JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

JPET# 67561

Title Page

Selective Induction of Apoptosis by PBOX-6 in Leukemia Cells occurs via the JNK Dependent Phosphorylation and Inactivation of Bcl-2 and Bcl-$_{XL}$

MARGARET M MC GEE[1], LISA M GREENE[1], SUSAN LEDWIDGE[1], GIUSEPPE CAMPIANI[2], VITO NACCI[2], MARK LAWLER[3], D CLIVE WILLIAMS[1] and DANIELA M ZISTERER[1].

Department of Biochemistry, Trinity College, Dublin 2, Ireland

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

JPET# 67561

Running title page

Running title: PBOX-6 inactivates Bcl-2 and Bcl-$_{XL}$ in leukemia cells

Corresponding Author:

Daniela Zisterer, Department of Biochemistry, Trinity College, Dublin 2, Ireland.

Tel: 00 353 1 6081855; Fax: 00 353 1 6772400; Email: dzistrer@tcd.ie

Number of text pages	35
Number of tables	0
Number of figures	10
Number of references	40
Number of words in abstract	220
Number of words in introduction	750
Number of words in discussion	2020

Abbreviations: PBOX, pyrrolo-1,5-benzoxazepine; CML, chronic myelogenous leukemia; PBMC, peripheral blood mononuclear cell; MAP, mitogen-activated protein; JNK, c-Jun NH_2-terminal kinase; JIP, JNK-interacting protein; ATF-2, activating transcription factor-2; AIF, apoptosis-inducing factor; UV, ultra-violet; LDH, dehydrogenase; MLK: Mixed lineage kinase.

Recommended section assignment: Chemotherapy, Antibiotics & Gene Therapy

ABSTRACT

Overexpression of the Bcl-2 proto-oncogene in tumour cells confers resistance against chemotherapeutic drugs. In this study we describe how the novel pyrrolo-1,5-benzoxazepine compound, PBOX-6, selectively induces apoptosis in Bcl-2-overexpressing cancer cells whereas it shows no cytotoxic effect on normal peripheral blood mononuclear (PBMC) cells. PBOX-6 overcomes Bcl-2-mediated resistance to apoptosis in chronic myelogenous leukemia (CML) K562 cells by the time- and dose-dependent phosphorylation and inactivation of anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-$_{XL}$. PBOX-6 also induces Bcl-2 phosphorylation and apoptosis in wild type T leukemia CEM cells and cells overexpressing Bcl-2. This is in contrast to chemotherapeutic agents such as etoposide, actinomycin D and ultraviolet irradiation, whereby overexpression of Bcl-2 confers resistance against apoptosis. In addition, PBOX-6 induces Bcl-2 phosphorylation and apoptosis in wild type Jurkat acute lymphoblastic leukemia cells and cells overexpressing Bcl-2. However, Jurkat cells containing a Bcl-2 triple mutant, whereby the principal Bcl-2 phosphorylation sites are mutated to alanine, demonstrate resistance against Bcl-2 phosphorylation and apoptosis. PBOX-6 also induces the early and transient activation of JNK in CEM cells. Inhibition of JNK activity prevents Bcl-2 phosphorylation and apoptosis, implicating JNK in the upstream signalling pathway leading to Bcl-2 phosphorylation. Collectively, these findings identify Bcl-2 phosphorylation and inactivation as a critical step in the apoptotic pathway induced by PBOX-6 and highlight its potential as an effective antileukemic agent.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

Hematopoietic disorders such as leukemia and lymphoma represent a large portion of human malignancies and are often characterised by acquired genetic changes including the expression of anti-apoptotic genes. For example, chronic myeloid leukemia cells express the transforming oncogene, *bcr-abl*, which confers resistance against apoptosis. Although a number of Bcr-Abl substrates have been identified, the precise signalling events that elicit the anti-apoptotic effects of Bcr-Abl in CML cells remain unclear and the activation of individual pathways may depend on cell type (Amarante-Mendes et al., 1998; Deininger et al., 2000). For instance, it has been reported that Bcr-Abl acts through expression of anti-apoptotic proteins Bcl-2 and Bcl-$_{XL}$ (Sanchez-Garcia and Grutz, 1995; Oetzel et al., 2000). Members of the Bcl-2 family together with mitochondria and cysteine proteases are essential components of many intracellular signalling pathways although the mechanisms by which they do so remains uncertain.

Bcl-2, the prototypic member of the Bcl-2 family of proteins, was cloned as a result of the t(14; 18) chromosomal translocation in human follicular B cell lymphoma, which results in its over expression (Baell and Huang, 2002). At least 16 Bcl-2 homologues are found in humans, as defined by sequence similarity to some or all four Bcl-2 homology domains in Bcl-2, and the family comprises both pro- and anti-apoptotic regulators (Tsujimoto and Shimizu, 2000). Anti-apoptotic Bcl-2 proteins can protect cells from death induced by many stimuli suggesting that they control a crucial step in the final common pathway for cell death (Martin et al., 1995; Keogh et al., 2000), however, the molecular mechanism involved remains incompletely defined. A striking feature of Bcl-2 proteins is their ability to form homodimers and heterodimers and heterodimerisation between opposing family members is thought to inhibit the biological activity of their partners (Sattler et al., 1997). In addition to heterodimerisation, alternative independent mechanisms of cell death regulation also exist including inhibition of caspase activity (Chinnaiyan et al., 1996) and the release of mitochondrial proteins such as cytochrome C and AIF (Susin et

al., 1999). The pro-apoptotic Bcl-2 family member, Bax, normally resides in the cytoplasm and translocates to the mitochondria where it undergoes oligomerization and/or a conformational change in response to apoptotic stimuli (Nouraini et al., 2000; Nomura et al., 1999). Bcl-2 counteracts the pro-apoptotic effect of Bax in some systems by preventing its translocation to the mitochondrial membrane (Mikhailov et al., 2001). In addition, emerging data implies that Bcl-2 proteins are targets for phosphorylation suggesting another mechanism of control although the functional significance of phosphorylation remains controversial (Tsujimoto, 1998). Phosphorylation of Bcl-2 occurs within an unstructured loop and involves several sites, the principal sites being serine 70, serine 87 and threonine 69 (Yamamoto et al., 1999; Srivastava et al., 1999). In some instances, phosphorylation of Bcl-2 results in its inactivation, rendering the cells more sensitive to the induction of apoptosis, whereas other reports suggest that Bcl-2 phosphorylation can enhance its anti-apoptotic effect (Ruvolo et al., 1998).

Much interest has focussed on the specific signalling pathway leading to Bcl-2 phosphorylation during drug-induced apoptosis. A family of mitogen-activated protein (MAP) kinase signalling proteins transduce signals from the cell membrane to the nucleus during apoptosis. One family member, known as c-jun NH_2-terminal kinase (JNK), becomes activated following its phosphorylation by a variety of upstream kinases including MEKK and members of the mixed-lineage protein kinase (MLK) family (Xu et al., 2001). Activated JNK phosphorylates downstream substrates such as ATF-2, c-jun and members of the Bcl-2 family, thereby regulating their expression (Barr and Bogoyevitch, 2001; Fan et al., 2000).

We have previously described how some members of a novel series of pyrrolo-1,5-benzoxazepine compounds potently induce apoptosis in a variety of cancerous cells including chemotherapy resistant CML cells, and the human breast carcinoma, MCF-7 cells, indicating their potential in the treatment of both diffuse and solid tumours (Zisterer

et al., 2000; Mc Gee et al., 2002a) We have shown that PBOX-6 induces apoptosis in K562 cells in a mechanism that bypasses the apoptotic suppressor Bcr-Abl (Mc Gee et al., 2001) We have shown that JNK activation is essential during PBOX-6-induced apoptosis, whereas the activation of caspases is dispensable (Mc Gee et al., 2002b). In this study, we show that PBOX-6 targets anti-apoptotic Bcl-2 proteins resulting in their phosphorylation and inactivation leading to the induction of apoptosis in resistant tumour cells, whereas it has no cytotoxic effect on normal peripheral blood mononuclear cells (PBMC's). Furthermore, we confirm the involvement of JNK in the signalling pathway leading to Bcl-2 phosphorylation and we have identified further potential targets for therapeutic intervention.

Methods

Materials. RPMI-1640, L-glutamine, fetal calf serum and gentamicin solutions were obtained from Sigma (Poole, Dorset, UK). The pyrrolobenzoxazepine 7-[{dimethylcarbamoyl}oxy]-6-(2-naphthyl)pyrrolo-[2,1-*d*] (1,5)-benzoxazepine (PBOX-6) was synthesised as previously described (Zisterer et al., 2000). The JNK inhibitor CEP11004 was generously provided by Cephalon, Inc. (West Chester, PA 19380, USA). Dicoumarol, Etoposide, Actinomycin D and MTT were all purchased from Sigma. Anti-Bcl-2 and anti-JNK antibodies were purchased from Santa Cruz (California, USA), anti-Bcl-$_{XL}$ antibody was purchased from Pharmingen and anti-Bax antibody was purchased from Transduction Laboratories. The phospho-specific JNK antibody and GST-c-Jun substrate were purchased from New England Biolabs (Hertfordshire, UK). Lymphoprep was purchased from Unitech. The RapiDiff kit was obtained from Diagnostic Development (Burscough, Lancashire, UK) and the Lactate Dehydrogenase kit was obtained from Promega (Madison, USA). The enhanced chemiluminesence reagent and the [γ-^{32}P] ATP were obtained from Amersham Biosciences (Aylesbury, UK).

Cell Lines. K562 cells were described previously (Mc Gee et al., 2001). CEM-Neo and CEM-Bcl-2 cells were generously supplied by Professor Seamus Martin (Department of Genetics, Trinity College Dublin, Ireland). Jurkat-Neo, Jurkat-WT Bcl-2 and Jurkat AA/A Bcl-2 cells were a kind gift from Professor Stanley Korsmeyer (Harvard Medical School and Dana-Farber Cancer Institute, Boston).

Cell Culture and Apoptosis Assay. Cells were grown in RPMI-1640 medium supplemented with fetal calf serum (10%), gentamicin (0.1mg/ml) and L-glutamine (2mM). Cells were maintained at 37^{o}C in a humidified incubator with 95% air and 5% CO_2. The

apoptosis assay was performed as described previously (Mc Gee et al., 2002b). Briefly, cells were seeded at $3x10^5$ cells/ml and following the relevant treatment, an aliquot of cells (150µl) was cytocentrifuged onto a glass slide and stained using the RapiDiff kit as described by the manufacturer. The degree of apoptosis and necrosis was determined by counting ~300 cells under a light microscope. At least three fields of view per slide, with an average of ~100 cells per field, were counted and the percent apoptosis and necrosis was calculated.

Normal Peripheral Blood Mononuclear Cells (PBMC's). Peripheral blood was obtained with informed consent from normal volunteers, in syringes containing preservative-free heparin, diluted 1:2 with sterile PBS, and layered over a cushion of 15 ml of lymphoprep in sterile 50ml centrifuge tubes. After centrifugation for 30 min at 400 g at room temperature, the interface layer, consisting of mononuclear cells, was extracted with a sterile Pasteur pipette and diluted in fresh RPMI 1640 medium containing 10 % FCS. Trypan blue dye exclusion was used to check viability. Only samples containing greater than 90 % viable cells were included in the study.

