Pyrrlo-1,5-benzoxazepines Induce Apoptosis in Chronic Myelogenous Leukemia (CML) Cells by Bypassing the Apoptotic Suppressor Bcr-Abl

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ABSTRACT

Expression of the transforming oncogene bcr-abl in chronic myelogenous leukemia (CML) cells is reported to confer resistance against apoptosis induced by many chemotherapeutic agents such as etoposide, ara-C, and staurosporine. In the present study some members of a series of novel pyrrolo-1,5-benzoxazepines potently induce apoptosis, as shown by cell shrinkage, chromatin condensation, DNA fragmentation, and poly(ADP-ribose) polymerase (PARP) cleavage, in three CML cell lines, K562, KYO.1, and LAMA 84. Induction of apoptosis by a representative member of this series, PBOX-6, was not accompanied by either the down-regulation of Bcr-Abl or by the attenuation of its protein tyrosine kinase activity up to 24 h after treatment, when approximately 50% of the cells had undergone apoptosis. These results suggest that down-regulation of Bcr-Abl is not part of the upstream apoptotic death program activated by PBOX-6. By characterizing the mechanism in which this novel agent executes apoptosis, this study has revealed that PBOX-6 caused activation of caspase 3-like proteases in only two of the three CML cell lines. In addition, inhibition of caspase 3-like protease activity using the inhibitor z-DEVD-fmk blocked caspase 3-like protease activity but did not prevent the induction of apoptosis, suggesting that caspase 3-like proteases are not essential in the mechanism by which PBOX-6 induces apoptosis in CML cells. In conclusion, this study demonstrates that PBOX-6 can bypass Bcr-Abl-mediated suppression of apoptosis, suggesting an important potential use of these compounds in the treatment of CML.

Chronic myelogenous leukemia (CML) is a member of a group of diseases called as myeloproliferative disorders, which account for 20% of all leukemias. CML is a clonal disorder that is usually easily recognized because the leukaemia cells of more than 95% of patients suffering from CML have a distinctive cytogenetic abnormality, the Philadelphia chromosome. This results from a reciprocal translocation between the long arms of chromosomes 9 and 22. This translocation results in the transfer of the Abelson (abl) oncogene on chromosome 9 to an area of chromosome 22 that includes the breakpoint cluster region (bcr) gene. This results in the presentation of a leukemia-specific fusion gene (bcr-abl) that gives rise to an abnormal tyrosine kinase protein, p210, with increased activity (Clarkson et al., 1997; Cortez et al., 1997).

The tyrosine phosphorylation status of a protein is controlled by two antagonistic families of enzymes, protein tyrosine kinases and phosphatases. Phosphorylation and dephosphorylation of cellular proteins are implicated in many important processes such as cell growth and differentiation (Weng et al., 1998). The products of several proto-oncogenes, such as bcr-abl, are tyrosine kinases, and kinase activities are either increased or constitutively activated in the corresponding oncogenes (Bergamaschi et al., 1993).

Bcr-Abl expressing leukemic blasts are highly resistant to different classes of chemotherapeutic drugs. K562 cells, derived from patients with CML in blast crisis (Lozzio and Lozzio, 1975), which express p210 Bcr-Abl, have been shown to be highly resistant to apoptosis induced by many chemotherapeutic agents (McGahon et al., 1994; Amarante-Mendes et al., 1998). Overexpression of Bcr-Abl has been implicated in inhibiting apoptosis induced by cytokine deprivation, DNA damage, and a variety of chemotherapeutic drugs (Cortez et al., 1997). Thus, the Bcr-Abl fusion protein has been sug-
gested to function as does bcl-2, as an antiapoptotic factor and overexpression of the Bcr-Abl protein in K562 cells may in part account for the resistance of these cells to apoptosis, thereby leading to the accumulation of leukemic blasts in patients with chronic myeloid leukemia (Urbano et al., 1998).

