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Article

Emetine Regulates the Alternative Splicing of *Bcl-x* through a Protein Phosphatase 1-Dependent Mechanism

Kritsanapol Boon-Unge,[1,2,3] Qingming Yu,[1,3] Tie Zou,[1] An Zhou,[1] Piyarat Govitrapong,[2] and Jianhua Zhou[1,*]
[1]Department of Medicine, Program in Neuroscience, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA
[2]Neuro-Behavioural Biology Center, Mahidol University, Salaya, Nakornpathom 73170, Thailand
[3]These authors contributed equally to this work.
*Correspondence: jianhua.zhou@umassmed.edu
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SUMMARY

Exon 2 of the *Bcl-x* gene undergoes alternative splicing in which the Bcl-xS splice variant promotes apoptosis in contrast to the anti-apoptotic splice variant Bcl-xL. In this study, the regulation of the alternative splicing of pre-mRNA of *Bcl-x* was examined in response to emetine. Treatment of different types of cancer cells with emetine dihydrochloride downregulated the level of *Bcl-xL* mRNA with a concomitant increase in the mRNA level of *Bcl-xS* in a dose- and time-dependent manner. Pretreatment with calyculin A, an inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), blocked emetine-induced alternative splicing in contrast to okadaic acid, a specific inhibitor of PP2A in cells, demonstrating a PP1-mediated mechanism. Our finding on the regulation of RNA splicing of members of the Bcl-2 family in response to emetine presents a potential target for cancer treatment.

INTRODUCTION

Alternative splicing occurs when the introns of a certain pre-mRNA are excised in more than one way, producing several possible mature mRNAs from one gene. Alternative pre-mRNA splicing is an essential mechanism for generating protein diversity [1–4] according to different regulatory programs. It is estimated that more than 60% of human genes undergo alternative splicing, leading to production of diversified functional isoforms [5]. Alternative splicing is precisely regulated. Aberrant splicing can lead to human disorders such as growth hormone deficiency, Frasier syndrome, Parkinson's disease, cystic fibrosis, retinitis pigmentosa, spinal muscular atrophy, and myotonic dystrophy [6, 7].

Apoptosis, or programmed cell death, plays an important role in normal tissue equilibrium by counterbalancing cell production and cell loss. Cancers and neurodegenerative disorders often show a defective cell death program [8], the former originating from too little apoptosis and the latter emerging from too much cell death. Deregulation of the balance between proliferation and cell death represents a protumorigenic principle in human carcinogenesis. Alternative splicing plays a critical role in the control of apoptosis. Several pre-mRNAs for cell death signals are alternatively spliced, yielding isoforms with opposing functions during programmed cell death [7].

One distinct example is the *Bcl-x* transcript, which is alternatively spliced in exon 2 to produce the proapoptotic Bcl-xS or the anti-apoptotic Bcl-xL [9]. The protein product of the larger Bcl-xL functions as a repressor of programmed cell death [10], whereas the smaller *Bcl-xS* encodes a protein that can accelerate cell death [11, 12]. Bcl-xL is highly expressed in several types of cancers, and overexpression of Bcl-xL inhibits apoptosis and promotes resistance to chemotherapy in tumors in vivo [13]. It has been found that Bcl-xL is overexpressed in high-grade prostate cancer and associated with hormone refractory phenotype [14]. Breast cancer also has a high level of Bcl-xL that is correlated with an increased risk of metastasis [15] and disallows apoptosis, gains resistance against cytokines, alters the relationship between cells and extracellular matrix, and probably renders a mechanism for cells to adapt to a new environment [16]. In ovarian cancers, Bcl-xL expression conferred resistance to chemotherapy-induced apoptosis resulting from treatment with cisplatin, paclitaxel, topotecan, and gemcitabine [17]. On the other hand, reduction of Bcl-xL protein and/or increase of production of Bcl-xS by specific antisense oligonucleotide (ISIS 16009) treatment or other approaches enhanced the chemosensitivity or radiosensitivity of colon cancer cells, breast cancer cells, HepG2 hepatoblastoma cells, and other tumor cell lines [18–23]. Because Bcl-xS overexpression can induce apoptosis in tumoral cell lines [24], the ability to alter the Bcl-xL/Bcl-xS ratio thus has promising therapeutic potential for cancer treatment.

