The Proapoptotic Benzodiazepine Bz-423 Affects the Growth and Survival of Malignant B Cells

Anthony Boitano, Jonathan A. Ellman, Gary D. Glick, and Anthony W. Oppari, Jr.

Abstract

Bz-423 is a novel proapoptotic 1,4-benzodiazepine that induces cell death via a superoxide signal. Previous work has shown that Bz-423 ameliorates disease in animal models of systemic lupus erythematosus that also have features of lymphoproliferative disease. Here we describe the effects of Bz-423 against a group of malignant B-cell lines derived from Burkitt’s lymphoma. These experiments demonstrate that Bz-423 has cytotoxic activity against all B-cell lines tested, regardless of EBV status or Bcl-2 and Bcl-xL expression levels. In addition to its cytotoxic properties, we found that Bz-423 is also a potent antiproliferative agent that induces a G1-phase arrest independent of p53. Mechanistically, both the cytotoxicity and growth arrest are mediated by increased reactive oxygen species levels and appear independent of binding to the peripheral benzodiazepine receptor. This work further defines the biological activities of Bz-423 that are consistent with those of other compounds in clinical development for antineoplastic therapies.

Introduction

Benzodiazepines are widely used therapeutic agents best known for their action as anxiolytics, anticonvulsants, antispasmodics, and hypnotics. Their physiological effects are mediated by binding to the central benzodiazepine receptor, which is associated with the γ-aminobutyric acid receptor in the central nervous system (1). Benzodiazepines also bind to an 18-kDa protein known as the PBR (2, 3). The PBR is present in nearly all cell types and is primarily located in mitochondria, but it can also be found on the plasma membrane and within the nucleus (4, 5). In mitochondria, the PBR forms part of the PTP, a multiprotein complex that forms junctions between the inner and outer membranes (6). The PBR has been implicated in numerous biological processes (e.g., proliferation, calcium channel activity, immune responses, porphyrin transport, heme biosynthesis, anion transport, and regulation of both steroid biosynthesis and phosphorylation) and has been extensively characterized pharmacologically and biochemically (7). Nevertheless, the precise function of the PBR remains an enigma (8).

We previously identified a novel 1,4-benzodiazepine (designated Bz-423; Fig. 1) that induces cellular responses independent of the central benzodiazepine receptor or PBR. Bz-423 possesses significant and selective cytotoxicity toward lymphocytes in vivo (9, 10). The mechanism of cytotoxicity involves a rapid increase in intracellular superoxide, and this ROS response is essential for inducing the subsequent apoptotic process characterized by cytochrome c release, mitochondrial depolarization, and caspase activation. Cell fractionation and inhibitor experiments reveal that the superoxide response results from the interaction of Bz-423 with a target in mitochondria that is not the PBR (9). To examine the therapeutic potential of this compound, Bz-423 was administered to mice with autoimmune disease dominated by lymphoproliferative changes. In the (NZB × NZW)F1 (NZB/W) mouse model, Bz-423 eliminated the pathological expansion of GC B cells (9). In MRL/MpJ-FasΔ/FasΔ (MRL- Δpr) mice, where peripheral autoreactive T cells drive a lupus-like process, Bz-423 caused a shift in the Th1/Th2 cell balance and decreased specific T-cell subsets (10). In both models, treatment decreased autoimmune damage to the kidneys and other organs and increased apoptosis in secondary lymphoid tissues. The reductions in pathogenic lymphocytes in Bz-423-treated mice were consistent with the ability of this compound to kill lymphoid cells in vitro. However, because lymphoid cell homeostasis is a balance between proliferation and cell death, an antiproliferative response to Bz-423 might also contribute to disease improvement in these models.