MTT Assay. The cytotoxic activity of PBOX-6 on normal PBMC's was measured using the MTT dye-reduction assay. The assay is based on the principle that only viable cells are able to reduce MTT to a colored formazan product that can be determined spectrophotometrically (Pieters). Optical density is linearly related to the number of viable cells. Unfractionated PBMC's (100µl) consisting of a mixed population of mononuclear cell types including lymphocytes, monocytes and natural killer cells, were seeded in 96-well plates at a density of $2x10^6$ cells/ml. Cells were treated with either medium alone, vehicle (1% (v/v) ethanol) or PBOX6 (10µM) for 48 h. Following treatment, MTT-solution

(5mg/ml in PBS) was added (20µl/well) and plates were incubated for a further 3 h at 37^{o}C. The purple formazan crystals were dissolved in 200µl of DMSO. After 20 min, the plates were read on an automated microplate spectrophotometer (Molecular Devices) at 595nm, reference filter 690nm. Assays were performed in triplicate on 3 independent blood donors. Complete medium (100µl), MTT stock (20µl) and DMSO (200µl) was used as a blank solution. Statistical analysis was performed using a two-tailed Student *t* test.

JNK Kinase Assay. JNK activity was measured by immunocomplex assay with anti-JNK1 antibody and GST-c-Jun as a substrate. Cells (6x10^{6}) were lysed in 400µl IP buffer (50mM Hepes, pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 0.1% (w/v) Tween 20, 1mM dithiothreitol, 1mM sodium fluoride) supplemented with 10µg/ml leupeptin, 10µg/ml aprotinin, 0.1mM phenylmethylsulfonyl fluoride and 0.1mM sodium orthovanadate. Protein concentration of the cell lysates was normalised to contain 300µg of protein and incubated for 3 h at 4^{o}C in the presence of anti-JNK antibody. Immune complexes were recovered with the aid of protein A Sepharose beads followed by two washes with IP buffer and one wash with kinase reaction buffer (50mM Hepes, pH 7.5, 10mM $MgCl_2$, 2.5mM EGTA and 0.1mM dithiothreitol, 0.1mM sodium orthovanadate). A kinase reaction was performed by resuspending the beads with 30µl kinase reaction buffer containing 2.5µg GST-c-jun, 20µM ATP and 2µCi of [γ-^{32}P] ATP, for 30 min at room temperature. The reaction was terminated by boiling the samples in Laemmli sample buffer for 3 min. Proteins were resolved by SDS-PAGE and the incorporation of [^{32}P] was visualised by autoradiography.

Subcellular Fractionation of Cells. Cells (2x10^{6}) were collected by centrifugation at 500g for 5 min and resuspended in isotonic lysis buffer (100µl) (200mM mannitol,

70mM sucrose, 1mM EGTA and 10mM Hepes, pH 6.9). Cells were ruptured by drawing repeatedly through a 21G needle followed by incubation on ice for 10 min. Unbroken cells, nuclei and heavy membranes were separated by centrifugation at 1000g for 5 min. The supernatant was centrifuged at 12,000g for 20 min at 4^{o}C to produce cytosolic and mitochondrial enriched fractions. The supernatant was collected and represents the cytosolic fraction. The mitochondria-enriched pellet was washed once with isotonic lysis buffer (200µl) followed by centrifugation at 12,000g for 5 min and resuspended in hypotonic lysis buffer (20µl) (10mM Tris-Cl, pH7.5, 5mM $MgCl_2$, 1mM EGTA and 1mM dithiothreitol) supplemented with protease inhibitors (5µg/ml pepstatin A, 2µg/ml leupeptin, 2µg/ml aprotinin) and the protein concentration was determined using a Bradford assay.

Western Blotting. Cells ($2x10^6$) were collected by centrifugation at 500g for 5 min and whole cell lysates were prepared by resuspending the pellet in SDS Lysis buffer (100µl) (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 50 mM DTT, 0.1 % (w/v) bromophenol blue). Extracts were sonicated for 15 sec and heated to 95^{o}C for 5 min. An equal amount of sample (20µl) was resolved on an SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. The membrane was blocked overnight at 4^{o}C in TBS containing 5% (w/v) dried milk followed by incubation with primary antibody for 1 h at room temperature. After incubation, the membrane was washed with TBS containing 0.1% (w/v) Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and enhanced chemiluminesence was used to visualise the proteins.

Lactate Dehydrogenase (LDH) Assay. The assay was performed under the conditions described by the manufacturer.

Results

PBOX-6 Induces Apoptosis in CEM-Bcl-2 Cells that are Resistant to Etoposide, Actinomycin D and UV irradiation. It has previously been reported that CEM cells transfected with empty vector (CEM-Neo) are susceptible to the induction of apoptosis by etoposide, actinomycin D and UV irradiation, however, overexpression of Bcl-2 in these cells (CEM-Bcl-2) confers resistance against apoptosis induced by these agents (Martin et al., 1995). We have verified that CEM-Bcl-2 cells overexpress Bcl-2 by Western blot analysis (Fig.1A). In agreement with Martin et al. (1995) we have found that CEM-Neo cells are susceptible to apoptosis induced by etoposide, actinomycin D and UV irradiation, whereas CEM-Bcl-2 cells are not (Fig.1B and C). In contrast, we have found that PBOX-6 induces apoptosis in both CEM-Neo and CEM-Bcl-2 cells (Fig.1B and C respectively). Apoptosis was induced with similar potency in CEM-Neo and CEM-Bcl-2 cells, and occured in a dose- and time-dependent manner following PBOX-6 treatment (Fig.2A and B). The morphological features of apoptosis, which includes cell shrinkage, chromatin condensation, nuclear fragmentation and the appearance of apoptotic bodies were absent from vehicle treated cells but were visible in CEM-Neo and CEM-Bcl-2 cells following treatment with PBOX-6 (Fig.2C). These results suggest that PBOX-6 can overcome Bcl-2-mediated resistance to apoptosis and highlights its potential in the treatment of tumour cells that overexpress Bcl-2.

Lack of Cytotoxicity Towards Normal PBMC's following PBOX-6 Treatment. To evaluate the effect of PBOX-6 on normal mononuclear cells, PBMC's were isolated from normal healthy volunteers and treated with either vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) up to 48 h. Cell viability was assessed using an MTT assay as well as by cytocentrifugation of the cells onto slides and examination of cell morphology. The MTT

assay measures the reduction of a tetrazolium salt by viable cells and absorbance is directly proportional to cell viability. Therefore, reduced cell viability due to apoptosis or necrosis is reflected by reduced absorbance.

An MTT assay was performed on PBMC's obtained from three volunteers. Statistical analysis of data obtained from all samples was carried out using the Instat computer program and it was found that PBOX-6 (10μM) did not display any significant cytotoxicity towards normal PBMC's up to 48 h after treatment. Results from a representative sample are shown in Fig 3A. RapiDiff staining of cells has shown that normal PBMC's remained healthy following treatment with PBOX-6 for 24h (data not shown). In agreement with this finding from the MTT assay, examination of PBMC morphology following treatment with ethanol or PBOX-6 for 48 h does not display any characteristic features of apoptosis or necrosis (Fig.3B and C respectively). Collectively these results illustrate the non-cytotoxic effect of PBOX-6 towards normal mononuclear cells and suggest that the apoptotic action demonstrated by PBOX-6 may be selective towards tumour cells.

PBOX-6 Induces Phosphorylation of Bcl-2 in K562 and CEM cells, Which Occurs in a Dose- and Time-dependent Manner. Phosphorylation of Bcl-2 has been shown to result in its inactivation (Fan et al., 2000). To investigate the mechanism by which PBOX-6 overcomes Bcl-2-mediated resistance to apoptosis, we examined the effect of the compound on Bcl-2 phosphorylation. Cells were treated with either a range (0.1-10μM) of PBOX-6 concentrations for 16 h or 10μM PBOX-6 for various lengths of time (0-16 h). Phosphorylation of Bcl-2 is detected by the presence of a slower migrating band by SDS-PAGE. Results from Western blotting show that PBOX-6 induces a dose- and time-dependent phosphorylation of Bcl-2 in K562 cells (Fig.4A and B respectively), CEM-

Neo cells (Fig.4C and D respectively) and CEM-Bcl-2 cells (Fig.4E and F respectively). In the three cell lines examined, Bcl-2 phosphorylation first becomes apparent at 1μM PBOX-6 and increases as drug concentration increases. In addition, Bcl-2 phosphorylation becomes visible following a 1 h treatment with PBOX-6 (10μM) and increases gradually up to 16 h. Collectively these results suggest that phosphorylation and inactivation of Bcl-2 may be part of the mechanism by which this novel compound overcomes Bcl-2-mediated resistance to apoptosis.

Jurkat-AA/A Bcl-2 Cells Demonstrate Resistance Against PBOX-6-induced Apoptosis and Bcl-2 Phosphorylation When Compared to Parental Cells. To investigate further the role of Bcl-2 phosphorylation following PBOX-6 treatment, we assessed its effect on Jurkat clones stably expressing empty vector (Jurkat-Neo), wild type Bcl-2 (Jurkat-WT Bcl-2) or a triple Bcl-2 mutant, whereby the phosphorylation sites serine 70, serine 87 and threonine 69 have been mutated to alanine (Jurkat-AA/A Bcl-2). It has been previously reported that Jurkat-AA/A Bcl-2 cells demonstrate increased resistance to Taxol-induced cell death compared to parental cells (Yamamoto et al., 1999). In agreement with this report we found that Jurkat-Neo and Jurkat-WT Bcl-2 cells undergo >70% cell death following treatment with Taxol (1μM) for 24 h, whereas significantly less death was induced in Jurkat-AA/A Bcl-2 cells (Fig.5A). Treatment of Jurkat-Neo and Jurkat-WT Bcl-2 cells with PBOX-6 (10μM) for 24 h induces approximately 75% cell death, however, the Jurkat-AA/A Bcl-2 cells display increased resistance against PBOX-6-induced apoptosis with > 50% protection observed following 24 h treatment (Fig.5B). This suggests that phosphorylation of Bcl-2 by PBOX-6 results in its inactivation. In addition, we assessed the ability of PBOX-6 to phosphorylate Bcl-2 in Jurkat cells expressing WT and mutant Bcl-2. It was found that PBOX-6 phosphorylates Bcl-2 in the Jurkat-Neo and

Jurkat-WT Bcl-2 cells however only a faint band indicative of endogenous Bcl-2 phosphorylation was present in the mutant Jurkat-AA/A Bcl-2 cells (Fig.5C). This suggests that PBOX-6-induced phosphorylation of Bcl-2 occurs within the unstructured loop on one or more of the mutated phosphorylation sites.

PBOX-6 Induces a Dose- and Time-dependent Phosphorylation of Bcl-$_{XL}$ in K562 cells. Upregulation of Bcl-$_{XL}$ has been implicated in the anti-apoptotic properties of CML cells (Amarante-Mendes et al., 1998) and its phosphorylation is believed to result in its inactivation (Halder et al., 1996). We assessed the effect of PBOX-6 on the phosphorylation status of Bcl-$_{XL}$ in K562 cells. Whole cell lysates were isolated from K562 cells following treatment with either a range (0.1-10μM) of PBOX-6 concentrations for 16 h, or 10μM PBOX-6 for various lengths of time (0-16 h) and the phosphorylation status of Bcl-$_{XL}$ was examined by Western blotting. Results reveal that PBOX-6 induces a dose- and time-dependent phosphorylation of Bcl-$_{XL}$, as shown by the presence of a slower migrating band by SDS-PAGE (Fig.6A and B respectively). The phosphorylation of Bcl-$_{XL}$ in K562 cells occurs at a later time than Bcl-2 phosphorylation. However, these results support the hypothesis that phosphorylation and inactivation of Bcl-$_{XL}$ may be part of the mechanism by which PBOX-6 induces apoptosis in chemotherapy resistant CML cells.