Numerous reports have demonstrated that *Bcl-x* alternative splicing can be regulated by small molecules such as ceramide in cancer cells [25], as well as by other biological molecules including IL-6, GM-CSF, amphetamine, and TPA [26, 27]. To identify additional small molecules that may be potentially used in cancer treatment by regulating *Bcl-x* splicing, we performed RT-PCR experiments in cells treated with 1040 Food and Drug Administration

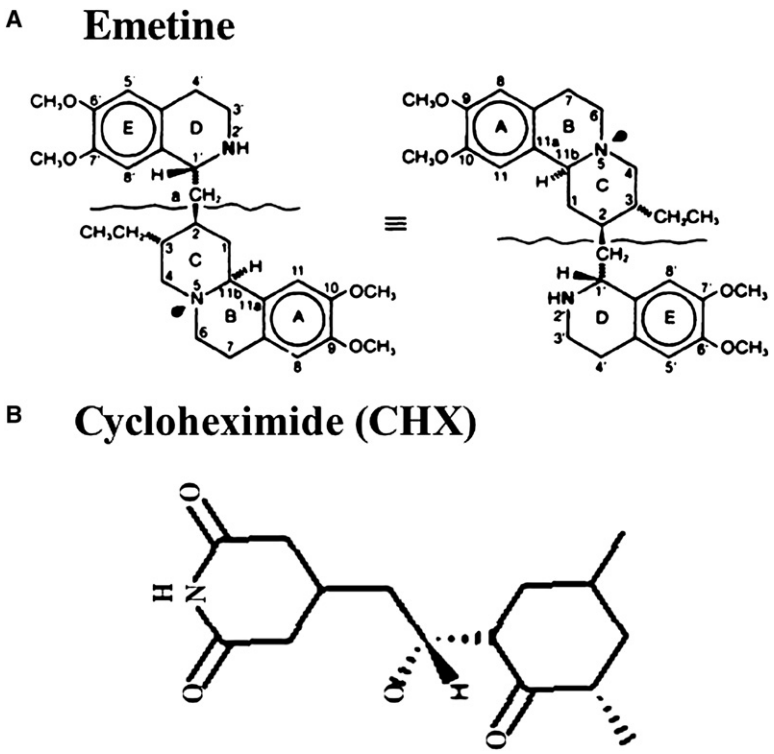

Figure 1. Chemical Structures
(A) Emetine.
(B) Cycloheximide (CHX).

(FDA)-approved drugs and compounds (National Institute of Neurological Disorders and Stroke [NINDS] Custom Collection, MicroSource Discovery Systems). We found that emetine, a potent protein synthesis inhibitor in eukaryotes [28], downregulated *Bcl-xL* and upregulated *Bcl-xS*. We further demonstrate that this defined emetine function is mediated by phosphorylation.

RESULTS

Emetine Regulates the Alternative Splicing of *Bcl-x* Pre-mRNA

It has been hypothesized that modulation of the *Bcl-xL*/*Bcl-xS* ratio by regulating alternative splicing of exon 2 in the *Bcl-x* gene may have potential for cancer treatment. To identify small molecules that regulate *Bcl-x* splicing, we screened 1040 FDA-approved drugs and compounds (NINDS Custom Collection, MicroSource Discovery Systems) using RT-PCR in C33A cells, a cervical cancer line. We found that emetine (see chemical structure in Figure 1) reduced *Bcl-xL* mRNA with a concomitant increase in *Bcl-xS* (Figures 2B and 2D). To further validate our findings, C33A cells were treated overnight with various concentrations of emetine. There was a decrease in the ratio of *Bcl-xL*/*Bcl-xS* from 9.6 to 2.6, 2.2, 1.8, and 1.5 with emetine 0.1, 0.3, 1.0, and 10.0 μM, respectively (Figure 2B). Interestingly, emetine did not alter splicing of *tau*, *SMN*, and *BACE1* genes, indicating its relative specificity for *Bcl-x* splicing. Emetine ($C_{29}H_{40}N_2O_4$), the ipecac alkaloid, is an amoebicidal agent that inhibits polypeptide chain elongation in parasites [29–31]. Emetine is a potent protein synthesis inhibitor in mammalian cells, plants, and yeasts [32]. Grollman has shown that emetine and cycloheximide, another protein synthesis inhibitor, have a similar site and mode of action for inhibition of protein synthesis, and his studies of the conformational, configurational, and electrostatic properties of the emetine molecule suggest that emetine and cycloheximide share certain structural properties around two nitrogen atoms that are essential for their activity (Figure 1) [32]. Consistent with this notion, we also demonstrate that cycloheximide regulates alternative splicing of exon 2 in the *Bcl-x* gene (Figures 2C and 2D). On the other hand, several other protein synthesis inhibitors such as anisomycin and puromycin in the NINDS collection of 1040 FDA-approved drugs and compounds have no or little effect on splicing of exon 2 in the *Bcl-x* gene, suggesting a relative specificity of emetine for *Bcl-x* splicing. All experiments were repeated at least three times.