Programmed cell death or apoptosis is an evolutionary conserved and genetically regulated biological process that plays an important role in the development and homeostasis of multicellular organisms (Wyllie et al., 1980). It is widely reported that mitochondria play a critical role in apoptosis. Mitochondria are the primary site of reactive oxygen species production within the cell. Many reports suggest an increase in the production of reactive oxygen species such as peroxides during apoptosis (McGowan et al., 1996), which may be prevented by antioxidants such as N-acetylcysteine.

The execution of apoptosis requires specific molecular machinery, the central component of which is a family of proteases called caspases. Caspases are cysteine proteases that cleave proteins after specific aspartate residues, in response to proapoptotic signals (Nicholson and Thornberry, 1997). During apoptosis caspases are thought to be activated in an amplifying proteolytic cascade, cleaving one another in sequence (Raff, 1998). One of the most widely studied caspases, caspase 3, is classed as an effector caspase and cleaves death substrates such as the structural proteins lamin and fodrin, and the nuclear protein poly(ADP-ribose) polymerase (PARP) (McGowan et al., 1996).

Recently a novel series of pyrrolo-1,5-benzoxazepines were synthesized (Campiani et al., 1996) and some of these compounds induce apoptosis in a number of cancerous cells such as the human promyelocytic leukemia HL-60 cell line, the human lymphoma Hut-78 cell line, and the human lymphoblast Jurkat cell line (Zisterer et al., 2000). The search for novel compounds that induce apoptosis in CML cells and that may be useful in the therapy of patients with CML is of importance. In the present study, a number of these novel pyrrolo-1,5-benzoxazepines (Fig. 1) were found to induce apoptosis in CML cells, including the extremely resistant K562 cell line. The bcr-abl oncogene is believed to be responsible for mediating the resistance of CML cells to the induction of apoptosis. We examined whether Bcr-Abl becomes down-regulated during PBOX-6-mediated apoptosis, and whether protein tyrosine kinase activity is altered. Zisterer et al. (2000) has recently shown that activation of caspase 3-like proteases is essential in the mechanism by which PBOX-6 induces apoptosis in HL-60 and Jurkat cells. In the current study, we tested the hypothesis that caspase 3-like proteases might play a role in PBOX-6-induced apoptosis in CML cells. We also examined the involvement of reactive oxygen intermediates in the apoptotic death program activated by PBOX-6. Finally, we discuss the potential of PBOX-6 as a novel anticancer drug for the treatment of chronic myeloid leukemia.

**Experimental Procedures**

**Materials.** The CML cells K562, KYO.1, and LAMA 84 were kindly supplied by Dr. Jane Apperley and Dr. Junia Melo, Department of Hematology, Imperial College, London, UK. These cell lines represent the different stages in the progression of CML. K562 and KYO.1 cells are derived from patients in blast crisis stage, whereas the LAMA 84 cells are derived from a patient in the accelerated stage. Human promyelocytic leukemia (HL-60), and human T-cell lymphoma (Jurkat T) cells were obtained from the European Collection of Animal Cell Culture, Salisbury, UK. CML and Jurkat T cells were grown in RPMI-1640 supplemented with 10% fetal calf serum, gentamicin (0.1 mg/ml), and L-glutamine (2 mM), all obtained from Sigma (Poole, Dorset, UK). HL-60 cells were maintained in similar medium containing 20% fetal calf serum. All cells were maintained in a humidified incubator with 95% air and 5% CO₂ at 37°C. The PBOX compounds were prepared as 1 mM stocks in ethanol and stored at −20°C. A 20 mM stock of etoposide (Sigma) was dissolved in ethanol:dimethyl sulfoxide (1:1) and stored at −20°C. Mitoxantrone, 50 μM stock, and ara-C, 10 mM stock (Sigma), were dissolved in H₂O and stored at −20°C. N-Acetylcysteine (NAC) (100 mM) and vitamin E (1 mM) (Sigma) were dissolved in 25 mM Tris (pH 7.2) and PBS, respectively, whereas H₂O₂ (Sigma) was diluted in growth medium. The caspase 3-like fluorogenic substrate Ac-DEVD-AMC was obtained from Alexis (Nottingham, UK). The caspase 3-like inhibitor z-DEVD-fmk, and the caspase 6-like fluorogenic substrate Ac-VEID-AMC were supplied by Calbiochem-Novabiochem (Nottingham, UK). The RapiDiff kit was purchased from Diagnostic Developments (Burscough, Lancashire, UK). The enhanced chemiluminescence reagent was from Amersham Pharmacia Biotech (Aylesbury, UK). The anti-c-abl, anti-phosphotyrosine, and anti-PARP antibodies were supplied by Calbiochem-Novabiochem. The pro-caspase 3 and pro-caspase 7 antibodies were obtained from Transduction Laboratories (Lexington, KY). Anti β-actin was obtained from Sigma.