Expression Levels and Subcellular Localisation of Bax are Unaltered in K562 Cells Following PBOX-6 Treatment. It has been suggested that Bcl-2 prevents apoptosis by inhibiting the translocation of Bax from the cytosol into the mitochondria where it can carry out its pro-apoptotic function (Nomura et al., 1999). The hypothesis that inactivation of Bcl-2 and Bcl-$_{XL}$ by phosphorylation may allow mitochondrial translocation of Bax was investigated. Levels of Bax expression were monitored in mitochondrial and cytosolic

fractions from K562 cells prepared following treatment with either vehicle or PBOX-6 for 16 h. Equal amounts of protein were resolved by SDS-PAGE and levels of Bax expression were determined by Western blotting. In order to determine the purity of each fraction, Bcl-2 was measured as a mitochondrial marker as previously shown (Gross et al., 1998; Khaled et al., 1999). The cytosolic enzyme, lactate dehydrogenase (LDH) was used as a marker of the cytosolic fractions and was expressed as a percentage of the total enzyme activity measured from whole cell extracts. Results from Western blotting reveal that Bax is present in the cytosolic and mitochondrial fractions of vehicle treated cells, and treatment with PBOX-6 does not alter the distribution of Bax in these cells (Fig.7A). The membrane was stripped and re-probed with Bcl-2 and results show the absence of Bcl-2 from the cytosolic fraction indicating the purity of the fraction (Fig.7B). Furthermore, results from the LDH assay indicates that the majority of LDH activity remains in the cytosolic fraction whereas < 1% was detected in the mitochondrial fraction, indicating that extensive contamination of the mitochondrial fraction with cytosolic components did not occur (Fig.7C). These results suggest that Bax is present in both the cytosolic and mitochondrial fraction of K562 cells and this cannot be accounted for by cross contamination of subcellular fractions. However, the expression levels and subcellular localisation of Bax remained unchanged following PBOX-6 treatment.

Activation of JNK Occurs Upstream of Bcl-2 Phosphorylation in CEM-Neo and CEM-Bcl-2 Cells Following PBOX-6 Treatment. It has been reported that the JNK MAP kinase phosphorylates Bcl-2 following taxol-induced apoptosis in HL60 cells (Shiah et al., 2001). We have recently shown that activation of JNK is essential in the apoptotic pathway induced by PBOX-6 in K562 cells (Mc Gee et al., 2002b) In order to determine the sequence of events that occur during PBOX-6 induced apoptosis, JNK activity was assessed following PBOX-6 treatment of CEM cells. Using GST-c-jun as a JNK substrate,

a kinase assay was performed and results show that PBOX-6 induces the transient activation of JNK in CEM-Neo and CEM-Bcl-2 cells (Fig.8A and B respectively). JNK activation becomes visible following a 15 min treatment with PBOX-6 in the two cell lines. The early activation of JNK following PBOX-6 treatment of CEM cells is similar to data obtained earlier using K562 cells (Mc Gee et al., 2002b) and suggests that JNK activation occurs upstream of Bcl-2 phosphorylation and therefore may be an important intermediate leading to inactivation of Bcl-2.

The JNK Inhibitors, Dicoumarol and CEP-11004, Prevent PBOX-6-induced Apoptosis in K562, CEM and Jurkat cells. The quinone reductase inhibitor, dicoumarol, specifically inhibits activation of JNK in response to a variety of stimuli (Cross et al., 1999; Krause et al., 2001). In agreement with these studies, we have previously shown that dicoumarol abolishes PBOX-6-induced JNK activation and apoptosis in K562 cells outlining the importance of JNK in the signalling pathway induced (Mc Gee et al., 2002b). In the present study we have found that dicoumarol blocks PBOX-6-induced apoptosis in CEM-Bcl-2 and Jurkat-WT Bcl-2 cells demonstrating that JNK activation plays an equally important role during apoptosis in these cells (Fig.9A). An alternative JNK inhibitor, CEP-11004, has been shown to inhibit JNK signalling by directly targeting and inhibiting members of the MLK family (Murakata et al., 2002). In agreement with this, using an *in vitro* kinase assay we have found that pretreatment of K562 cells with CEP-11004 (5μM) completely inhibits PBOX-6-induced JNK activation (Fig.9B). In addition, we have found that pretreatment of K562, CEM-Bcl-2 and Jurkat-WT Bcl-2 with CEP-11004 (5μM) for 1 hr prior to treatment with PBOX-6 (10μM) for a further 16 h completely inhibits PBOX-6-induced apoptosis (Fig.9C). The ability of the two JNK inhibitors to block PBOX-6-

induced apoptosis in the three cell lines further emphasises the importance of JNK activation during PBOX-6-induced apoptosis.

Dicoumarol and CEP-11004 Prevent Bcl-2 Phosphorylation Induced by PBOX-6. To investigate whether JNK activation leads to Bcl-2 phosphorylation, the effect of dicoumarol and CEP-11004 on the phosphorylation status of Bcl-2 was assessed. Results show that pretreatment of K562, CEM-Bcl-2 and Jurkat-WT Bcl-2 cells with dicoumarol (200μM) or CEP-11004 (5μM) for 1 h prior to treatment with PBOX-6 (10μM) for a further 16 h, completely blocks PBOX-6-induced phosphorylation of Bcl-2 (Fig.10 A and B respectively). These results suggest that phosphorylation of Bcl-2 occurs as a direct consequence of JNK activation in these cells.

Discussion

A characteristic feature of greater than 80% of human malignancies is overexpression of the Bcl-2 proto-oncogene, which confers resistance against cell death induced by a range of cytotoxic agents. In the present study we report that a representative compound from a novel series of pyrrolo-1,5-benzoxazepine compounds, PBOX-6, potently induces apoptosis in a variety of Bcl-2-overexpressing tumour cells while showing no cytotoxic effects on normal peripheral blood mononuclear cells. We have identified the inactivation of Bcl-2 as the critical step by which PBOX-6 induces apoptosis in chemotherapy resistant tumour cells, and we have uncovered components of the signalling pathway involved. Firstly, we assessed the effect of PBOX-6 on wild type CEM cells (CEM-Neo) and cells overexpressing Bcl-2 (CEM-Bcl-2). Martin et al. (1995) has previously shown that overexpression of Bcl-2 in these cells confers resistance against apoptosis induced by Etoposide, Actinomycin D and UV irradiation and we have confirmed these findings. However, in contrast to these agents, we found that PBOX-6 induces apoptosis in CEM-Neo and CEM-Bcl-2 cells with similar potency. The induction of apoptosis, which occurs in a dose- and time-dependent manner, suggests that PBOX-6 can overcome Bcl-2-mediated resistance to apoptosis. The morphological criteria associated with apoptosis, which include cell shrinkage, chromatin condensation, nuclear fragmentation and the appearance of apoptotic bodies was visible in the two cell lines following PBOX-6 treatment. In direct contrast to data obtained using tumour cells, it was found that an equivalent concentration of PBOX-6 has no cytotoxic effect on normal PBMC's isolated from healthy volunteers. Induction of apoptosis in tumour cells was detected following a 4-hour treatment with PBOX-6, however, it has no effect on normal PBMC's up to 48 hours post treatment. This data illustrates the ability of PBOX-6 to

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

selectively induce apoptosis in tumour cells and highlights its potential in the treatment of cancer.

The mechanism by which anti-apoptotic Bcl-2 proteins exert their effect remains uncertain and a number of hypotheses have been proposed. These include dimer formation between opposing family members, prevention of Bax translocation and inhibition of mitochondrial events. Post-translational modification of Bcl-2 proteins, such as proteolysis and phosphorylation, also control their function although the functional significance of these modifications remains controversial. For example, the chemotherapeutic drug, Taxol, induces Bcl-2 phosphorylation and apoptosis in lymphoid cells and prostate cancer cells (Halder et al., 1995; 1996), whereas phosphorylation of Bcl-2 by protein kinase C α enhances its anti-apoptotic function and it has been suggested that the role of Bcl-2 phosphorylation may depend on the phosphorylation site (Ruvolo et al., 1998).

CML cells express the chimeric oncogene, *bcr-abl*, and show elevated levels of Bcl-2 and Bcl-$_{XL}$, which may be responsible for their anti-apoptotic properties (Amarante-Mendes et al., 1998; Salomoni et al., 2000). We have previously reported that PBOX-6 induces apoptosis in the K562 CML cell line, in a pathway that bypasses the apoptotic suppressor Bcr-Abl (Mc Gee et al., 2001). In this study we have found that PBOX-6 induces phosphorylation of Bcl-2 and apoptosis in K562, CEM-Neo and CEM-Bcl-2 cells. Bcl-2 phosphorylation occurs in a dose- and time-dependent manner and this finding is consistent with others who report Bcl-2 inactivation by phosphorylation.

In addition to Bcl-2, Bcl-$_{XL}$ has been implicated in the anti-apoptotic phenotype of Bcr-Abl-positive K562 cells. The Bcr-Abl tyrosine kinase inhibitor, CGP57148, better known as STI571, causes down regulation of Bcl-$_{XL}$ and apoptosis in K562 cells (Oetzel et al., 2000). In contrast to this, we found that PBOX-6 induces a dose- and time-dependent phosphorylation of Bcl-$_{XL}$ in K562 cells. Phosphorylation of Bcl-$_{XL}$ occurs at a later time than Bcl-2 phosphorylation in K562 cells and closely parallels the induction of apoptosis

previously reported in these cells following PBOX-6 treatment (Mc Gee et al., 2001). In agreement with others, these findings support a role of Bcl-2 and Bcl-$_{XL}$ in the anti-apoptotic properties of K562 cells and suggest that phosphorylation and inactivation of anti-apoptotic Bcl-2 proteins may be part of the apoptotic pathway induced by PBOX-6.

To further investigate the role of Bcl-2 phosphorylation following PBOX-6 treatment, we assessed its effect on Jurkat clones stably expressing empty vector, wild type Bcl-2 or a triple Bcl-2 mutant whereby the primary phosphorylation sites, Ser70, Ser87 and Th69, have been mutated to alanine. It has been found that while PBOX-6 potently induces apoptosis in Jurkat-Neo and Jurkat-WT Bcl-2 cells, the mutant Bcl-2 expressing cells demonstrate increased resistance against PBOX-6-induced apoptosis. This finding further suggests that phosphorylation of Bcl-2 following PBOX-6 treatment results in its inactivation and is in agreement with Yamamoto et al. (1999) who reported that Jurkat cells expressing the triple Bcl-2 mutant were more resistant to cell death induced by Taxol, which we have also verified. Furthermore, it was found that PBOX-6 phosphorylates Bcl-2 in Jurkat-WT Bcl-2 cells, however, Bcl-2 phosphorylation of the Bcl-2 triple mutant cells was greatly reduced. A faint band indicative of phosphorylated Bcl-2 was present in the Jurkat-Neo and mutant cells, which represents phosphorylation of endogenous Bcl-2. This data suggests that phosphorylation of Bcl-2 by PBOX-6 occurs within the unstructured loop region on one or more of the mutated phosphorylation sites. A small extent of cell death was detected in the Bcl-2 triple mutant cells following PBOX-6 treatment. This finding, which has also been reported by others (Yamamoto et al., 1999), may suggest that phosphorylation at Ser70, Ser87 and Th69 is not sufficient to antagonize all the anti-apoptotic function of Bcl-2 and that PBOX-6 may induce modifications beyond phosphorylation. Collectively, these findings indicate that the phosphorylation and inactivation of Bcl-2 may be a critical upstream event leading to the induction of apoptosis by PBOX-6 in chemotherapy resistant tumour cells.

A recent review suggests that Bcl-2 phosphorylation cannot accelerate cell death in apoptosis-resistant cell lines (Blagosklonny, 2001). In direct contrast to this, these results show that phosphorylation of Bcl-2 by PBOX-6 leads to the induction of apoptosis in a number of chemotherapy resistant cell lines. Bcl-2 was phosphorylated on two residues in some instances, whereas we could only detect phosphorylation on one residue in other instances. This may be due to poorer resolution upon development of the Western blot, or the phosphorylation state of Bcl-2 may depend on the level of upstream kinase activation. However, these findings are consistent with others who report the phosphorylation of Bcl-2 on one or more residues. For instance, up to five distinct bands were visible by Western blotting following Bcl-2 phosphorylation in COS cells (Maundrell et al., 1997). In addition, we did not detect any evidence of Bcl-2 degradation following phosphorylation by PBOX-6.

