A Novel Benzodiazepine Increases the Sensitivity of B Cells to Receptor Stimulation with Synergistic Effects on Calcium Signaling and Apoptosis*

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Bz-423 is a 1,4-benzodiazepine with selective lymphotoxic properties and potent therapeutic activity against lupus-like disease in autoimmune mice. In NZB/W lupus-prone mice, Bz-423 specifically kills germinal center B cells, which are the cells that drive disease both in this model and in human systemic lupus erythematosus. In this report, the mechanistic basis for the selective action of Bz-423 is investigated. We show that Bz-423 induces superoxide as an immediate early response and that this reactive oxygen species is more effective as a second messenger death signal in B cells activated by B cell receptor stimulation compared with resting cells. As a result, low [Bz-423] that are not cytotoxic to non-stimulated cells kill stimulated cells in synergy with anti-immunoglobulin M antibodies. Subsequent experiments demonstrated that Bz-423 extends the rise in intracellular calcium that accompanies anti-immunoglobulin M stimulation, and this effect mediates the synergistic death response. Because B cell hyperactivation and altered calcium signaling is a distinguishing feature of autoreactive lymphocytes in lupus, the mechanism by which Bz-423 induces apoptosis preferentially targets disease-causing cells on the basis of their activation state. Thus, molecules like Bz-423 could form the basis for new and selective anti-lupus agents.

Systemic lupus erythematosus (SLE) is characterized by a spectrum of auto-antibodies that are the products of B cells that escape peripheral tolerance (1). Although immunosuppressive, lymphotoxic drugs are effective for many patients, these drugs cause life-threatening side effects that account for a notable portion of lupus-related deaths (2). Therefore, agents with greater selectivity against disease-causing lymphocytes could significantly advance the treatment of SLE and related disorders.

Because lymphocyte toxicity is an established treatment modality, it offers a starting point to develop new classes of therapeutic molecules. Toward this end, a library of 1,4-benzodiazepines was previously screened for cytotoxic members against Ramos B cells, a neoplastic B cell line with a germinal center (GC) phenotype (3). These studies led to the identification of Bz-423 (Fig. 1), a pro-apoptotic molecule whose mechanism of action depends upon an increase in intracellular superoxide ($O_2^-$), produced as a result of the interaction of Bz-423 with a mitochondrial molecular target (3). Comparison of Bz-423 with benzodiazepines used clinically and ligands of the peripheral benzodiazepine receptor reveals the unique cytotoxicity of this compound against B cells in vitro (3).

Based upon its lymphotoxic properties in vitro, we explored the cytotoxic properties of Bz-423 in two animal models of SLE, the MRL-lpr and the (NZB x NZW)/F1 (NZB/W) mouse strains. Lupus-like disease in MRL-lpr mice is T-cell-dominated and is linked to defective Fas signaling. These defects allow a population of autoreactive CD4+ T cells to expand instead of undergoing apoptosis in response to physiologic cues (4, 5). In these animals, treatment with Bz-423 specifically reduced activated CD4+ cells (6).

In contrast, an expanded population of activated B cells within GCs mediates disease in NZBW mice. These activated B cells drive autoantibody production and pathogenicity that ultimately results in lupus nephritis (7, 8). Administering Bz-423 to NZBW mice selectively targeted activated GC B cells and reduced the number and size of GCs. These mice also had reduced autoantibody levels and improved glomerulonephritis. Although a complete molecular explanation for the abnormal GC persistence in NZBW mice is not yet known, current evidence implicates defects in normal tolerance mechanisms, including defective B cell receptor (BCR)-mediated activation-induced cell death (AICD) (9). In normal immune BALB/c mice, Bz-423 neither decreased viability nor increased apoptosis of splenic lymphocytes nor affected physiologic GC responses. Thus, at therapeutic doses, Bz-423 selectively kills disease-causing cells.