**Apoptosis and DNA Fragmentation Assays.** Cells were seeded at 3 × 10⁴ cells/ml and after treatment with the indicated compound, an

![Fig. 1. Structures of the pyrrolo-1,5-benzoxazepines.](image-url)
aliquot (150 μl) was removed and cytoreprocessed onto poly-L-lysine-coated glass slides. The slides were stained using the RapidDiff kit following manufacturer’s instructions. The extent of apoptosis and necrosis was determined by counting approximately 300 cells under 40× magnification using a light microscope. At least three fields of view per slide, with approximately 100 cells/field, were counted. Apoptotic cells were characterized by cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. Cell swelling and loss of cell membrane identified necrotic cells. For the DNA fragmentation assay 1 × 10⁶ cells were seeded in 20 ml of growth medium and treated with ethanol (1% v/v) or PBOX-6 (10 μM) for the required time period at 37°C. Cells were centrifuged at 500 g for 5 min and the supernatant removed. The pellet was resuspended in lysis buffer (1 ml) (20 mM EDTA, 100 mM Tris, pH 8.0, 0.8% (w/v) sodium laurel sarcosinate) and incubated at 37°C for 1 h. After incubation RNase A was added to each tube (0.5 mg/ml) and left at 37°C for a further 2 h. Proteinase K (6 mg/ml) was then added to each sample and tubes were incubated overnight at 37°C. An aliquot of each sample (45 μl) was mixed with 5 μl of loading dye (0.25% bromophenol blue, 30% glycerol in Tris borate) and samples were resolved on a 1.5% agarose gel in Tris borate for 4 h and DNA ladders were visible under UV light using a UVP gel documentation system.

Measurement of Bcr-Abl and Protein Tyrosine Phosphorylation by Western Blotting. Cells (6 × 10⁵) were harvested by centrifugation at 500 g for 5 min followed by washing in ice-cold PBS. Pellets were resuspended in 200 μl of Harvesting buffer for assay of Bcr-Abl (20 mM HEPES, pH 7.5, 10% (w/v) sucrose, 0.1% (w/v) thiothreitol, 0.1% (w/v) Nonidet P-40, 1 mM sodium EDTA, and 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitors (1 μg/ml pepstatin A and 1 μg/ml leupeptin). For the measurement of protein tyrosine phosphorylation the cell pellet was suspended in 100 μl of ice-cold lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin]. In both cases, cells were incubated on ice for 10 min followed by passage 12 times through a 21-gauge needle. After a further 10-min incubation on ice the homogenates were centrifuged at 20,000 g for 20 min at 4°C. Supernatants were removed and protein concentration was determined using a Bradford assay (Bradford, 1976). Equal amounts of protein were resolved on an 8% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride. After transfer, the membranes were blocked in PBS containing 5% (w/v) dried milk and probed with anti-c-abl or anti-phosphotyrosine antibodies. Blots were washed and probed with goat anti-mouse horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence detection according to manufacturer’s instructions. The Bcr-Abl blots were stripped of the manufacturer’s instructions. The extent of apoptosis and necrosis was determined by counting approximately 300 cells under 40× magnification using a light microscope. At least three fields of view per slide, with approximately 100 cells/field, were counted. Apoptotic cells were characterized by cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. Cell swelling and loss of cell membrane identified necrotic cells. For the DNA fragmentation assay 1 × 10⁶ cells were seeded in 20 ml of growth medium and treated with ethanol (1% v/v) or PBOX-6 (10 μM) for the required time period at 37°C. Cells were centrifuged at 500 g for 5 min and the supernatant removed. The pellet was resuspended in lysis buffer (1 ml) (20 mM EDTA, 100 mM Tris, pH 8.0, 0.8% (w/v) sodium laurel sarcosinate) and incubated at 37°C for 1 h. After incubation RNase A was added to each tube (0.5 mg/ml) and left at 37°C for a further 2 h. Proteinase K (6 mg/ml) was then added to each sample and tubes were incubated overnight at 37°C. An aliquot of each sample (45 μl) was mixed with 5 μl of loading dye (0.25% bromophenol blue, 30% glycerol in Tris borate) and samples were resolved on a 1.5% agarose gel in Tris borate for 4 h and DNA ladders were visible under UV light using a UVP gel documentation system.