Bcl-2 normally resides in various subcellular locations such as endoplasmic reticulum, mitochondrial and nuclear membranes (Blagosklonny, 2001). From subcellular fractionation studies we found that PBOX-6 phosphorylates mitochondrial Bcl-2. The unphosphorylated Bcl-2 present on Western blots from whole cell lysates may represent Bcl-2 at other subcellular locations that is not phosphorylated by PBOX-6. This hypothesis is supported by findings from IL-3-induced phosphorylation, where phosphorylation only affects a minor pool of soluble Bcl-2 and is not visible by immunoblotting (Blagosklonny, 2001).

One proposed mechanism of Bcl-2 function is the inhibition of Bax translocation from the cytosol to the mitochondria where is carries out it pro-apoptotic function (Nomura et al., 1999; Gross et al., 1998). We assessed the effect of PBOX-6 on the subcellular localisation of Bax in K562 cells. In agreement with previous reports (Amarante-Mendes et al., 1998) it was found that Bax resides in both the cytosolic and mitochondrial fraction of viable cells. Levels of Bax expression in the two subcellular fractions remained unchanged

following PBOX-6 treatment. In a manner similar to Fadeel et al. (1999) and others, the purity of the various fractions was confirmed by measuring the activity of the cytoplasmic enzyme, lactate dehydrogenase (LDH), as a cytosolic marker. In addition, Western blotting was used to determine levels of cross contamination of subcellular fractions. Bcl-2 was detected in its phosphorylated form, in the mitochondrial fraction following PBOX-6 treatment, but was absent from the cytosolic fraction. These results demonstrate that the anti-apoptotic effects of Bcl-2 and Bcl-$_{XL}$ in K562 cells are not exerted through their inhibition of Bax translocation. Alternative mechanisms exist whereby anti-apoptotic Bcl-2 and Bcl-$_{XL}$ exert their function such as their ability to dimerise with Bax and repress cell death (Yang et al., 1995). It has been shown that inactivation of Bcl-2 and Bcl-$_{XL}$ by phosphorylation, following Taxol treatment of PC3 prostate cancer cells, reduces the formation of Bax heterodimers, and increases the formation of Bax-Bax homodimers, thus inducing apoptosis (Halder et al., 1996). We found that Bax is present in the mitochondrial fraction of untreated K562 cells, therefore, it is possible that phosphorylation of Bcl-2 and Bcl-$_{XL}$ by PBOX-6 alters the dimerisation properties of Bax at this location, thus promoting apoptosis. Further studies will be carried out to investigate this hypothesis.

The stress-activated kinase, JNK, has been identified as an upstream kinase responsible for phosphorylation of Bcl-2 in some instances (Fan et al., 2000). We have previously shown that JNK becomes activated in K562 cells following PBOX-6 treatment. Inhibition of JNK using the JIP-1 signalling inhibitor and dicoumarol abolished PBOX-6-induced activation of JNK and apoptosis, outlining an essential role of JNK in the apoptotic pathway induced by PBOX-6 in CML cells (Mc Gee et al., 2002b). In this study we found that PBOX-6 induces the transient activation of JNK in CEM-Neo and CEM-Bcl-2, suggesting that a similar apoptotic pathway is triggered in these cells. The early activation of JNK, which occurs within 15 minutes, implicates it in the upstream pathway leading to Bcl-2 phosphorylation. In addition, the two JNK inhibitors, dicoumarol and CEP-11004,

inhibit Bcl-2 phosphorylation and apoptosis in K562, CEM and Jurkat cells, providing further evidence that JNK activation occurs upstream of Bcl-2 phosphorylation. These findings are in agreement with others who report JNK as an upstream kinase responsible for the phosphorylation of Bcl-2 family members. For instance, inhibition of JNK using antisense oligonucleotides prevented the phosphorylation of Bcl-2 and Bcl-$_{XL}$ in KB-3 human carcinoma cells following treatment with vinblastine (Barr et al., 2001). In direct contrast to this, some stimuli that activate JNK such as DNA damaging agents, fail to phosphorylate Bcl-2 (Blagoskloony et al., 1997). These conflicting reports suggest that under certain cellular conditions substrate accessibility may be a key factor enabling JNK to phosphorylate Bcl-2. The indolocarbazole analogue, CEP-11004, inhibits JNK signalling by directly targeting and inhibiting members of the upstream MLK family. CEP-11004 has been shown to prevent JNK activation and apoptotic neuronal cell death in rat pheochromocytoma PC12 cells, primary cortical neurons and human SH-SY5Y cells following treatment with a variety of apoptotic stimuli (Murakata et al., 2002). In addition, CEP-11004 protects against LPS-induced activation of JNK and c-jun in microglia cultivated from neonatal rats (Hidding et al., 2002). In agreement with these reports, we have found that CEP-11004 prevents JNK activation and apoptosis in K562 cells following PBOX-6 treatment, which correlates with results obtained previously using JIP-1 and Dicoumarol (Mc Gee et al., 2002b). This finding suggests that members of the MLK family may be part of the upstream signalling pathway leading to JNK activation by PBOX-6 and further aids our understanding of the mechanism of action of this novel compound.

Identification of novel drug targets that may be exploited in therapeutic intervention is of high importance, however it is limited due to an incomplete knowledge of the core cell death machinery (Baell and Huang, 2002). Therefore, understanding the mechanism of apoptosis has important implications in the prevention and treatment of cancer. Although significant advances have been made in recent years following the treatment of CML

patients with the Abl tyrosine kinase inhibitor, STI571, the development of drug resistance remains a problem and highlights the continued need for the development of novel drugs with different mechanisms of action. We have previously reported that the novel pyrrolo-1,5-benzoxazepine compound, PBOX-6, induces apoptosis in Bcr-Abl-positive and Bcr-Abl-negative cells. In this study we have identified Bcl-2 as a critical target of PBOX-6. Phosphorylation and inactivation of Bcl-2 abrogates its protective effect in a number of tumour cells following PBOX-6 treatment. Importantly, the non-cytotoxic effect of PBOX-6 on normal PBMC's highlights its selectivity for tumour cells and its potential as a novel target-based drug for the treatment of cancer.

Acknowledgements

We would like to thank Professor Stanley Korsmeyer and Professor Seamus Martin for kindly donating cells used in this study. We also thank Professor Luke O' Neill (Trinity College) for advice and scientific discussion.

References

Amarante-Mendes GP, McGahon AJ, Nishioka WK, Afar DE, Witte ON and Green DR (1998) Bcl-2-independent Bcr-Abl-mediated resistance to apoptosis: protection is correlated with up regulation of Bcl-xL. *Oncogene* 16: 1383-1390.

Baell JB and Huang DCS (2002) Prospects for targeting the Bcl-2 family of proteins to develop novel cytotoxic drugs. *Biochem Pharmacol* 64: 851-863.

Barr RK and Bogoyevitch MA (2001) The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). *Int J of Biochem & Cell Biology* 33: 1047-1063.

Blagosklonny MV (2001) Unwinding the loop of Bcl-2 phosphorylation. *Leukemia* 15: 869-874.

Blagosklonny MV, Giannakakou P, El-Deiry WS, Kingston DG I, Higgs PI, Neckers L and Fojo T (1997) Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res* 57: 130-135.

Chinnaiyan AM, Orth K, O'Rourke K, Duan H, Poirier GG and Dixit VM. (1996) Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. *J Biol Chem* 271: 4573-4576.

Cross JV, Deak JC, Rich EA, Qian Y, Lewis M, Parrott LA, Mochida K, Gustafson D, Vande Pol S and Templeton DJ (1999) Quinone reductase inhibitors block SAPK/JNK and NFkappaB pathways and potentiate apoptosis. *J Biol Chem* 274: 31150-31154.

Deininger MW, Goldman JM and Melo JV (2000) The molecular biology of chronic myeloid leukemia. *Blood* 96: 3343-3356.

Fadeel B, Hassan Z, Hellstrom-Lindberg E, Henter JI, Orrenius S and Zhivotovsky B (1999) Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of

human myeloid leukemia cells. *Leukemia* 13: 719-728.

Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA and Chambers TC (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *J Biol Chem* 275: 29980-29985.

Gross A, Jockel J, Wei MC and Korsmeyer SJ (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *Embo* 17: 3878-3885.

Haldar S, Chintapalli J and Croce CM (1996) Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res* 56: 1253-1255.

Haldar S, Jena N and Croce CM (1995) Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci U S A* 92: 4507- 4511.

Hidding U, Mielke K, Waetzig V, Brecht S, Hanisch U, Behrens A, Wagner E and Herdegen T (2002) The c-Jun N-terminal kinases in cerebral microglia: immunological functions in the brain. *Biochem Pharmacol* 64: 781-788.

Keogh SA, Walczak H, Bouchier-Hayes L and Martin SJ (2000) Failure of Bcl-2 to block cytochrome c redistribution during TRAIL-induced apoptosis. *FEBS Lett* 471: 93-98.

Khaled AR, Kim K, Hofmeister R, Muegge K and Durum SK (1999) Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc Natl Acad Sci U S A* 96: 14476-14481.

Krause D, Lyons A, Fennelly C and O'Connor R (2001) Transient activation of Jun N-terminal kinases and protection from apoptosis by the insulin-like growth factor I receptor can be suppressed by dicumarol. *J Biol Chem* 276: 19244-19252.

Martin SJ, Takayama S, McGahon AJ, Miyashita T, Corbeil J, Kolesnick RN, Reed JC and Green DR (1995) *Cell Death and Diff* 2: 253-257.

Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C,

Boschert U, Vial-Knecht E, Martinou J.C and Arkinstall S (1997) Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J Biol Chem* 272: 25238-25242.

Mc Gee MM, Campiani G, Ramunno A, Fattorusso C, Nacci V, Lawler M, Williams DC and Zisterer DM (2001) Pyrrolo-1,5-benzoxazepines induce apoptosis in chronic myelogenous leukemia (CML) cells by bypassing the apoptotic suppressor bcr-abl. *J Pharmacol Exp Ther* 296: 31-40.

Mc Gee MM, Campiani G, Ramunno A, Nacci V, Lawler M, Williams DC and Zisterer DM (2002b) Activation of the c-Jun N-terminal kinase (JNK) signaling pathway is essential during PBOX-6-induced apoptosis in chronic myelogenous leukemia (CML) cells. *J Biol Chem* 277: 18383-18398.

Mc Gee MM, Hyland E, Campiani G, Ramunno A, Nacci V and Zisterer DM (2002a) Caspase-3 is not essential for DNA fragmentation in MCF-7 cells during apoptosis induced by the pyrrolo-1,5-benzoxazepine, PBOX-6. *FEBS Lett* 515: 66-70.

Mikhailov V, Milhailova M, Pulkrabek DJ, Dong Z, Venkatachalam MA and Saikumar P (2001) Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane *J Biol Chem* 276: 18361-18374.

Murakata C, Kaneko M, Gessner G, Angeles TS, Ator MA, O'Kane TM, McKenna BAW, Thomas BA, Mathiasen JR, Saporito MS, Bozyczko-Coyne D and Hudkins RL (2002) Mixed lineage kinase activity of indolocarbazole analogues. *Bioorganic & Medical Chem Lett* 12: 147-150.

Nomura M, Shimizu S, Ito T, Narita M, Matsuda H and Tsujimoto Y (1999) Apoptotic cytosol facilitates Bax translocation to mitochondria that involves cytosolic factor regulated by Bcl-2. *Cancer Res* 59: 5542-5548.

Nouraini S, Six E, Matsuyama S, Krajewski S, Reed JC (2000) The putative pore-forming

domain of Bax regulates mitochondrial localization and interaction with Bcl-X(L). *Mol Cell Biol* 20: 1604-1615.