Other benzodiazepines have been shown to have antiproliferative activity, and this effect has been correlated with PBR binding characteristics only in some cases (11–13). However, because the antiproliferative effects of PBR ligands occur only at concentrations dramatically above those necessary to saturate PBR binding (e.g., 1000-fold), it is likely that binding to the PBR does not mediate this response (14, 15). For example, PK11195 [1-(2-chlorophenyl)-N-(1-methylpropyl)-3-isoquinolinicarboxamide] and 4-CIDz bind to the PBR with nanomolar dissociation constants, but the antiproliferative effects of these agents are usually observed at micromolar concentrations. In addition, genetically silencing PBR expression does not alter normal cellular proliferation (16). Therefore, the mechanistic basis of the antiproliferative effects of benzodiazepines remains uncertain.

Because Bz-423 selectively targeted pathologically expanded GCs in NZB/W mice and kills Ramos cells derived from BL, a disease that originates from the malignant conversion GC B cells, we hypothesized that Bz-423 would have activity against other BL-derived cells. BL occurs as a EBV-associated malignancy among Africans, as a common neoplasm in HIV-infected patients, and in sporadic cases not associated with EBV. All BL cells carry reciprocal chromosomal translocations that activate the c-myc oncogene through juxtaposition to one of the immunoglobulin loci and many carry mutations in the p53 tumor suppressor gene (17, 18). In vitro, the expression of EBV-latent gene products in BL cells is associated with a lymphoblastoid phenotype, the expression of cellular survival genes, and increased resistance to apoptosis (19–21). Although intensive chemotherapy results in long-term survival for nearly 70% of children with BL, survival for adults remains less than 25%, and innovative therapeutic approaches are an urgent priority for this disease (22).

In this report, we extend our characterization of Bz-423, demonstrating and comparing its cytotoxic function among a group of malignant B-cell lines, including cells transfected to express high levels of the antipapoptotic proteins Bcl-2 and Bcl-xL. In addition to cytotoxic activity, we demonstrate potent antiproliferative effects of Bz-423 that are independent of cell death. In fact, Bz-423 is signifi-
cantly more effective at inhibiting cell growth than other antiproliferative benzodiazepines and PK11195. Mechanistic experiments demonstrate that Bz-423-induced ROS are essential for growth inhibition and that the magnitude of this response may arbitrate between death and G1 growth arrest.

MATERIALS AND METHODS

Reagents. Bz-423 was synthesized as described previously and dissolved in aqueous DMSO as the vehicle (23). DMSO was present at a final concentration of 0.5% (v/v) in all experiments.

Cells and Culture. Ramos, ST486, Raji, Daudi, Namwala, and CA46 were purchased from American Type Culture Collection (Manassas, VA). Cells (10^6 cells/ml) were maintained in RPMI 1640 (Mediatech, Herndon, VA) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (290 μg/ml), and heat-inactivated FBS (HyClone, Logan, UT). Viability experiments were conducted in this same media at 37°C in a CO_{2} (5%) atmosphere.

Transfection and Selection of Cells Expressing Bcl-2 and Bcl-x_{L}. Expression plasmids for Bcl-2 and Bcl-x_{L} were provided by G. Nunez (24). Ramos cells (10^5) were transfected with pSFFVneo (vector only control), pSFFVneo-Bcl-2, and pSFFVneo-Bcl-x_{L} by electroporation. Stably transfected cells were selected and maintained in complete media containing Geneticin (1.5 mg/ml; Life Technologies, Inc., Rockville, MD).

Assessment of Viability and Cell Number. Cells were incubated with PI (1 μg/ml, 15 min), and viability was assessed by flow cytometry based on PI exclusion (25). Data were collected and analyzed using a FACS Calibur (BD Biosciences, San Diego, CA). For each sample, 10,000 events were recorded, and the data were analyzed excluding aggregates. In experiments to determine growth, the number of viable and dead cells was determined after indicated treatments using trypan blue (0.4% w/v) staining, and cells were quantified by flow cytometry using a hemocytometer. In experiments where Bz-423 was washed out, cells were treated with this compound or vehicle for 3 days, washed by centrifugation and three media exchanges, and then replated in fresh media at 100,000 cells/ml.