Fluorogenic Assay of Caspase-3-Like Proteases. The fluorogenic assay was performed as previously described (Zisterer et al., 2000). The fluorogenic substrate Ac-VEID-AMC was used to measure caspase 6-like protease activity.

Results

Induction of Apoptosis in CML Cells by Pyrrolo-1,5-benzoxazepines. Some of a series of pyrrolo-1,5-benzoxazepines were found to induce apoptosis in K562 cells. The characteristic morphological features of apoptosis such as cell shrinkage, chromatin condensation, DNA fragmentation, and membrane blebbing were observed in these cells (Fig. 2). To make a direct comparison of apoptotic potencies, a number of PBOX compounds were tested at the same time point (16 h) and at the same concentration (10 μM). Of the PBOX compounds tested, PBOX-3, -4, -5, -6, and -7 were found to induce apoptosis, exhibiting between 40 and 50% cell death, whereas other members of the PBOX series had no effect on cell viability, suggesting a structure-activity relationship (Fig. 3). The subsequent experiments described in this study were performed using PBOX-6 as the representative proapoptotic pyrrolobenzoazepine. It has previously been reported that K562 cells are resistant to many chemotherapeutic drugs (Martins et al., 1997; Amarante-Mendes et al., 1998). In this study it was confirmed that K562 cells are resistant to the induction of apoptosis by etoposide, mitoxantron, and ara-C, all commonly used chemotherapeutic agents (Fig. 4). In the three CML cell lines examined, K562, LAMA 84, and KYO.1, PBOX-6 induced apoptosis in a dose-dependent (Fig. 5A) and a time-dependent (Fig. 5B) manner. The morphological features of apoptosis became apparent after a 4-h treatment with PBOX-6 (10 μM) and levels rose until 16 h, where approximately 40 to 50% of cells displayed...
some proteins was detected after a 24-h treatment with PBOX-6, at which point approximately 50% of the cells had undergone apoptosis. These results further suggest that down-regulation of Bcr-Abl or inhibition of the Abl tyrosine kinase activity is not part of the upstream events associated with PBOX-6-induced apoptosis.

**PBOX-6-Induced Apoptosis in K562 Cells Does Not Involve the Production of Reactive Oxygen Intermediates (ROIs).** Several observations suggest an involvement of ROIs in the signal transduction pathway leading to apoptosis (Jacobson, 1996). To determine whether the induction of apoptosis in K562 cells by PBOX-6 involved the production of ROIs, cells were pretreated with vitamin E or N-acetylcysteine for 1 or 24 h, respectively, before treatment with PBOX-6 for a further 16 h. The extent of apoptosis was determined by cytopinning the cells onto slides and staining with the RapiDiff kit. These compounds did not protect against PBOX-6-induced apoptosis (Fig. 8, A and B), suggesting that the production of ROIs is not involved in the mechanism by which PBOX-6 induces apoptosis in K562 cells. As a positive control HL-60 cells were pretreated with the antioxidants vitamin E or N-acetylcysteine before treatment with the apoptosis-inducing agents, UV irradiation, or H2O2, respectively. Results showed that pretreatment of HL-60 cells with these antioxidants before either UV-irradiation or H2O2 treatment reduced the level of apoptosis induced in these cells (Fig. 8, C and D).

