY5Y Cells—Overexpression of Bcl-2 or BclXL is able to block cell death induced by many forms of death stimuli, e.g., radiation and most chemotherapeutic drugs (2, 27). The limited concentrations of endogenous factors serving the apoptotic signaling pathway preceding the mitochondria step enable BclXL overexpression to block these signals. On the other hand, if a compound acts directly on BclXL, it should be able to overcome the effect of overexpression of the protein easily since cellular protein concentration, even in a state of overexpression, is limited in comparison to concentrations achievable with small molecular weight compounds. To test our hypothesis, we generated SH-SY5Y cells that overexpress BclXL. In these cells the level of BclXL is greatly enhanced, while other members of the Bcl-2 family such as Bax, Bak, and Bid stay relatively constant with a moderate down-regulation of Bcl-2 level (Fig. 4A). Treatment of BclXL-overexpressing cells with staurosporine up to 1 μM did not induce cell death as indicated by the lack of mitochondrial potential change (Fig. 4B, data not shown) as well as the absence of sub-G1 DNA (Fig. 4C). In contrast, the vector line was very sensitive to staurosporine-induced apoptosis. Nearly 100% of the cells exhibited mitochondrial potential change, and 80% of the cells contained sub-G1 DNA when only a 100 nM concentration of the drug was added (Fig. 4, B and C). Similarly the apoptotic effects of etoposide (Fig. 4, B and C) and H7 (data not shown) were abolished by BclXL overexpression. Interestingly, although the staurosporine- and etoposide-treated BclXL-overexpressing cells did not undergo apoptosis, they were arrested at the G1 and S phase of the cell cycle, respectively (see the supplemental figure). These observations are consistent with previous reports indicating that cell cycle arrests induced by genotoxic drugs are not affected by BclXL overexpression (28). Overexpression of BclXL was able to confer resistance to the killing effect of chelerythrine at low concentration of up to 2 μM. At higher concentrations, chelerythrine overcame the protective effect of BclXL and induced apoptosis in these cells effectively (Fig. 4D). Similar results were obtained with a Bcl-2-overexpressing, mouse interleukin-3-dependent, promyelocytic cell line, FDC-P1, in which chelerythrine at concentrations higher than 1.25 μM was able to overcome the protective effect of Bcl-2 (data not shown). The data suggest that chelerythrine, unlike staurosporine, H7, and etoposide, induces apoptosis by inhibiting BclXL/Bcl-2 directly.

Enhanced expression of antiapoptotic Bcl-2-related proteins in cancer cells has been implicated in resistance to currently available antineoplastic agents (2, 27). Chelerythrine has been shown to exhibit cytotoxic activity against radioresistant and chemoresistant squamous carcinoma cells and p53-deficient cells (29). It delays tumor growth in an experimental model with relatively mild toxicity to the animal (29). Our results indicate that chelerythrine may act as a BH3 mimetic that is able to circumvent the upstream antiapoptotic barriers in transformed cells and thus can be explored as a potential anticancer therapeutic.

The inhibitors of BclXL-BH3 interaction identified so far are all proapoptotic in nature. However, the diverse structural differences among these compounds suggest that they may act through multiple mechanisms in affecting the Bcl-2 family proteins. Interestingly BclXL overexpression confers slight protection against chelerythrine- and BH3I-1-induced apoptosis, while it sensitizes the cells toward antimycin A3 (8). The identification of chelerythrine as a novel inhibitor of BclXL-BH3 interaction adds to the repertoire of reagents that are invaluable in defining the molecular mechanisms by which proteins of the Bcl-2 family mediate their functions.

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References

Supplemental figure for Chan et al.

Data from FACS analysis are presented. Vector and BclXL overexpressing SH-SY5Y cell lines were treated with increasing concentrations of the indicated compounds for 48 h. The percentage of cells with Sub-G1 DNA is shown.
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