Titration of BSA to Culture Media. Certified FBS used in this study contains BSA at a concentration of 27 mg/ml, meaning an approximate BSA concentration of 2.7 mg/ml in culture media containing 10% FBS (v/v). To achieve a total BSA concentration equivalent to that found in media containing 5%, 7.5%, or 10% FBS, media with 2% FBS were supplemented with purified BSA (Sigma, St. Louis, MO) added at 0.81, 1.49, and 2.16 mg/ml, respectively. Before use, media were sterilized by filtration.

Monitoring Cell Divisions using PKH67. PKH67 was used according to the manufacturer’s protocol (Sigma). Briefly, cells (2 × 10^5) were washed in serum-free media and suspended in PKH67 (1 ml of a 0.5 μm solution) for 5 min at room temperature. Cells were then washed suspended in complete media at 0.5 × 10^5 cells/ml. Immediately after staining, and after indicated treatment for 3 days, fluorescence intensity was determined by flow cytometry on a FACSCalibur. Analysis excluded any dead cells based on forward/side scatter characteristics.

Detection of Intracellular ROS. DCHF-DA (Molecular Probes, Eugene, OR) was prepared as a 10 mM stock solution in DMSO before each use. Cells (10^6 cells/ml) were loaded (30 min, 37°C) with DCHF-DA (100 μM), washed by centrifugation, and resuspended in fresh media before treatment. After the indicated treatments, the fluorescence of the oxidized product DCFa was monitored on a FACSCalibur.

RESULTS

Bz-423 Kills BL-Derived B-Cell Lines. The apoptotic properties of Bz-423 were first evaluated using Ramos cells, a human malignant B-cell clone derived from BL that shares many characteristics with activated GC B cells. Other benzodiazepines are significantly less cytotoxic than Bz-423 despite binding more tightly to the PBR (Table 1). Other BL cell lines were also sensitive to Bz-423 (Fig. 2, A and B). The sensitivity of the different cell lines to Bz-423-induced death was fairly similar, with the LC_{50} ranging from 6 μM (ST486) to 10 μM (Namwala cells). To determine the potential impact of survival genes encoded or induced by latent EBV infection on the effectiveness of Bz-423, these experiments included EBV-negative cells and EBV-positive cells known to express latent viral gene products. The BL cells that do not contain EBV (Fig. 2A, Ramos, CA46, and ST486) are only slightly more sensitive than the group III EBV-positive cells (Fig. 2B, Daudi, Namwala, and Raji). Experiments assessing colony formation by Ramos and Raji cells in methylcellulose-based semisolid media for 1 week after treatment with Bz-423 for 24 h confirmed that treatment dose-dependently reduces clonogenic survival (data not shown).

Previous work has established that Bz-423-induced cell death is executed through mitochondria-dependent events beginning with superoxide production within 1 h of treatment. Superoxide production is the first detectable signal that triggers subsequent collapse of the mitochondrial electrochemical gradient and cell death events consistent with opening of the PTP (9). To determine whether the antiapoptotic proteins Bcl-2 and Bcl-x_{L}, which contribute to the regulation of mitochondrial pore function and cellular redox balance, limited the effectiveness of Bz-423, Ramos cells were generated that overexpress these gene products (Fig. 2C). The gene product-overexpressing cells acquired significant resistance to the toxic effects of CDDP (Fig. 2D).

Binding to Serum Albumin Modulates the Cytotoxic Effects of Bz-423. It is well established that nonspecific binding to serum albumin regulates the bioavailability of benzodiazepines by altering the free concentration of drug (26). In tissue culture media containing 10% (v/v) FBS with 10 μM Bz-423, nearly 99% of the compound is bound to BSA (data not shown). Therefore, initial studies with Bz-423 were performed with culture media containing 2% (v/v) FBS. However, media with less serum have lower levels of growth factors and other components that could also affect cell survival. To investigate