Time and Dosage Dependence of Emetine on *Bcl-x* Splicing in Cancer Cells

To further validate whether emetine regulates exon 2 splicing in the *Bcl-x* gene and to study whether regulation of exon 2 splicing has potential relevance to cancer therapy, we examined the effects of emetine on the pre-mRNA processing of *Bcl*-x in several tumor cell lines. We treated cells with different time durations and/or different concentrations of emetine. Semiquantitative RT-PCR was used to determine the effects of emetine. We found that in MCF-7 (a breast cancer cell line) (Figures 3A and 3C) and PC3 (a prostate cancer cell line) cells (Figures 3B and 3C), regulation of *Bcl-x* splicing is slightly more pronounced. There was a decrease in the ratio of *Bcl-xL*/

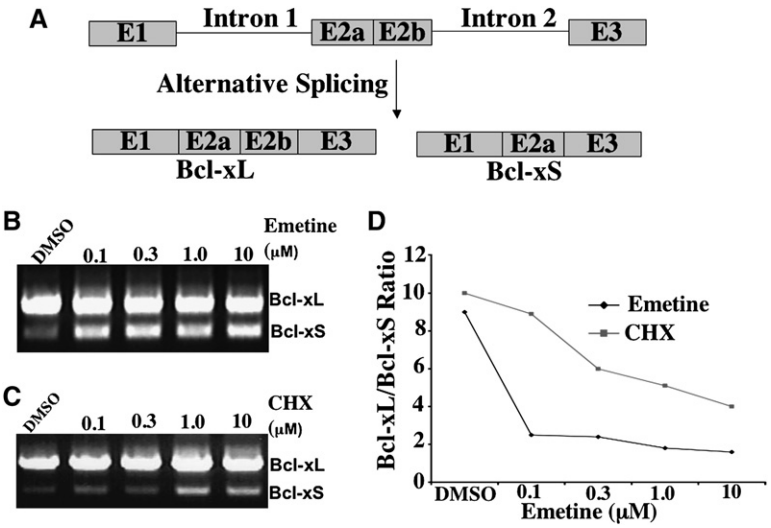

Figure 2. Emetine Regulates *Bcl-x* Splicing in C33A Cells

C33A cells were treated with emetine. Total RNA was extracted and analyzed by RT-PCR for the alternative splicing of *Bcl-x*.

(A) Alternative splicing of exon 2 in the *Bcl-x* gene produces the larger Bcl-xL and smaller Bcl-xS.

(B) Decrease of *Bcl-xL* and increase of *Bcl-xS* are correlated with emetine concentration.

(C) Cycloheximide (CHX) also regulates exon 2 splicing in the *Bcl-x* gene.

(D) Ratio of *Bcl-xL*/*Bcl-xS*. Each experiment was repeated at least three times. Results from all experiments consistently show that emetine and cycloheximide (CHX) modulate exon 2 splicing in the *Bcl-x* gene.