Oetzel C, Jonuleit T, Gotz A, van der Kuip H, Michels H, Duyster J, Hallek M and Aulitzky WE (2000) The tyrosine kinase inhibitor CGP57148 (ST1 571) induces apoptosis in BCR-ABL-positive cells by down-regulating BCL-X. *Clin Cancer Res* 6:1958-1968.

Ruvolo PP, Xingming D, Boyd KC and Stratford May W (1998) A functional role for Mitochondrial Protein Kinase Cα in Bcl-2 phosphorylation and suppression of apoptosis. *J Biol Chem* 273: 25436-25442.

Salomoni P, Condorelli F, Sweeney SM and Calabretta B (2000) Versatility of BCR/ABL-expressing leukemic cells in circumventing proapoptotic BAD effects. *Blood* 96: 676-684.

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB and Fesik SW (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 275: 983-986.

Sanchez-Garcia I and Grutz G (1995) Tumorigenic activity of the Bcr-Abl oncogene is mediated by BCL-2. *Proc Natl Acad Sci USA* 92: 5287-5291.

Shiah S-G, Chuang S-E and Kuo M-L (2001) Involvement of Asp-Glu-Val-Asp-directed, caspase-mediated mitogen-activated protein kinase kinase 1 Cleavage, c-Jun N-terminal kinase activation, and subsequent Bcl-2 phosphorylation for paclitaxel-induced apoptosis in HL-60 cells. *Mol Pharmacol* 59: 254-262.

Srivastava RK, Mi QS, Hardwick JM and Longo DL (1999) Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc Natl Acad Sci U S A* 96: 3775-3780.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot

E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM and Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397: 441-446.

Tsujimoto Y (1998) Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes to Cells* 3: 697-707.

Tsujimoto Y and Shimizu S (2000) Bcl-2 family: life-or-death switch. *FEBS lett* 446: 6-10.

Xu Z, Maroney AC, Dobrzanski P, Kukekov NV and Greene LA (2001) The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol* 21: 4713-4724.

Yamamoto K, Ichijo H and Korsmeyer SJ (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol* 19: 8469-8478.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB and Korsmeyer SJ (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80: 285-291.

Zisterer DM, Campiani G, Nacci V and Williams DC (2000) Pyrrolo-1,5-benzoxazepines induce apoptosis in HL-60, Jurkat, and Hut-78 cells: a new class of apoptotic agents. *J Pharmacol Exp Ther* 293: 48-59.

Footnotes

This work was supported by BioResearch Ireland and Enterprise Ireland.

Reprint requests to Daniela Zisterer, Biochemistry Department, Trinity College, Dublin 2, Ireland.

[1]*Department of Biochemistry, Trinity College, Dublin 2, Ireland.*

[2]*Dipartimento Farmaco Chimico Tecnologico, Universita'delgi Studi di Siena, Siena, Italy*

[3]*Department of Haematology, Institute of Molecular Medicine, Trinity Centre, St. James's Hospital, Dublin, Ireland*

Legends for figures

Fig.1 PBOX-6 induces apoptosis in CEM-Bcl-2 cells that are resistant to apoptosis induced by etoposide, ultraviolet irradiation and actinomycin D. (A) CEM-Neo and CEM-Bcl-2 cells (5×10^6) were harvested by centrifugation at 500g for 5 min and cell lysates were prepared as described. Protein (60μg) was resolved by SDS-PAGE, transferred onto PVDF membrane and probed with anti-Bcl-2 antibody (upper panel). The membrane was stripped and re-probed with anti-β-actin as a loading control (lower panel). Mean arbitary unit from densitometry analysis of 3 replicates is included. (B) CEM-Neo and (C) CEM-Bcl-2 cells were seeded at 3×10^5 cells/ml and either left untreated, UV-irradiated for 2 min and incubated at 37^{o}C for a further 3 h, treated with etoposide (200μM) for 24 h, actinomycin D (5μM) for 24 h or PBOX-6 (10μM) for 24 h. The extent of apoptosis was determined by centrifuging an aliquot of cells (150μl) onto a glass slide followed by RapiDiff staining. Values represent the mean ± S.E.M. of three separate experiments.

Fig. 2 PBOX-6-induces apoptosis in CEM-Neo and CEM-Bcl-2 cells in a dose- and time-dependent manner. CEM-Neo and CEM-Bcl-2 cells were seeded at 3×10^5 cells/ml and treated with either (A) a range (0-10μM) of PBOX-6 concentrations for 8 h or (B) PBOX-6 (10μM) for a period of 4, 8, 16, and 24 h. The extent of apoptosis was determined by cytospinning an aliquot of cells (150μl) onto a glass slide and staining with the RapiDiff kit. Values represent the mean ± S.E.M. of three separate experiments. (C) Morphological features of CEM-Neo and CEM-Bcl-2 cells undergoing PBOX-6-induced apoptosis following treatment with vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 16 h. Vehicle treated cells are characterised by a continuous plasma membrane and intact nucleus. PBOX-6 treated cells display the morphological features of apoptosis, which include cell

shrinkage, chromatin condensation, nuclear fragmentation and the production of apoptotic bodies.

Fig. 3 PBOX-6 has no cytotoxic effect on normal PBMC's. Normal PBMC's were isolated from normal volunteers and plated at a density of 2 x 10^6/ml. Cells were left untreated or treated with either vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 48 h. In (A) an MTT assay was performed as described in Materials and methods. Statistical analysis was carried out using a 2 tailed students t-test, p=0.1069, considered not significant. In (B) and (C), an aliquot of cells was cytocentrifuged onto a glass slide and stained using the RapiDiff kit. Results are representative of 3 separate experiments.

Fig. 4 PBOX-6 induces a dose- and time-dependent phosphorylation of Bcl-2 in K562, CEM-Neo and CEM-Bcl-2 cells. K562 cells (A and B), CEM-Neo cells (C and D) and CEM-Bcl-2 cells (E and F) were seeded at 5x10^6 cells/flask and treated with vehicle (V) (1% (v/v) ethanol) and either a range (0.1-10μM) PBOX-6 for 16 h (A, C, E), or PBOX-6 (10μM) for 1, 4, 8 and 16 h (B, D, F). Whole cell lysates were prepared as outlined and an equal amount of protein (60μg) was resolved by SDS-PAGE and probed with anti-Bcl-2 antibody. Results are representative of three separate experiments. Arrows outline the presence of phosphorylated bands.

Fig. 5 Effect of PBOX-6 on apoptosis and Bcl-2 phosphorylation in Jurkat-Neo, Jurkat-WT-Bcl-2 and mutant Jurkat-Bcl-2 AA/A cells. Jurkat-Neo, Jurkat-WT-Bcl-2 and Jurkat-AA/A Bcl-2 cells were seeded at 3 x 10^5 cells/ml and treated with (A) Taxol (1μM) or (B) PBOX-6 (10μM) for up to 24 h. Percentage apoptosis was determined by centrifuging an aliquot of cells (150μl) onto a glass slide followed by RapiDiff staining, results are

reported as % Viability of vehicle (1% (v/v) ethanol) treated cells which was 100%. Values represent the mean ± S.E.M. of three separate experiments. (C) Jurkat clones stably expressing alanine-substituted phosphorylation sites of Bcl-2 consisting of Ser70, Ser87 and Thr69 (AA/A), WT-Bcl-2 (WT) and empty vector (Neo) were treated with either vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 16 h. Whole cell lysates were prepared as outlined and an equal amount of protein (60μg) was resolved by SDS-PAGE and probed with anti-Bcl-2 antibody. Results are representative of two separate experiments. Arrows outline the presence of phosphorylated bands.

Fig. 6 PBOX-6 induces a dose- and time-dependent phosphorylation of Bcl-$_{XL}$ in K562 cells. K562 cells were seeded at $5x10^6$ cells/flask and treated with (A) vehicle (V) (1% (v/v) ethanol) and either a range (0.1- 10μM) of PBOX-6 concentrations for 16 h or (B) PBOX-6 (10μM) for 1, 4, 8 and 16 h. Whole cell extracts were prepared and an equal amount of protein (60μg) was resolved by SDS-PAGE and probed with anti-Bcl-$_{XL}$ antibody. Results are representative of two separate experiments. Arrows outline the presence of phosphorylated bands.

Fig.7 PBOX-6 has no effect on the subcellular localization and expression levels of Bax in K562 cells. K562 cells ($2x10^6$ cells/ml) were treated with either vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 16 h. Whole cell extracts, or subcellular fractions consisting of cytosolic (Cyto) and mitochondrial (Mito) proteins, were prepared as described in Materials and methods. An equal amount of protein (60μg) from cytosolic, mitochondrial and whole cell extracts was resolved by SDS-PAGE followed by Western blotting. Membranes were incubated with anti-Bax antibody (A) and protein was visualised by ECL. Membranes were stripped and re-probed with anti-Bcl-2 antibody, which is used as a

mitochondrial marker (B). To test the purity of the fractions, an LDH assay was performed on an equal amount of protein (1μg) from each subcellular fraction and enzyme activity in each fraction was expressed as percent of total LDH measured from whole cell extracts (C). Results are representative of three separate experiments.

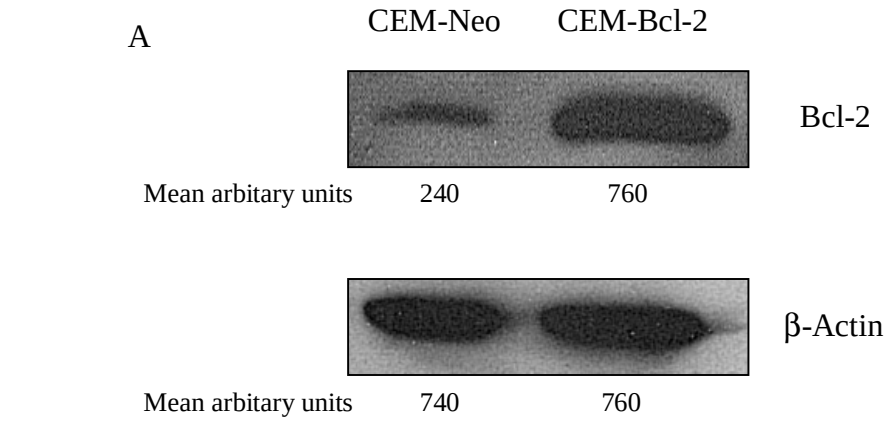
Fig. 8 PBOX-6 induces JNK activation within 15 minutes in CEM-Neo and CEM-Bcl-2 cells. (A) CEM-Neo and (B) CEM-Bcl-2 cells were seeded at a density of $6x10^6$ cells /flask and treated with vehicle (V) (1% (v/v) ethanol) or PBOX-6 (10μM) for 5, 15, 30 and 45 min. Cells were lysed as outlined and incubated with anti-JNK antibody for 3 h at 4°C. Immune complexes were recovered with protein A Sepharose beads, which were washed twice with IP buffer and once with kinase reaction buffer. A kinase reaction containing 2.5μg GST-c-jun, 20μM ATP and 2μCi of [γ-^{32}P] ATP was performed at room temperature for 30 min. The reaction was stopped by boiling in Laemmli sample buffer (15μl) and protein was resolved by SDS-PAGE. The incorporation of [^{32}P] was visualised by autoradiography. Results are representative of three separate experiments. Mean arbitary unit from densitometry analysis of 3 replicates is included.