### Table 1: Comparison of Bz-423 with other benzodiazepines and high-affinity ligands of the PBR

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM)</th>
<th>LC_{50} (μM, 2% FBS)</th>
<th>GL_{50} (μM, 10% FBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-423</td>
<td>360</td>
<td>6.6 ± 0.3</td>
<td>11.5 ± 0.8</td>
</tr>
<tr>
<td>PK11195</td>
<td>8</td>
<td>69 ± 0.7</td>
<td>67 ± 2.3</td>
</tr>
<tr>
<td>4-ClDz</td>
<td>48</td>
<td>64 ± 1.4</td>
<td>62 ± 3.5</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>74300</td>
<td>&gt;80</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

**Statistical Analysis.** Statistical significance was assessed using Student’s t test unless otherwise noted. Ps are two-tailed, and data are given as mean ± SD of the mean.
the role of culture conditions on the activity of Bz-423, purified BSA was added to media containing 2% FBS in amounts to achieve a total BSA concentration equivalent to that contained in media with 5%, 7.5%, or 10% FBS. Titrating in BSA shifted the Bz-423 killing curve. When BSA was added to the culture medium, to yield the total amount contained in media supplemented with 10% FBS, the dose-response curve overlapped the dose response in media actually containing 10% FBS (Fig. 3). From these results, we conclude that binding to BSA accounts for the lower cytotoxicity (higher LC50) of Bz-423 observed in culture media with 10% versus 2% FBS.

**Higher Serum Conditions Reveal the Antiproliferative Effects of Bz-423.** To examine the effects of Bz-423 on proliferation, Ramos cells cultured in media with 10% FBS were continuously exposed to Bz-423 (20 μM) or vehicle control and analyzed over 3 days to determine the total number of live and dead cells by vital dye microscopy. Bz-423 dramatically reduced the increase in cell number (growth) over 3 days without causing a significant increase in dead cells over the same time (Fig. 4). To be certain that this effect on cell number truly resulted from reduced proliferation versus increased cell death balanced by ongoing proliferation, cell divisions were monitored using the membrane-specific fluorescent compound PKH67. An important feature of PKH67 is that after labeling a cell, the probe is partitioned equally between daughter cells upon cell division, making it possible to quantify divisions based on fluorescence intensity (27). PKH67 itself does not affect the viability or proliferation of Ramos cells (data not shown). To determine how Bz-423 affects cell divisions, cells were labeled with PKH67, treated with Bz-423 for 3 days, and analyzed by flow cytometry. Over this time frame, the maximal concentration of Bz-423 used in these experiments (20 μM) killed <10% of cells. Mean fluorescence intensity (gating on live cells) was compared for cells immediately after labeling (e.g., time 0) with cells treated as indicated for 3 days after dye labeling. As seen in Fig. 5, the decrease in mean fluorescence intensity after 3 days from the time 0 value is greater in control cells than in cells treated with Bz-423, and in additional dose-response experiments, the magnitude of the difference from control is directly proportional to the concentration of Bz-423 used (data not shown). Thus, Bz-423 dose-dependently prevented the decrease in PKH67 fluorescence intensity, demonstrating that it blocks proliferation.

### Table 2

MnTBAP modulates Bz-423-induced death and antiproliferative responses

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LC50 (μM, 2% FBS)</th>
<th>GI50 (μM, 10% FBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Ramos</td>
<td>6.6 ± 0.3</td>
<td>11.5 ± 0.8</td>
</tr>
<tr>
<td>Ramos + MnTBAP</td>
<td>12.3 ± 0.1</td>
<td>19.1 ± 1.2</td>
</tr>
<tr>
<td>Neo transfectant</td>
<td>6.5 ± 0.1</td>
<td>10.7 ± 1.4</td>
</tr>
<tr>
<td>Bcl-2 transfectant</td>
<td>10.8 ± 0.5</td>
<td>12.2 ± 1.6</td>
</tr>
<tr>
<td>Bcl-xL transfectant</td>
<td>9.5 ± 0.4</td>
<td>12.6 ± 1.1</td>
</tr>
</tbody>
</table>

**Fig. 2.** Bz-423 has cytotoxic activity in BL cell lines. Dose-dependent killing in media with 2% FBS was determined by PI exclusion. A, ST486 ( ), Ramos ( ), and CA46 ( ) cells; B, Daudi ( ), Raji ( ), and Namalwa ( ) cell lines. C, Ramos cells stably transfected as indicated express Bcl-2 or Bcl-xL at levels substantially greater than wild-type or vector control-transfected cells. D, cell death determined after 24 h of exposure to CDDP in media with 2% FBS for nontransfected Ramos cells ( ), vector control-transfected cells ( ), Bcl-2-expressing cells ( ), and Bcl-xL-expressing cells ( ). E, cell death after 24 h caused by Bz-423 using the same cells and conditions as in C.