Bcl-xS from 8.6 to 7.2, 5.1, 3.8, 3.3, and 3.0 in MCF-7 cells and from 7.1 to 5.0, 4.8, 4.6, 3.3, and 3.1 in PC3 cells with emetine 0.1, 0.3, 1.0, 3.0, and 10.0 μM, respectively (Figure 3C). However, we see a more dramatic regulation of *Bcl-x* splicing in lung cancer cell line A549 cells by emetine (Figure 4). The effects of emetine on the pre-mRNA processing of *Bcl-x* in A549 cells were time course and dosage dependent. For dosage-dependent study, A549 cells were treated overnight with various concentrations of emetine. There was a decrease in the ratio of *Bcl-xL*/*Bcl-xS* from 7.5 to 3.9, 2.9, 2.0, 1.5, and 1.2 with emetine 0.1, 0.3, 1.0, 3.0, and 10.0 μM, respectively (Figures 4A and 4C). For time-course study, A549 cells were treated with 1 μM emetine. There was a decrease in the ratio of

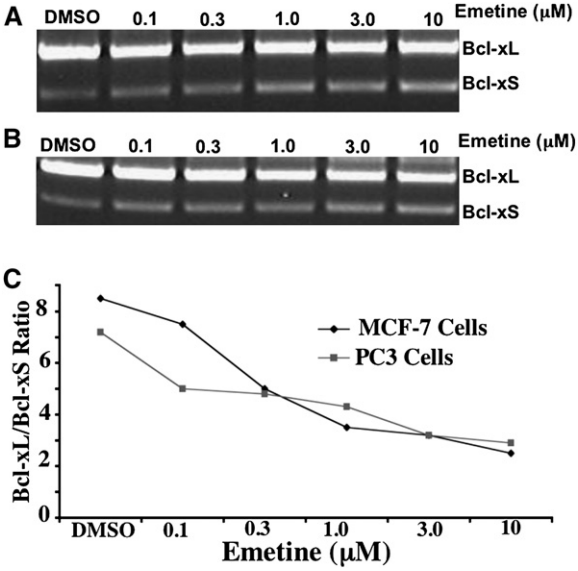

Figure 3. Emetine Regulates *Bcl-x* Splicing in MCF-7 Breast Cancer Cells and PC3 Prostate Cancer Cells

Total RNA was extracted from MCF-7 and PC3 cells that were treated with emetine. Splicing of exon 2 in the *Bcl-x* gene was analyzed by RT-PCR.

(A) RT-PCR results from MCF-7 cells treated with different concentrations of emetine.

(B) RT-PCR results from PC3 cells treated with different concentrations of emetine.

(C) Reduction of *Bcl-xL* and increase of *Bcl-xS* were observed and quantified. All experiments were repeated at least three times and consistently show that emetine regulates exon 2 splicing of the *Bcl-x* gene in MCF-7 and PC3 cells.

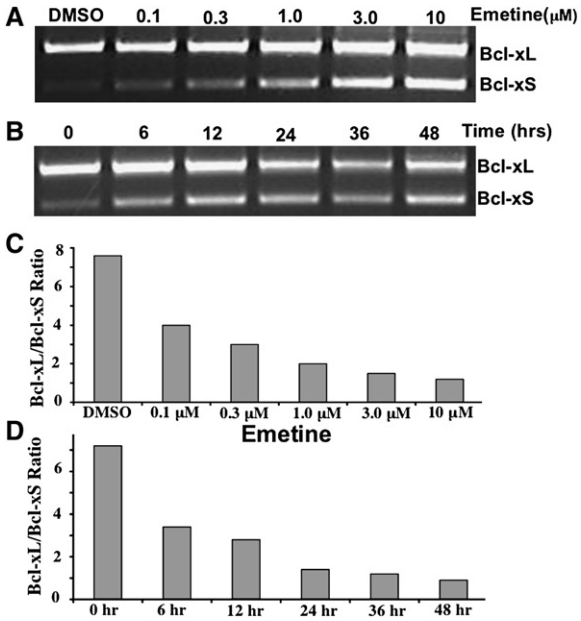

Figure 4. Emetine Regulates *Bcl-x* Splicing in A549 Lung Cancer Cells

A549 cells were treated with emetine. Total RNA was extracted and analyzed by RT-PCR for the alternative splicing of *Bcl-x*.

(A and C) Reduction of *Bcl-xL* and increase of *Bcl-xS* are correlated with emetine concentration.

(B and D) Cells were treated with 1 μM emetine for different durations. RT-PCR was carried out to quantify alternative splicing of *Bcl-x*. All experiments were repeated at least three times.