**PBOX-6 Treatment of CML Cells Results in PARP Cleavage.** In some cell lines, PARP (116 kDa) is cleaved during apoptosis into its 87- and 29-kDa fragments, which can be detected by Western blot analysis of cell lysates. Control and PBOX-6-treated cell lysates of CML cells were examined for evidence of PARP cleavage. Results show that PARP cleavage occurs to a small extent in all three CML cell lines in response to PBOX-6 (Fig. 9, A–C). This activation of PARP in these cells suggests that cysteine proteases known as caspases may be involved in the mechanism by which these cells undergo apoptosis (Gu et al., 1995).

**Caspase 3-Like Proteases Become Activated in K562 and LAMA 84 Cells but Not in KYO.1 Cells in Response to PBOX-6 Treatment.** Caspase 3 is one of the most intensely studied caspases and is believed to play a role as one of the downstream executioners in many apoptotic pathways. Control and apoptotic cell lysates from CML cells were examined for activated caspase 3-like protease status. After a 16-h treatment with PBOX-6, caspase 3-like proteases become activated to low levels in two CML cell lines, K562 and LAMA 84 (Fig. 10A). The timing of caspase 3-like protease activation correlates with the appearance of the morphological features of apoptosis as determined using the RapiDiff kit. However, caspase 3-like protease activation was not detected in KYO.1 cells (Fig. 10A), although apoptosis was induced to the same extent in this cell line. Because one cell line, the MCF-7 breast carcinoma cells, had previously been shown to be deficient in the caspase 3 proenzyme (Janicke et al., 1998), we determined whether the KYO.1 cells contain the pro-caspase 3 protein. Western blot analysis with an antibody directed against the pro-enzyme form of caspase 3 was used to demonstrate the presence of this protein in all three CML cell lines (Fig. 10B).
with PBOX-6 for a further 8 h. Results show that pretreatment of K562 and LAMA 84 cells with z-DEVD-fmk inhibited the activity of caspase 3-like proteases (Fig. 10C) but did not prevent the induction of apoptosis by PBOX-6 (Fig. 10D). These results would suggest that although during apoptosis a low level of caspase 3-like protease activation was detected in two of the CML cell lines, this activation is not an essential step in the pathway under which PBOX-6 induces apoptosis in any of the three cell lines.

**Discussion**

The efficacy of chemotherapeutic agents depends on their effectiveness at inducing apoptosis in malignant cells. Al-
though a variety of chemotherapeutic agents have been used in the treatment of leukemia, many forms such as chronic myeloid leukemia are resistant to chemotherapy-induced cell death (Lehne et al., 1998). K562 cells, which express p210 Bcr-Abl, have been shown to be highly resistant to apoptosis induced by many chemotherapeutic agents (McGahon et al., 1994; Amarante-Mendes et al., 1998). In the present study we describe how a novel series of pyrrolo-1,5-benzoxazepines potently induce apoptosis in three CML cell lines. The morphological criteria associated with apoptosis, which include cell shrinkage, chromatin condensation, DNA fragmentation, and membrane blebbing, were visible in all three cell lines. Of all the compounds tested PBOX-3, -6, and -7 were found to be the most potent, whereas PBOX-1 and -2 had no effect. This chemical selectivity, together with their lack of apoptotic activity in other cell lines (Zisterer et al., 2000), argues against a general toxic effect on cells. This may suggest their potential as novel chemotherapeutic agents for the treatment of chronic myeloid leukemia.