Fig. 9 The JNK inhibitors, dicoumarol and CEP-11004, prevent PBOX-6-induced apoptosis. (A) CEM-Bcl-2 and Jurkat-WT Bcl-2 cells (3×10^5 /ml) were treated with either vehicle (1% (v/v) ethanol) for 16 h, dicoumarol (200μM) for 17 h, PBOX-6 (10μM) for 16 h, or a pretreatment of dicoumarol (200μM) for 1 h prior to treatment with PBOX-6 (10μM) for a further 16 h. The extent of apoptosis was determined by centrifuging an aliquot of cells (150μl) onto a glass slide followed by RapiDiff staining. Values represent the mean +/- SEM of three separate experiments. (B) K562 cells ($6x10^6$) were treated with vehicle (0.1% (v/v) DMSO / 1% (v/v) ethanol), CEP-11004 (5μM) for 1 h, PBOX-6

(10μM) for 45 min or a pretreatment of CEP-11004 (5μM) for 1 h prior to PBOX-6 (10μM) for a further 45 min. Cells were lysed and a JNK kinase assay containing 2.5μg GST-c-jun, 20μM ATP and 2μCi of [γ-^{32}P] ATP was performed for 30 min at room temp as outlined. The reaction was stopped by boiling in Laemmli sample buffer (15μl) and protein was resolved by SDS-PAGE and the incorporation of [^{32}P] was visualised by autoradiography. Mean arbitary unit from densitometry analysis of 3 replicates is included. (C) K562, CEM-Bcl-2 and Jurkat-WT Bcl-2 cells (3 x 10^5 /ml) were treated with vehicle (0.1% (v/v) DMSO / 1% (v/v) ethanol), CEP-11004 (5μM) for 17 h, PBOX-6 (10μM) for 16 h or a pretreatment of CEP-11004 (5μM) for 1 h prior to PBOX-6 (10μM) for a further 16 h. The extent of apoptosis was determined by RapiDiff staining. Values represent the mean +/- SEM of three separate experiments.

Fig. 10 Dicoumarol and CEP-11004 prevent Bcl-2 phosphorylation induced by PBOX-6. K562, CEM-Bcl-2, Jurkat-WT Bcl-2 cells were seeded at 5 x 10^6 cells/flask. Cells were treated with either (A) vehicle (1% (v/v) ethanol) for 16 h, dicoumarol (200μM) for 17 h, PBOX-6 (10μM) for 16 h, or a pretreatment of dicoumarol (200μM) for 1 h prior to treatment with PBOX-6 (10μM) for a further 16 h or (B) vehicle (0.1% (v/v) DMSO / 1% (v/v) ethanol), CEP-11004 (5μM) for 17 h, PBOX-6 (10μM) for 16 h or a pretreatment of CEP-11004 (5μM) for 1 h prior to PBOX-6 (10μM) for a further 16 h. Cells were collected by centrifugation and whole cell lysates were prepared and an equal amount of protein (60μg) was resolved by SDS-PAGE and probed with anti-Bcl-2 antibody. Results are representative of two separate experiments. Arrows outline the presence of phosphorylated bands.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.