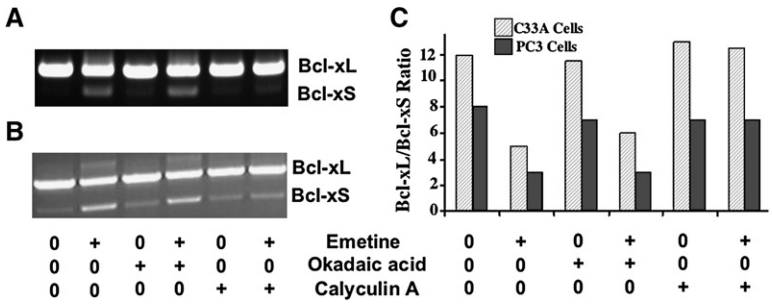

Figure 5. Calyculin A but Not Okadaic Acid Blocks Effects of Emetine on *Bcl-x* Splicing

Cells were pretreated with either 5 μM calyculin A, an inhibitor of both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), or 5 μM okadaic acid, a selective PP2A inhibitor, and then exposed to 1.0 μM emetine for 24 hr. RT-PCR was carried out. The results suggest that PP1, not PP2A, mediates the effects of emetine on the alternative splicing of *Bcl-x*.

(A) Effects of calyculin A in C33A cells.

(B) Effects of calyculin A in PC3 cells. All experiments were repeated at least three times.

(C) Effects of calyculin A or okadaic acid on *Bcl-x* splicing mediated by emetine were quantified and plotted. Ratios of *Bcl-xL/Bcl-xS* in C33A cells: no treatment, 12.0; with emetine, 4.5; with okadaic acid, 12.0; with emetine and okadaic acid, 5.8; with calyculin A, 12.0; with emetine and calyculin A, 11.8. Ratios of *Bcl-xL/Bcl-xS* in PC3 cells: no treatment, 7.9; with emetine, 3.0; with okadaic acid, 7.0; with emetine and okadaic acid, 3.1; with calyculin A, 7.0; with emetine and calyculin A, 7.0. +: with emetine, okadaic acid, or calyculin A; 0: without emetine, okadaic acid, or calyculin A.

Bcl-xL/Bcl-xS from 7.2 to 3.3, 2.8, 1.3, 1.1, and 0.9 with the duration of treatment of 6, 12, 24, 36, and 48 hr, respectively (Figures 4B and 4D). However, because emetine is a potent protein synthesis inhibitor, no synthesis of new protein with exon 2b exclusion should be expected after emetine treatment. Consistent with this notion, we observed only a slight change of *Bcl-xL/Bcl-xS* ratio at the protein level in A549 cells treated with emetine (see Figure S1 in the Supplemental Data available with this article online).

Emetine Exerts Its Effect on *Bcl-x* Splicing via Protein Phosphatase 1

Previous studies show that ceramide affects splicing of *Bcl-x* in a phosphorylation-dependent pathway. To examine whether emetine exerts the effects on *Bcl-x* splicing in a similar manner, we treated C33A and PC3 cells with phosphatase inhibitors calyculin A and okadaic acid.

We found that 5 μM calyculin A, an inhibitor of both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), completely blocked the emetine effects on *Bcl-x* alternative splicing in both C33A and PC3 cells (Figure 5, compare +emetine with +emetine/calyculin A). To establish whether PP1 or PP2A was the emetine-responsive protein phosphatase that regulates *Bcl-x* alternative splicing, C33A and PC3 cells were pretreated for 1 hr with 5 μM okadaic acid, a selective PP2A inhibitor. Pretreatment with okadaic acid had no effect on *Bcl-x* alternative splicing (Figure 5, compare +emetine with +emetine/okadaic acid). Taken together, these results suggest that PP1 mediates the effects of emetine on the alternative splicing of *Bcl-x*.