The resistance of K562 cells to the induction of apoptosis by chemotherapeutic agents has been attributed to overexpression of the bcr-abl gene (McGahon et al., 1994, 1995; Martins et al., 1997). It is widely agreed that the mechanism by which Bcr-Abl may cause the accumulation of leukemic blasts is by inhibition of apoptosis, rather than by increasing the rate of cell division (Kabarowski et al., 1994; Rowley et al., 1996). The mechanism or the step at which this antiapoptotic effect is exerted has not been clearly defined. However, it is reported that Bcr-Abl exerts its effect upstream of events that result in caspase activation (Amarante-Mendes et al., 1998). The 26S proteasome is a large multicatalytic protease that regulates several cell cycle and apoptosis regulatory proteins (Hopkin, 1997). A recent report suggests that inhibition of the proteasome activity, using selective inhibitors, causes the induction of apoptosis in K562 cells. Proteasome inhibition resulted in a reduction of Bcr-Abl protein expression, which occurred several hours before the onset of apoptosis. Furthermore, reduced Bcr-Abl expression resulted in significant attenuation of Bcr-Abl-mediated protein tyrosine phosphorylation (Ping Dou et al., 1999). CGP57148B, the ATP-competitive inhibitor of the Abl protein kinase, has also been shown to inhibit the tyrosine phosphorylation of Bcr-Abl and additional Bcr-Ab substrates within minutes, resulting in apoptosis (Gambacorti-Passerini et al., 1997). It has also been reported that the resistance of K562 cells can be reversed by down-regulation of Bcr-Abl using antisense oligonucleotides that inhibit synthesis of the Bcr-Abl kinase (Szczylisk et al., 1991; Martins et al., 1997). Studies by McGahon et al. (1994) using antisense oligonucleotides reveal that down-regulation of p210 Bcr-Abl expression rendered the cells more susceptible to apoptosis induced by chemotherapeutic agents. However, some studies have revealed conflicting reports. It has been reported that down-regulation is not caused by an antisense mechanism, but is due to a nonspecific effect brought about by the DNA sequence (Vaerman et al., 1995).

Our results show that a representative pyrrolo-1,5-benzo-
xazepine, PBOX-6, induces a time- and dose-dependent induction of apoptosis in three CML cell lines. In the present study, PBOX-6 was able to bypass Bcr-Abl-mediated resistance to apoptosis in the three CML cell lines. Any down-regulation of Bcr-Abl did not accompany, but rather followed apoptosis in these cells. Any down-regulation was not detected until after a 24-h treatment with PBOX-6 when approximately 50% of cells displayed the morphological features of apoptosis. This would suggest that down-regulation of Bcr-Abl is not involved in the upstream events associated with PBOX-6-induced apoptosis. The tyrosine phosphorylation status of proteins remained unchanged up to 24 h after treatment with PBOX-6, suggesting that inhibition of Bcr-Abl activity is not responsible for execution of the apoptotic cascade after PBOX-6 treatment. Results from this study suggest that a reduction in p210 expression, or inhibition of Bcr-Abl activity, is not the only mechanism by which cells can escape the antiapoptotic effect of the \textit{bcr-abl} gene.

Several observations suggest an involvement of ROIs in the signal transduction pathway leading to apoptosis. This mode of cell death is sometimes associated with increases in intracellular ROI levels and the addition of various exogenous antioxidants, such as NAC, can inhibit apoptosis (McGowan et al., 1996). This work has shown that PBOX-6-induced apoptosis in K562 cells was unaffected by the presence of NAC or vitamin E. This would suggest that PBOX-6-induced apoptosis is not mediated by ROIs. Similar findings were reported by Zisterer et al. (2000) after treatment of HL-60 cells with PBOX-6. This is in agreement with recent reports, which have indicated that ROIs are not necessarily a requirement for apoptosis (Jacobson and Raff, 1995).