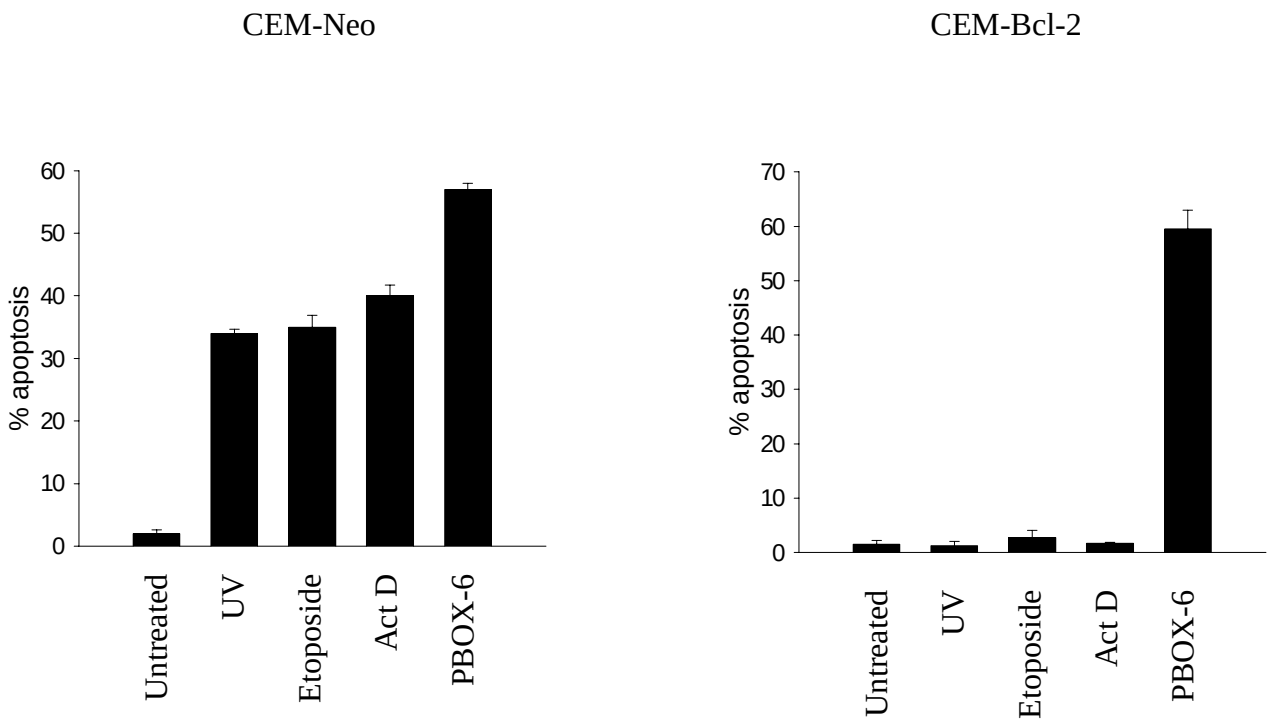


Fig. 1. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

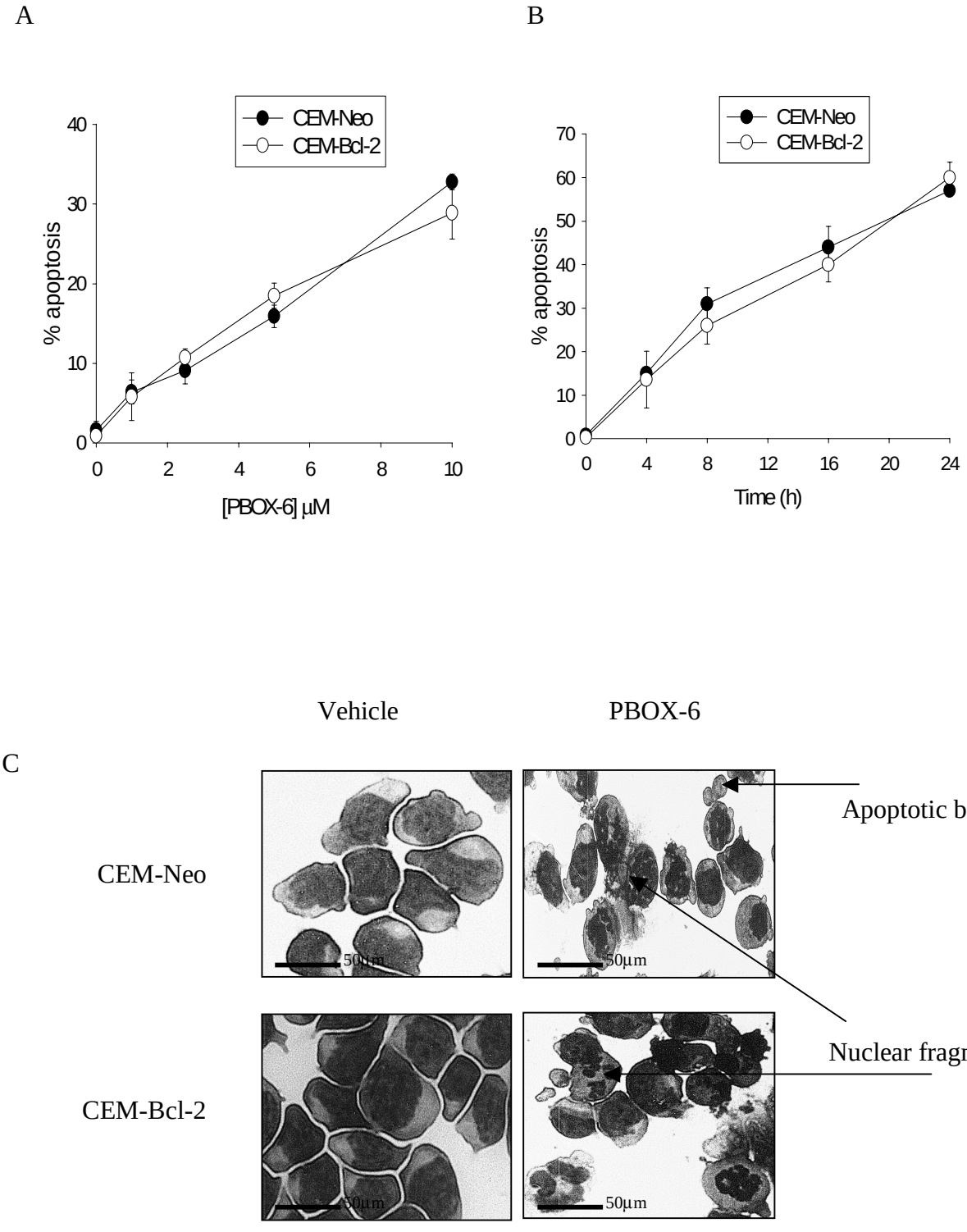


Fig. 2. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

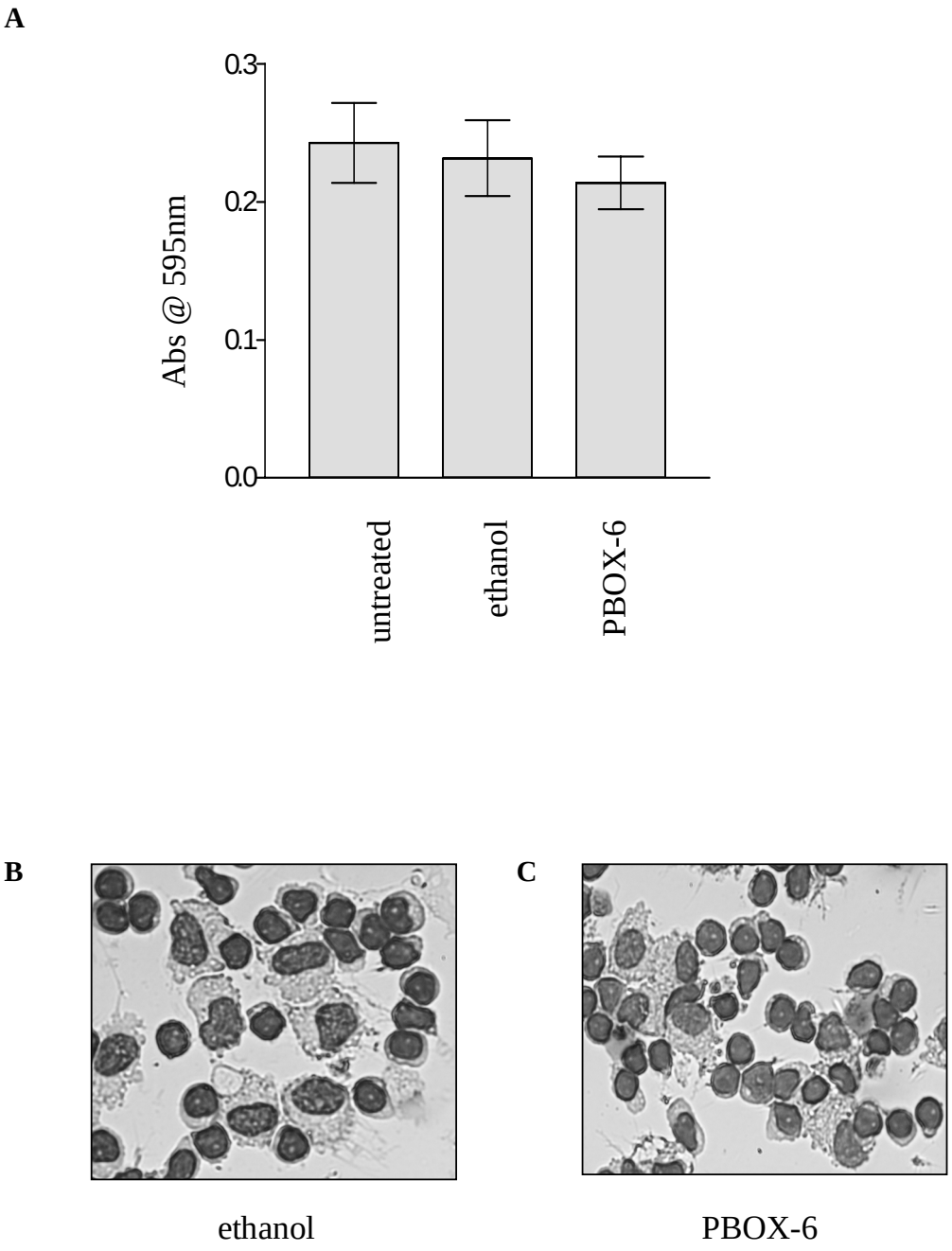


Fig. 3 Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

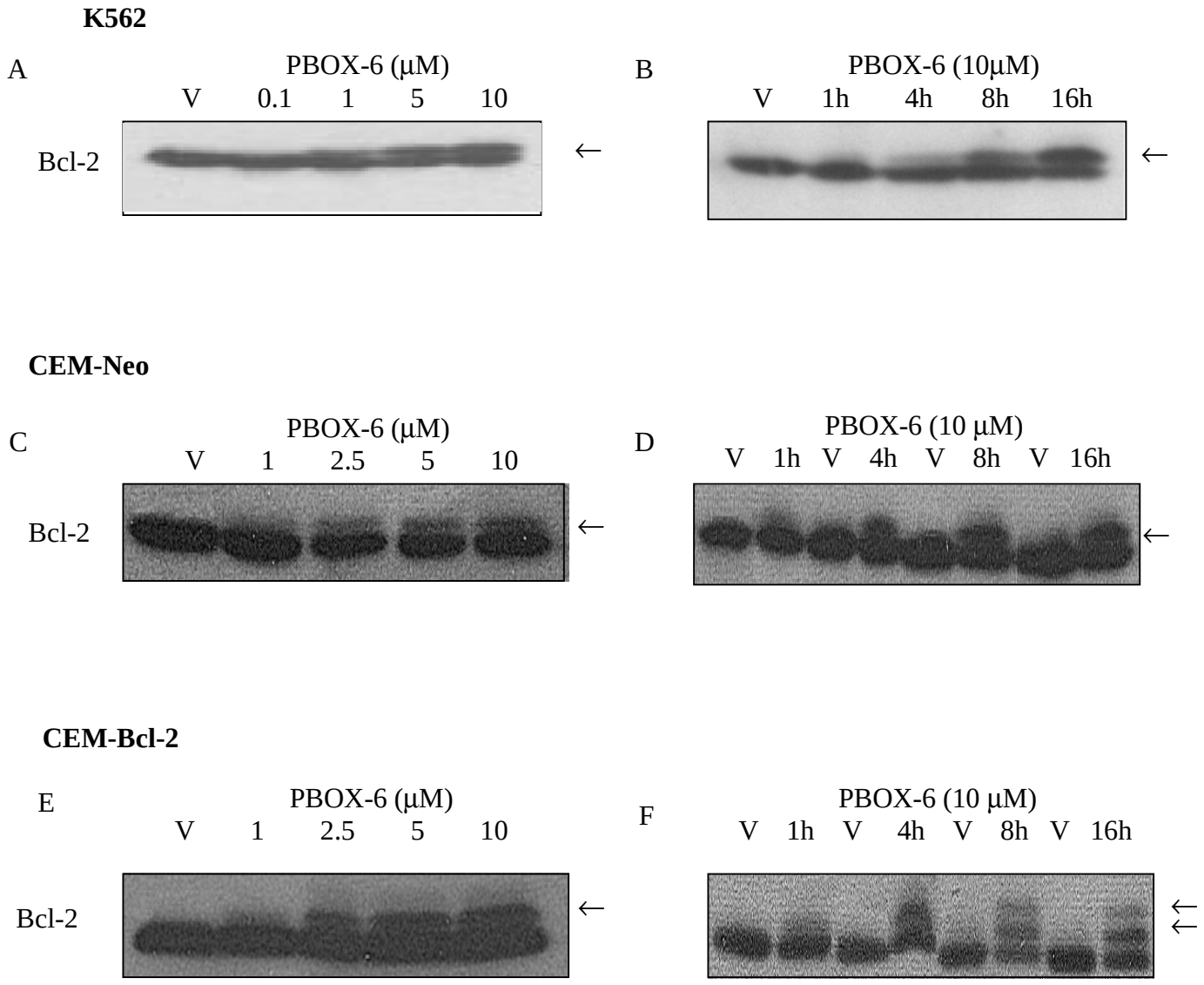
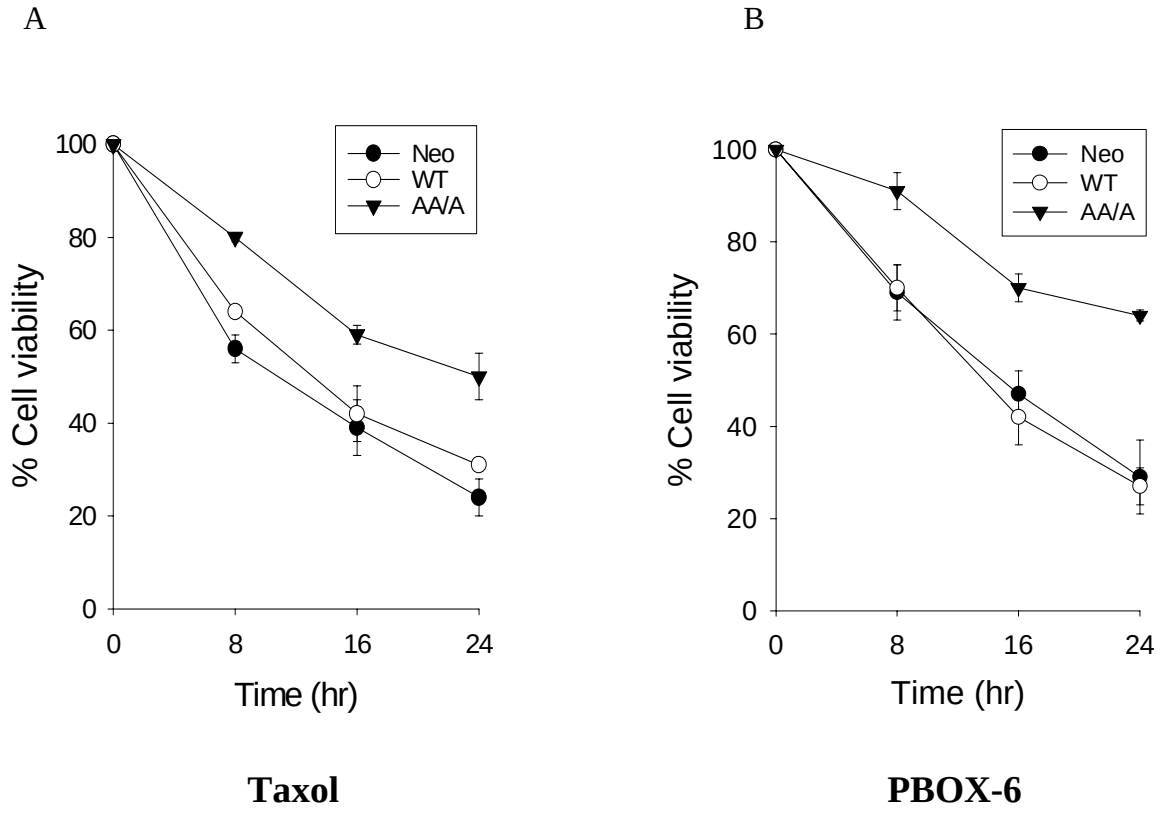


Fig. 4. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.