**Fig. 3.** Serum albumin limits the bioavailability of Bz-423. Dose-dependent cytotoxicity of Bz-423 against Ramos cells was determined in culture media with 2% FBS containing final BSA concentrations of 0.54 mg/ml ( ), 1.35 mg/ml ( ), 2.03 mg/ml ( ), 2.7 mg/ml ( ), or 10% FBS that also contains 2.7 mg/ml BSA ( ). Viability after 24 h was determined by PI exclusion.

Table 2 MnTBAP modulates Bz-423-induced death and antiproliferative responses

LC50 was determined in media with 2% FBS, by PI exclusion. GI50 is based on growth inhibition after 3 days in media with 10% FBS and was determined by cell counting.
of cells after treatment with an equal concentration (20 μM) of clonazepam or PK11195 is indistinguishable from that of vehicle control-treated cells (Fig. 5). Although 4-ClDz also blocks proliferation, it is significantly less potent than Bz-423. These data are consistent with the results of cell counting experiments summarized in Table 1, where the drug concentration reducing growth by 50% (GI50) for Bz-423 is significantly less than that for other benzodiazepines and PK11195.

We next used a clonogenic assay to determine whether growth inhibition is permanent or whether it is reversed when the compound is removed from cells. Ramos cells were treated with vehicle control or Bz-423 (10 and 20 μM) for 3 days, during which time cell number was monitored daily by staining with trypan blue and counting using a hemocytometer (Fig. 6A). After 72 h, cells were removed from culture, washed extensively, and replated at equal concentrations (1 × 105 cells/ml) in media without any compound or vehicle. During this washout phase, cell number was again determined daily, and, as indicated in Fig. 6A, no differences in growth were observed between control cells and those treated previously with Bz-423. These results demonstrate that the antiproliferative effects of Bz-423 depend on the continuous presence of the drug, thus arguing that an irreversible genotoxic mechanism does not underlie its antiproliferative effects.

To determine whether the antiproliferative effects involved arrest of the cell cycle at a specific checkpoint, cells treated with Bz-423 for 24, 48, and 72 h were analyzed for DNA content to reveal the distribution of cells within the cell cycle. By 24 h, treatment with Bz-423 caused a significant increase in cells with G1-G0 DNA content and corresponding reductions in S and G2 cells (Fig. 6, B and C). No increase in hypodiploid DNA, a marker of apoptosis, was observed under these treatment conditions. In the continuous presence of Bz-423, the G1-G0 arrest persisted for up to 72 h, the longest point at which this response was measured (data not shown). Together, these results demonstrate that Bz-423 blocks the proliferation of Ramos B cells independent of its cytotoxic effects through a mechanism that arrests the cell cycle at the G1 checkpoint.