Because GC-derived cells have also been shown to mediate disease pathogenesis in human SLE, we were particularly interested in understanding the mechanistic basis for the selectivity of Bz-423 in these cells. B cells acquire and maintain a GC phenotype through a process that depends upon stimulation of BCR. GC homeostasis also depends upon BCR-coupled apoptosis, which is defective in SLE and NZBW mice (10). These observations suggested that BCR stimulation might be
involved in enhancing sensitivity to Bz-423, resulting in the selectivity observed in the NZB/W studies. Therefore, we embarked on a line of investigation testing the hypothesis that BCR stimulation facilitates killing by Bz-423.

**Experimental Procedures**

Reagents—Bz-423 was synthesized as described previously (11). Unless noted, all reagents were obtained from Sigma. Dihydroethidium (DHE), was obtained from Molecular Probes (Eugene, OR). Manganese(III)meso-tetrakis(4-benzoic acid)porphyrin (MnTBAP) was purchased from Alexis Biochemicals (San Diego, CA). Polyclonal goat anti-mouse immunoglobulin M (IgM) was obtained from ICN (Aurora, OH); soluble goat Fab, anti-human IgM was obtained from Southern Biotechnology Associates (Birmingham, AL); anti-human CD40 monoclonal antibody clone 5C3 was obtained from Pharmingen (San Diego, CA).

**Animals, Primary B Cells, Cell Lines, and Culture**—6-week-old female Balb/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were euthanized and the spleens were removed for analysis. Splenocytes were obtained by mechanical disruption with isotonic lysis of red blood cells. B cell-rich fractions were prepared by negative selection using magnetic cell sorting with CD4, CD8a, and CD11b-coated microbeads (Miltenyi Biotec, Auburn, CA). Ramos cells were purchased from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and 1-glutamine (290 μg/ml). Media for primary cells also contained 2-mercaptoethanol (50 μM). In vitro studies were performed in media containing 2% FBS and 0.5% Me2SO.

**Analysis of Lymphoid Cell Markers**—Surface markers were detected using fluorescent-conjugated anti-Thy 1.2 (1 μg/ml, Pharmingen) and/or anti-B220 (1 μg/ml, Pharmingen). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**B Cell Stimulation**—Cell lines were stimulated with soluble goat Fab, anti-human IgM (1 μg/ml) and/or purified anti-human CD40 (2.5 μg/ml). Primary mouse B cells were stimulated with affinity-purified goat anti-mouse IgM (20 μg/ml, ICN) immobilized in culture wells and/or soluble purified anti-mouse CD40 (clone HM40-3, 2.5 μg/ml, Pharmingen). Bz-423 was added 10 min before stimuli were applied. Inhibitors were added 30 min prior to Bz-423.

**Determination of Synergy**—Synergistic effects upon cytotoxicity between Bz-423 and anti-IgM were evaluated using isologram analysis as described previously (12, 13). For this analysis, two series of dosage curves were obtained. First, using fixed concentrations of Bz-423, the EC50 values (i.e. the concentration at which 50% of the cells are dead) of anti-IgM were measured. Then, at fixed concentrations of anti-IgM, the EC50 values of Bz-423 were obtained. These EC50 values were used to construct isolograms.

**Detection of Cell Death and Intracellular ROS**—Cell viability, hypodiploid DNA, and O2 were measured by flow cytometry staining with propidium iodide (PI) and DHE, as described previously (3).