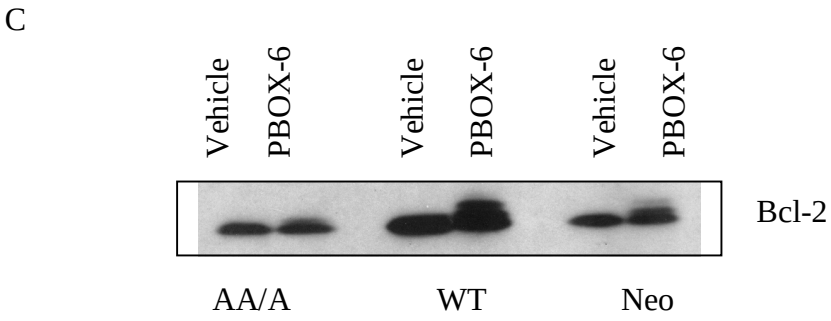


Fig. 5 Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

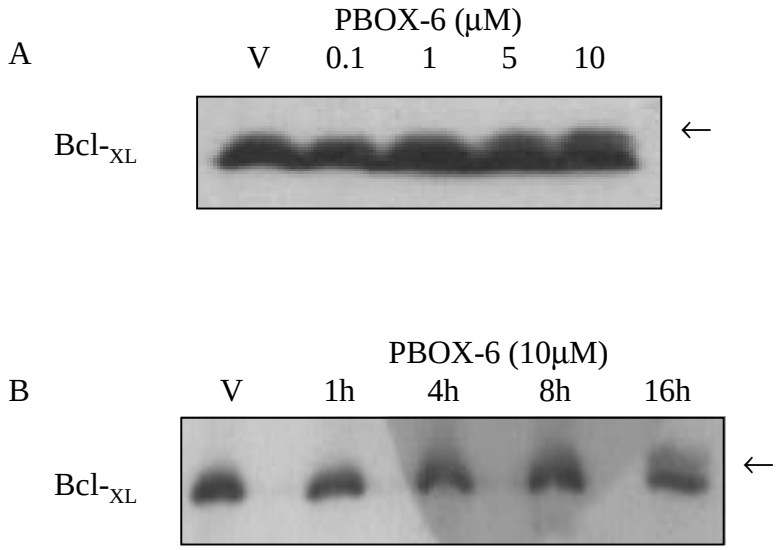


Fig. 6. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

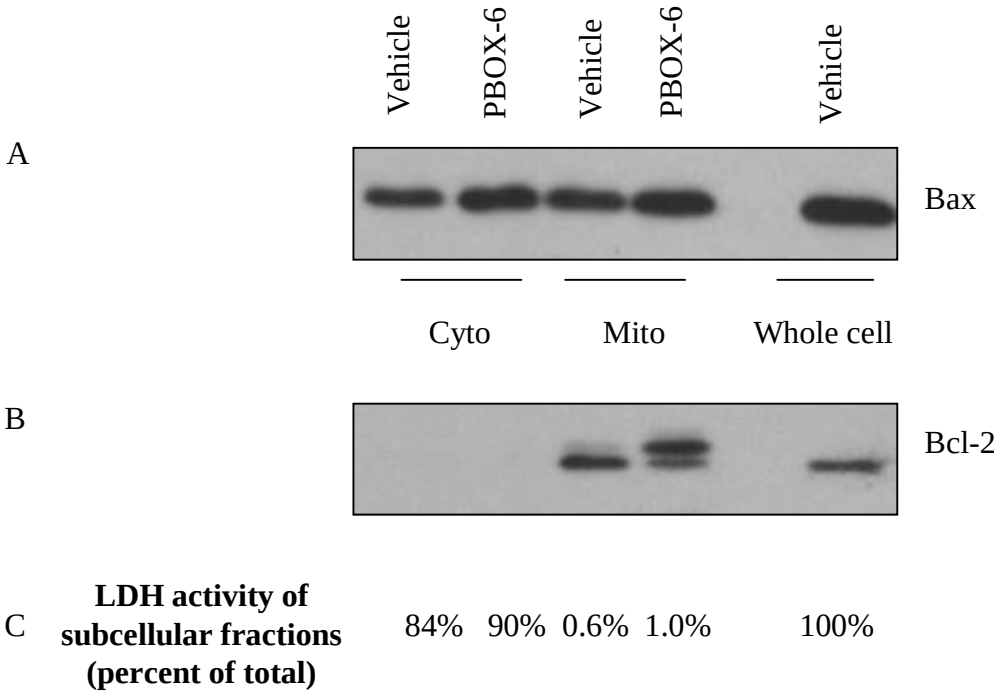


Fig. 7. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

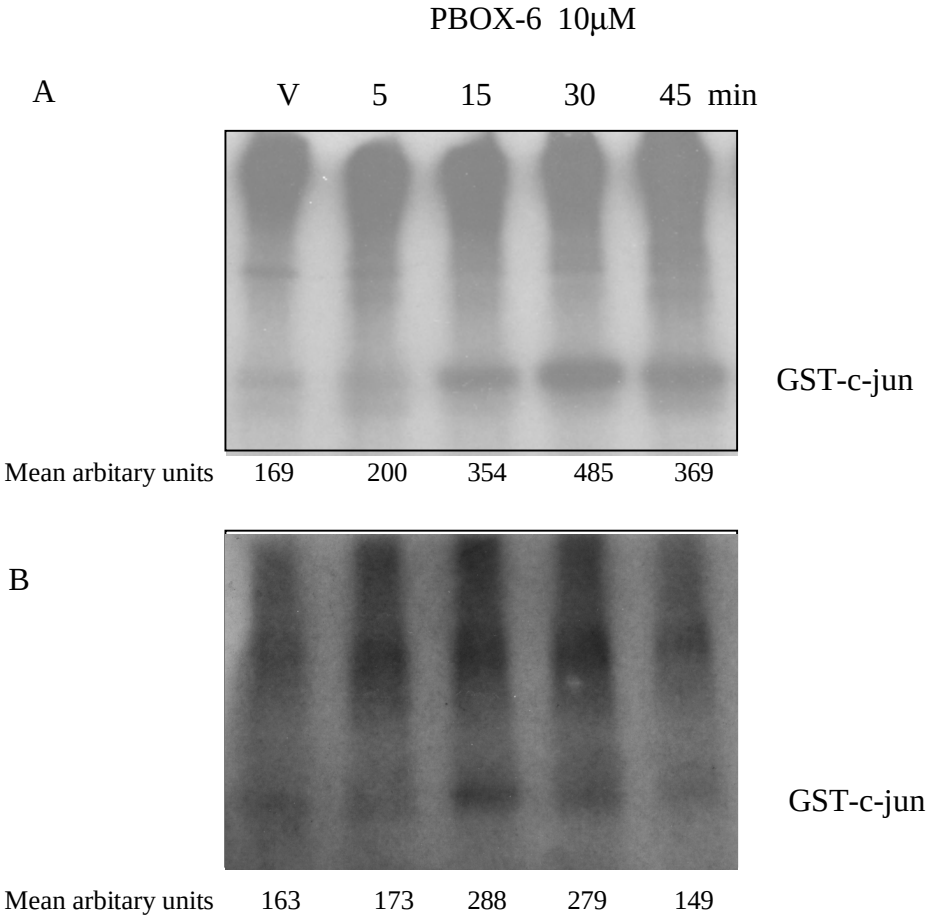


Fig. 8. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

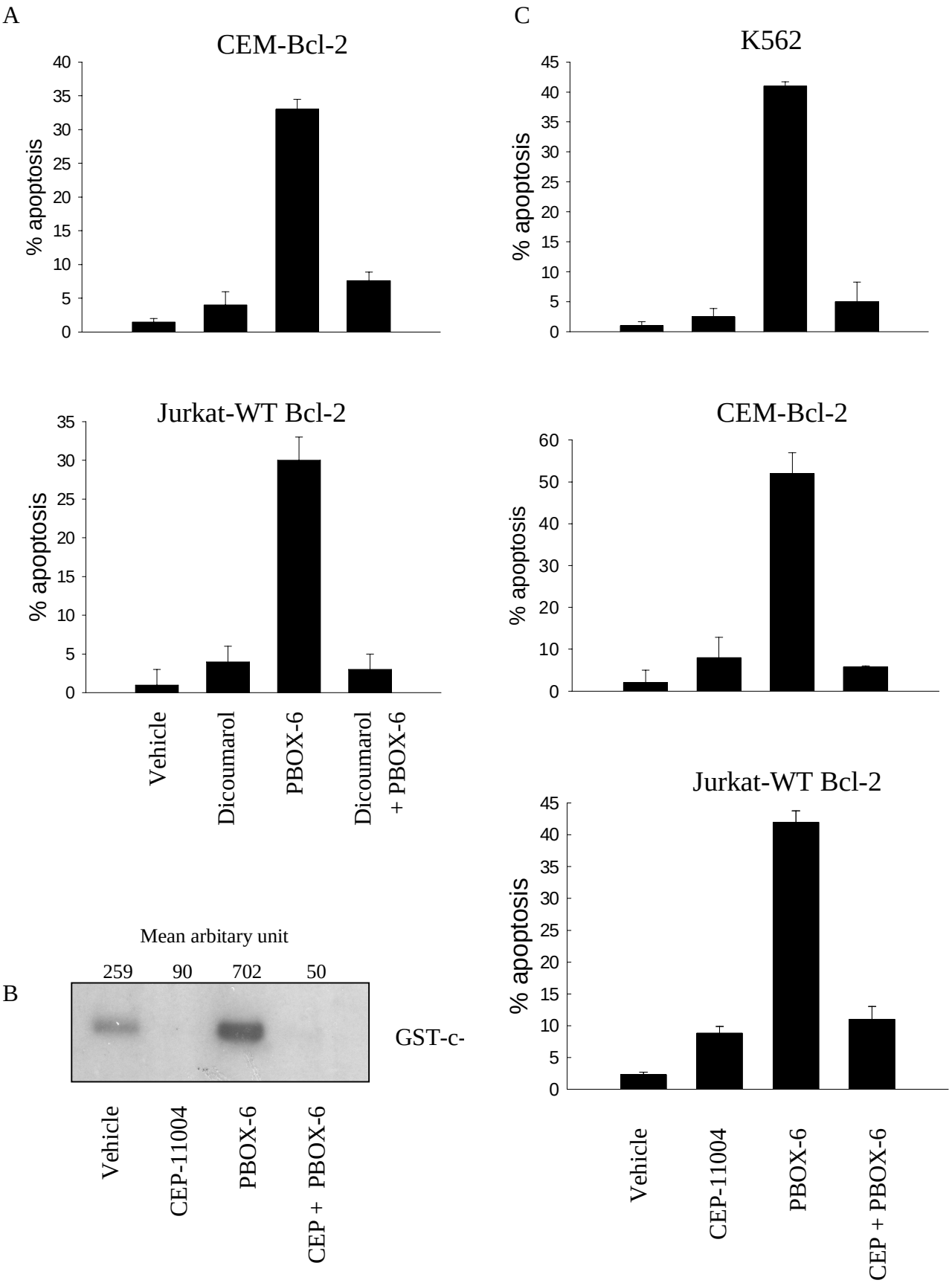


Fig. 9. Mc Gee et al.

JPET Fast Forward. Published on May 13, 2004 as DOI: 10.1124/jpet.104.067561
This article has not been copyedited and formatted. The final version may differ from this version.

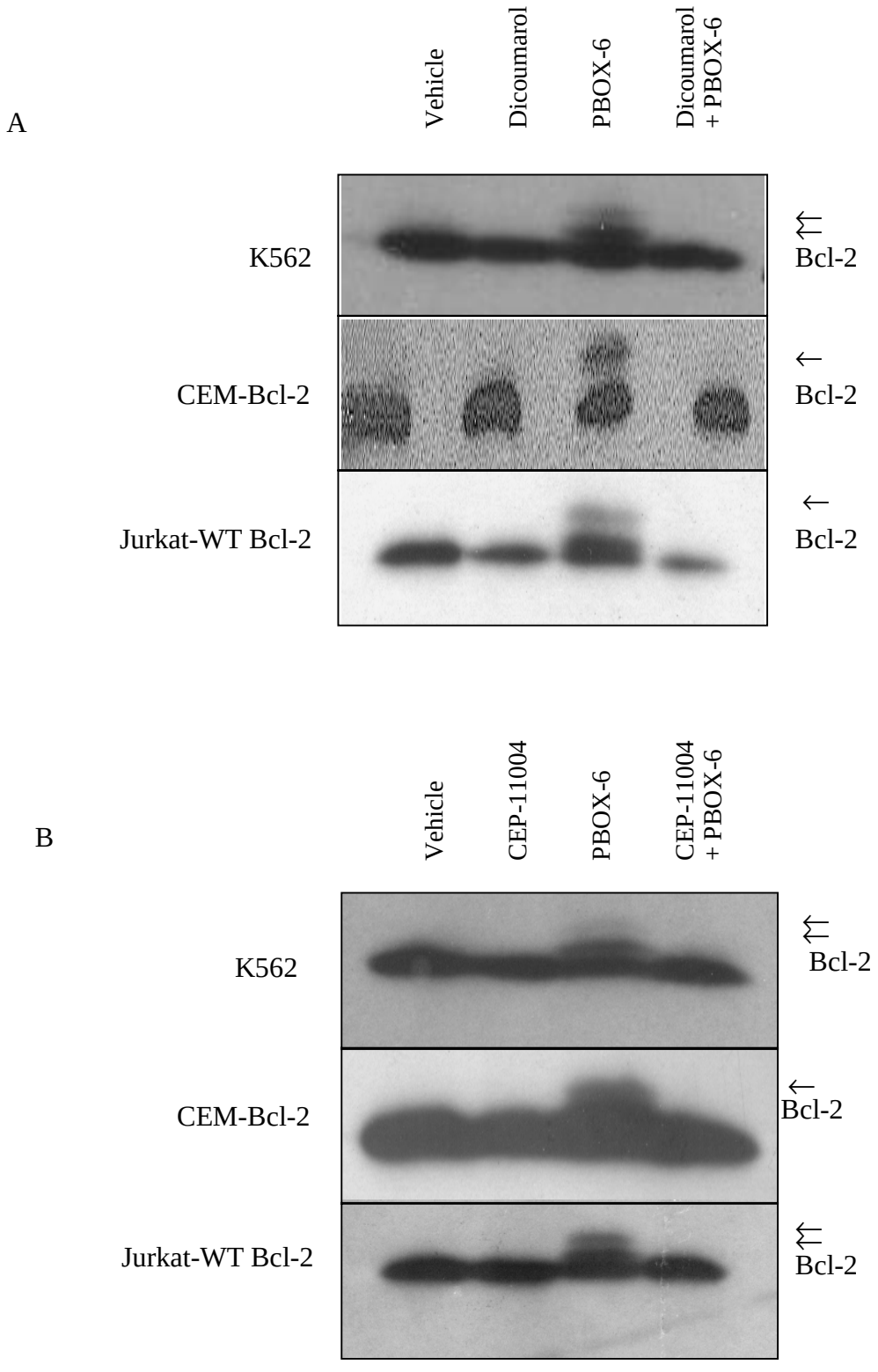


Fig. 10 Mc Gee et al.