DISCUSSION

Bcl-x belongs to the Bcl-2 family and plays an important role in apoptosis. *Bcl-x* produces anti-apoptotic Bcl-xL and proapoptotic Bcl-xS via alternative splicing of exon 2 (Figure 2A). It has been suggested that the expression of Bcl-xL in tumor cells is one of the important indicators of chemotherapeutic efficacy because Bcl-xL protects cells from a wide variety of apoptotic stimuli and confers a multidrug resistance phenotype [33]. In contrast, the smaller form, Bcl-xS, sensitizes cells to cell death inducers. These data indicate that manipulating levels of Bcl-xL and Bcl-xS proteins in tumors may provide a venue for cancer treatment with a combination of chemotherapeutic agents. Recent reports demonstrate that the alternative splicing of exon 2 in the *Bcl-x* gene can be altered for this purpose [20]. For instance, modifying the ratio of Bcl-xL to Bcl-xS in the cells with an antisense oligonucleotide allows cells to be sensitized to undergo apoptosis in response to ultraviolet B radiation and chemotherapeutic agent treatment [34]. On the other hand, downregulation of *Bcl-xL* by RNA interference was shown to suppress cell growth and induce apoptosis in human esophageal cancer cells [35]. In chemotherapy-resistant human colon cancer cells, *Bcl-xL* small interfering RNA was found to suppress cell proliferation. Therefore, *Bcl- xL* downregulation might provide a new target for human chemotherapy-resistant cancer therapy [36].

Emetine is a potent inhibitor of protein synthesis in mammalian cells, plants, and yeast [37]. It has been shown that (−)-emetine is an effective chemotherapeutic agent by increasing the life span in tumor-bearing mice [38, 39] and thus has possibilities for clinical advantage [40]. To this end, emetine has been evaluated in phase II clinical studies as a potential chemotherapeutic agent for the treatment of solid tumors [41]. However, to our knowledge, no previous study has explained the mechanism responsible for the antitumor effect of emetine. In this report, we demonstrate that emetine regulates alternative splicing of exon 2 in the *Bcl-x* gene, resulting in more production of proapoptotic *Bcl-xS* with a concomitant decrease in anti-apoptotic *Bcl-xL*, leading to our speculation that regulating *Bcl-x* splicing is one of the underlying mechanisms of the antitumor effect of emetine. Because several other protein synthesis inhibitors including anisomycin and puromycin in the 1040 FDA-approved drugs/compounds did not alter the splicing of *Bcl-x*, we reason that inhibition of new protein synthesis is unlikely the mechanism of action for emetine on splicing. To

further test this hypothesis, it will be crucial in the future to investigate emetine analogs that do not inhibit protein synthesis on the splicing of *Bcl-x*.

This defined mechanism was also shown to be dependent on protein phosphatase 1 activation. This conclusion was based on the use of the potent inhibitors of serine/threonine-protein phosphatases, okadaic acid, and calyculin A. Calyculin A, which inhibits both PP1 and PP2A, completely blocked emetine-induced alternative splicing of *Bcl-x*, whereas okadaic acid, a specific inhibitor of PP2A, had no effect on emetine-induced alternative splicing of *Bcl-x* (Figure 5). This therefore implies that the mechanism is dependent on PP1 activation. With the demonstration of PP1 as an emetine-activated protein phosphatase, potential PP1 substrates and mechanisms regulated by PP1 became candidate targets for emetine action. These findings are significant for several reasons. First, this mechanism of emetine-induced alternative splicing defines a mechanism of controlling the gene expression of proapoptotic factors in response to extracellular inducing agents. Second, a specific and direct mechanism mediated by an emetine-activated protein phosphatase has been established. Interestingly, the mechanistic action of emetine is similar to what has been described for ceramide, a small molecule that also induces, via alternative splicing, the expression of the proapoptotic splice variant Bcl-xS, with a concomitant loss in the anti-apoptotic splice variant Bcl-xL [25]. It appears that ceramide affects phosphorylation of SR proteins, a conserved family of serine/arginine-rich (SR) splicing factors which are involved in regulating splicing of eukaryotic mRNA. Therefore, it would be important to examine whether and how emetine affects splicing factors and whether ceramide is generated downstream of emetine. Moreover, it is also possible that emetine selectively affects the stability of either *Bcl-xL* or *Bcl-xS* mRNA, resulting in the change of the *Bcl-xL*/*Bcl-xS* ratio. This should be explored in the future. Finally, although cells treated with emetine at low concentrations do not undergo apoptosis, we observed that these cells are sensitized to high concentrations of emetine and to other death inducers (Figure S2), implying that regulation of the *Bcl-xL*/*Bcl-xS* ratio may indeed affect cell survival.