**ROS Are Involved in the Antiproliferative Response to Bz-423.**

Because intracellular ROS are increased by Bz-423 as an early, necessary step in the cytotoxic response, we hypothesized that growth arrest may also be mediated by ROS. To test this possibility, ROS was measured using DCHF-DA in cells treated with Bz-423 in media with 10% FBS (28). Similar to the data obtained in media with 2% FBS, treatment with Bz-423 leads to the dose-dependent production of ROS within 1 h (Fig. 7A). The fluorescent signal reflects the presence of superoxide because addition of the superoxide dismutase mimetic, MnTBAP (29), abrogates the fluorescence change (Fig. 7B).
In contrast, those BL lines that contain EBV and express the viral tendancy to easily undergo apoptosis to a variety of stimuli (32) maintain close phenotypic approximations of GC cells, including a proliferation rate similar to that of Bz-423, we show that, like primary GC cells in NZB/W mice and Ramos cells, these cell lines do not contain EBV (Ramos, ST486, and CA46) and express the viral latency-associated proteins (Daudi, Raji, and Namalwa) are resistant to apoptotic triggers, including calcium ionophores, serum depriviation, and IgM surface receptor cross-linking (19). Bz-423 triggers apoptosis in both groups of cells with only a modest increase in LC50 observed for the group III EBV-positive compared with the EBV-negative lines. Therefore, the resistance mechanisms resulting from the expression of EBV latent proteins do not significantly affect the mechanism of Bz-423.

Mutation of the p53 tumor suppressor gene is an important factor in the malignant transformation, progression, and therapeutic response to chemotherapy of BL (37, 38). In each of the cell lines tested, p53 function is abnormal. For example, in Ramos cells, one copy of the p53 gene is deleted, and the remaining allele encodes a mutant protein that is incapable of activating transcription or mediating apoptosis (39). Because Bz-423 is active in cells that express only mutant p53, its ability to induce apoptosis and inhibit cell proliferation is independent of p53 status.

The oncoproteins Bcl-2 and Bcl-xL can diminish therapeutic effectiveness of many cytotoxic drugs by preventing apoptosis (40, 41). Several mechanisms may be involved in the antiapoptotic effects of Bcl-2 and Bcl-xL. Paramount among these is their ability to keep the mitochondria PTP closed (42). Although their exact role in BL is not clear, Bcl-2 may be necessary to block the apoptotic effects of deregulated c-myc expression, which is a hallmark of BL (43, 44). We tested whether high-level expression of Bcl-2 or Bcl-xL blocked Bz-423 killing and observed only a slight increase in the LC50 and no change in the ability of Bz-423 to induce ROS (data not shown). By way of comparison, cells overexpressing these gene products are completely resistant to CDDP. Collectively, these experiments indicate that Bz-423 circumvents an important group of drug resistance mechanisms.

The mechanisms of action mediating apoptosis in response to 4-ClDz and PBR ligands such as PK11195 are not well understood. Some studies have, however, associated the cytotoxicity of these agents with increases in ROS or inhibition of coupled mitochondrial respiration. Using HL60 and KG1A leukemia cells, Fennell et al. (45) have shown that PK11195 induces a ROS response required for the subsequent collapse of mitochondrial transmembrane gradient, which is itself necessary for the apoptotic response. 4-ClDz and PK11195 reduce cellular oxygen consumption and coupled mitochondrial respiration, both effects that are often linked to increased ROS production (12, 46, 47). At concentrations substantially greater than their KJ values for binding to the PBR, both compounds also reduce oxygen consumption in isolated mitochondria under conditions similar to those in which we found that Bz-423 induces superoxide [i.e., respiratory state 3 (12)]. These similarities suggest that cytotoxic activity of Bz-423, PK11195, and 4-ClDz could arise from a common mechanism and possibly a common molecular target.

At subapoptotic concentrations, Bz-423 has profound antiproliferative effects of Bz-423 on ROS levels (Fig. 8). ROS in Ramos cells treated with 10 μM Bz-423 or 20 μM (○) in media containing 10% FBS as a function of time were monitored by DCF fluorescence. The results presented are the mean of five separate determinations. P < 0.05 for the differences between Bz-423 and control for each end point.
BBURKITT’S LYMPHOMA CELLS ARE SENSITIVE TO Bz-423
tive effects. Because the proportion of cells in the G1-G0 phase
significantly increased with treatment, Bz-423 appears to affect the G1
checkpoint. Experimental agents that cause cell cycle arrest are pres-
ently attracting considerable interest as an approach to control cancer.
For example, flavopiridol and 7-hydroxyxastrosporine block Cdk
functions causing G1 and G2 arrest; both are currently being tested as
novel anticancer drugs in human trials (48).