A unique family of cysteine proteases called caspases appear to play a critical role in initiating and sustaining the biochemical events that occur during apoptotic cell death. This family of proteases, which contain at least 12 human members, cleaves after aspartate residues (Martins et al., 1997). Janicke et al. (1998) and others have shown that activation of caspase 3 is not essential for tumor necrosis factor-, staurosporine-, or Fas-induced apoptosis. Although a low level of activation of caspase 3-like proteases was detected in K562 and LAMA 84 cells, inhibition of this caspase 3-like protease activity using the inhibitor z-DEVD-fmk did not inhibit the induction of apoptosis. In addition, caspase 3-like protease activity was not detected in KYO.1 cells undergoing apoptosis. The lack of activation of caspase 3-like proteases in KYO.1 cells was not due to the absence of the
proenzyme form of caspase 3, as demonstrated by Western blotting. Therefore, in this study it has been shown that activation of caspase 3-like proteases is not required for the induction of apoptosis by PBOX-6 in CML cells. Results from this study are in contrast to those reported by Zisterer et al. (2000), where caspase 3 activation was shown to be essential in the mechanism by which PBOX-6 induces apoptosis in HL-60 and Jurkat cells, suggesting that the pathway in which PBOX-6 induces apoptosis is cell-type specific.

Downstream effector caspases, which are thought to be involved in cleaving a number of death substrates, include caspases 3, 6, and 7 (Kaufmann and Earnshaw, 2000). A fluorogenic assay and Western blot analysis were used to determine the activity of caspases 6 and 7, respectively. Results showed that these caspases did not become activated in any of the three CML cell lines after treatment with PBOX-6 (data not shown). In these cells, alternative, as yet unknown effector caspases may be involved in the induction of apoptosis or a caspase-independent mechanism for commitment cell death may exist. For example, a recent report indicates that inhibition of the activity of the transcription factor nuclear factor-κB in human T lymphocytes resulted in apoptosis without detectable activation of caspase 1-, 3-, or 6-like proteases, suggesting either low level of activation is required or different caspases are involved (Kolenko et al., 1999). Caspase-independent pathways have also been reported, for example, overexpression of the proapoptotic protein Bax in mammalian cells induces DNA condensation and membrane alterations leading to cell death without caspase activation (Xiang et al., 1996).

In the present study Western blot analysis has shown only a small degree of PARP cleavage into its 87-kDa cleavage product. Although Western blotting may not provide an accurate quantitative assessment of cleavage, this low level of cleavage is consistent with low or no caspase 3-like protease activation because caspase 3 is thought to be the major protease cleaving this substrate (Cuvillier et al., 1998). As mentioned above, caspase-independent apoptosis can occur, which would in turn suggest that PARP cleavage is not essential for apoptosis in these cases. Our results suggest...
that in the three CML cell lines, either extensive PARP cleavage or PARP cleavage per se is not essential for apoptosis.

In agreement with previous reports, we have shown that K562 cells are resistant to apoptosis induced by many chemotherapeutic agents (Amarante-Mendes et al., 1998). Pyrrolo-1,5-benzoxazepines have recently been described as a novel class of apoptotic agent based on their ability to induce apoptosis in a number of leukemia and lymphoma cell lines derived from the hematopoietic system such as HL-60, Jurkat, and Hut 78 cells (Zisterer et al., 2000). In the present work it has been shown that a number of these novel pyrrolo-1,5-benzoxazepine derivatives are also potent inducers of apoptosis in drug-resistant CML cells. These compounds induce apoptosis with similar potencies to that reported in HL-60 cells, which are often used as a model for the induction of apoptosis (Zisterer et al., 2000). The resistance of CML cells has been attributed to the expression of the transforming oncogene bcr-abl. Results from the current study suggest that this novel compound, PBOX-6, can induce apoptosis in CML cells by bypassing Bcr-Abl-mediated resistance. This may suggest the potential of this compound as a novel anti-
cancer agent for the treatment of CML. More work is required to fully understand its mechanism of action and to identify events that trigger the apoptotic cascade.

References