In summary, we demonstrate that emetine reduces the expression of the cell survival factor Bcl-xL and increases the expression of proapoptotic factor Bcl-xS in MCF-7 breast cancer cells, PC3 prostate cancer cells, A549 lung cancer cells, and C33A cervical carcinoma cells. Further study is needed to examine whether phosphorylation of SR proteins is regulated by emetine-activated protein phosphatase 1 and what the consequences are of the dephosphorylation of SR proteins. Does this effect contribute to the apoptosis process? What is the critical percentage of *Bcl-x* splicing that determines chemotherapeutic sensitivity? How much *Bcl-xL* needs to be spliced to *Bcl-xS* in order to allow cells to become susceptible to chemotherapy? These studies will have direct relevance to chemotherapeutic sensitivity because specific control of the alternative splicing of *Bcl-x* is linked to the sensitization of cells to chemotherapeutic agents, initiating a new target for anticancer treatment.

SIGNIFICANCE

Emetine is a crystalline alkaloid, $C_{29}H_{40}N_2O_4$, derived from ipecac root. It is a potent protein synthesis inhibitor and is clinically used in the treatment of protozoan infection. Emetine has shown promise as an antitumor agent without bone marrow suppression.

In the present study, we have examined the effect of emetine on the alternative pre-mRNA processing of *Bcl-x*. We demonstrate that emetine downregulates the levels of *Bcl-xL* mRNA with a concomitant increase in the mRNA levels of *Bcl-xS* in a dose-and time-dependent manner. Mechanistically, emetine-induced alternative splicing was dependent on the activation of protein phosphatase 1. This conclusion is based on the use of the potent inhibitors of serine/threonine-protein phosphatases, okadaic acid, and calyculin A. To our knowledge, this is the first report on the regulation of RNA splicing of members of the *Bcl-2* family in response to emetine. This significant finding may have direct relevance to chemotherapeutic sensitivity, giving rise to a new target for anticancer therapies. Future study is needed to confirm whether chemosensitivity is associated with increased *Bcl-x* splicing and whether SR proteins are dephosphorylated by PP1-mediated action of emetine.

EXPERIMENTAL PROCEDURES

Compounds

All chemicals including emetine, cycloheximide, calyculin A, and okadaic acid were purchased from Sigma.

Cell Culture

Human cervical carcinoma C33A cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% (v/v) fetal bovine serum, L-glutamine, and penicillin-streptomycin. PC3 prostate cancer cells were cultured in RPMI media supplemented with 10% (v/v) fetal bovine serum, L-glutamine, and penicillin-streptomycin. Adenocarcinoma 549 lung cancer cells were grown in DMEM/nutrient mixture F-12 (Ham) supplemented with 10% (v/v) fetal bovine serum, L-glutamine, and penicillin-streptomycin. Human breast cancer cells MCF-7 and MCF-7/Adr were cultured in RPMI media supplemented with 10% (v/v) fetal bovine serum, L-glutamine, and penicillin-streptomycin. All cells were maintained at less than 80% confluence under standard incubator conditions.

Emetine Treatment

Emetine dihydrochloride hydrate with the stock solution concentration of 100 μM was used. Twenty-four hours prior to emetine treatment, cells were plated in 2 ml of medium in 6-well plates at a density of 200,000 cells/well. The cells were treated with different concentrations of emetine for 24 hr for dosage-dependence study. For time-course experiments, cells were treated with 1.0 μM emetine for various durations.

Protein Phosphatase Inhibitor Treatment

Cells were pretreated with calyculin A or okadaic acid for 1 hr. The media were removed. Fresh regular media with emetine were added

to treat cells for the duration of 24 hr. RT-PCR was then carried out to examine *Bcl-x* splicing.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out with 1 μg of total RNA using Improm II reverse transcriptase (Promega) and oligo (dT) as the priming agent. After 1 hr incubation at 42°C, the reactions were terminated by heating at 70°C for 15 min. To analyze alternative splicing of exon 2 in the *Bcl-x* gene, an upstream 5′ primer to *Bcl-x* (5′-GAGGCAGGCGACGAGTTTGAA-3′) and a downstream 3′ primer (5′-TGGGAGGGTAGAGTGGATGGT-3′) were used for PCR amplification (32 cycles, 94°C, 30 s; 55°C, 30 s; 72°C, 1 min) with Choice Taq Blue Mastermix (Denville). PCR products were separated and analyzed on agarose gels.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/12/1386/DC1/.

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