Under conditions where Bz-423 induces cell cycle arrest (and not
apoptosis), we observed a sustained ROS. This signal is functionally
significant in the antiproliferative mechanism because antioxidants
((MnTBAP) attenuate the efficacy of Bz-423-mediated growth inhibi-
tion. Thus, just as ROS are necessary for Bz-423-mediated apoptosis,
they also appear to regulate Bz-423-induced cell cycle arrest. ROS can
cause withdrawal from the cell cycle at the G1 checkpoint. For
example, peroxides suppress S-phase entry in a dose-dependent man-
ner by inhibiting cyclin E/Cdk2 activity, which controls the G1
checkpoint (49). In addition, the ataxia telangiectasia mutated protein
(pATM) is thought to function, in part, as a sensor of oxidative stress.
pATM is required for both the G1 and G2 checkpoint responses and
causes induction of p53 in cells treated with tert-butyl hydroperoxide
(50). p53 transcriptionally activates genes including p21 (Cip1/Waf1),
which binds to and inhibits the activity of cyclin E/Cdk2 (51). Be-
cause the mutated p53 present in Ramos cells is transcriptionally
inactive, other mechanisms must be involved in Bz-423-mediated growth arrest (39). In this regard, it is interesting to note that p21 can
be induced independently of p53 in response to certain chemicals (52).

Other benzodiazepines and ligands of the PBR have been reported
to affect cell cycle progression, resulting in growth arrest via unde-
limited mechanisms (53–56). However, several lines of evidence sug-
gest that direct PBR binding is not involved in this response. First,
Leydig cells in which PBR expression is silenced by antisense trans-
faction survive and continue to proliferate normally (16). Second, to
affect proliferation or survival, PBR ligands must be present at con-
centrations greatly exceeding those necessary to saturate the PBR
[e.g., 1000 times the Kd (14, 15)]. Third, among the PBR ligands,
binding affinity for the PBR has not been consistently shown to
correlate with effectiveness at inhibiting cell growth (13). Thus, these
reports and our data with Bz-423 suggest that benzodiazepines have
antiproliferative effects via an alternative target.

Second-wave signaling responses that intervene between benzodi-
azepines binding and processes leading to growth arrest are also not
well understood. One possibility is that polyamine metabolism is
involved in this signaling. The antiproliferative effects of 4-ClDz are
associated with a rapid reduction in ODC activity in Nb2 lymphoma
cells (57). This response is expected to reduce intracellular poly-
amines, including putrescine, spermine, and spermidine, which play
key roles in the regulation of cell proliferation. Consequently, anti-
proliferative signals induced by benzodiazepines may stem from
reduced ODC expression or increased expression of antizyme 1, a
negative regulator that targets ODC for proteosomal degradation. Of
particular interest, under conditions that favor increased ROS, ODC
activity is reduced, in part through optimization of active site thiol groups
(58, 59). Thus, these observations suggest that polyamine pathways
may be the critical mediators between Bz-423-induced ROS and
growth arrest.

In conclusion, Bz-423 is a novel 1,4-benzodiazepine that has both
cytotoxic and antiproliferative properties against transformed Bcells.
Increased ROS are essential for mediating both cell death and growth
arrest, and the magnitude of the ROS response may determine the fate
of a Bz-423-treated cell. Low concentrations of Bz-423 produce levels
of ROS that ultimately result in growth arrest. Higher amounts of
ROS may cause proportionally greater levels of ROS that trigger
apoptosis. Compounds that act on mitochondria to induce apoptosis
are currently being explored as pharmacological candidates to use
against cancer and related disease processes (60). Bz-423 is a prom-
ising therapeutic candidate because cells respond to it regardless of
Bcl-2 or Bcl-XL levels or EBV and p53 status, factors that limit the
effectiveness of current chemotherapeutics. Further work is now
needed to identify the molecular target that responds to Bz-423 and
orchestrates the cellular response.
The Proapoptotic Benzodiazepine Bz-423 Affects the Growth and Survival of Malignant B Cells


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