**Measurement of Intracellular Calcium Concentration (Ca2+)**—Using Fura 2—The cell-permeable acetoxyethyl ester form of Fura 2 (2.5 μM, Fura 2-AM) was added to Ramos cells (10^7/ml) in loading buffer (1 mM CaCl2, 1 mM MgCl2, 1% FBS). Fura 2-AM is retained in the cell after being de-esterified by esterases in the cytoplasm; when excited at two alternate wavelengths (340 and 380 nm), the ratio of fluorescence emission at 510 nm is related to [Ca2+], independently of intracellular dye concentration. After incubating 30 min at 37 °C to load with Fura 2, cells were washed and then resuspended in loading buffer at 10^7/ml; 200 μl (2 × 10^6 cells) were added per well to a 96-well plate. As indicated, cells were pre-incubated with Bz-423 or solvent control for 2 min, warmed to 37 °C, and anti-IgM (1 μg/ml) was added, immediately after which fluorescence (510 nm) was monitored every 15 s over 8 min using a microplate spectrofluorometer (Molecular Devices). Fluorescence specific to Fura 2 was calculated as the difference between the fluorescence intensities from Fura 2-loaded cells and unloaded cells. The maximal fluorescence at 380 nm determined by the addition of ionophore Br-A23187 (2 μg/ml) and minimum fluorescence at 340 nm determined by the addition of EGTA (35 mM) to Bz-423-treated cells were used to calculate [Ca2+], as described (14). Detection of [Ca2+]i within Single Cells using Flow Cytometry—Ramos cells (10^7/ml) were incubated (30 min, 37 °C) with the cell-permeable fluorescent dye Fluo-3 AM (4 μg/ml) in loading buffer (1 mM CaCl2, 1 mM MgCl2, 1% FBS) containing Pluronic (0.02%) and Probenecid (4 μM). The cells were washed with phosphate-buffered saline and resuspended in loading buffer at 10^7/ml. Cell suspensions were incubated with Bz-423 for 10 min at room temperature, warmed to 37 °C, then analyzed on the flow cytometer continuously for 10 min with the temperature maintained at 37 °C. ([Ca2+]i) was calculated as described previously based upon maximum Fluo-3 fluorescence (determined by the addition of 2 μg/ml Br-A23187) and minimum fluorescence (determined by addition of 35 mM EGTA to Br-A23187-treated cells).

**Statistical Analysis**—Statistical analysis was conducted by using the SPSS software package. All data are presented as mean ± S.D.

**Results**

**Bcr-stimulation Sensitizes Cells to Bz-423—**B cells acquire and maintain a GC phenotype through a process that depends upon stimulation of BCRs (10). To account for the selective GC reduction in the treated NZB/W mice, we postulated that BCR stimulation facilitates killing by Bz-423. To test this hypothesis, primary B cells were isolated by negative selection from splenocytes harvested from Balb/c mice. B cell-enriched isolates (>95% B220^−/Thy1.2^−) were incubated with immobilized polyclonal anti-IgM to extensively cross-link BCRs. This strong stimulus expectedly provoked AICD in ~40% of cells (Fig. 2, white bars). When added alone, Bz-423 (4 μM) killed 20% of the cells (black bars). When Bz-423 (4 μM) was combined with anti-IgM, killing was greater than with either agent alone (gray bars). Cells were also co-stimulated during these treatments with antibody specific for CD40, because CD40 stimulation is a GC cell-surface signal linked with lupus pathogenesis (15, 16). In the presence of anti-CD40, killing by anti-IgM alone was completely abrogated (40% killing decreased to 5%; see Fig. 2) and the response to Bz-423 was also decreased (20 to 9%). Interestingly, however, the response to Bz-423 plus anti-IgM was not reduced to the same extent by CD40 (anti-IgM alone, 5%; Bz-423 alone, 9%; anti-IgM plus Bz-423, 38% kill-
ing). These data indicate that in primary B cells given a survival stimulus through CD40, BCR and Bz-423 cooperate to increase cytotoxic effects.

Because primary B cells can not be maintained in culture over long periods of time, we chose to further investigate synergy in the immortalized, follicular B cell lymphoma Ramos cell line. Ramos cells are an Epstein-Barr virus-negative B cell lymphoma line and express surface markers and Bel-6 consistently with a GC phenotype (17, 18). More importantly, because Ramos cells mount an apoptotic response to BCR ligation like GC cells (19, 20), this line was suitable to examine the synergy between Bz-423 and BCR stimulation on cell death.

Soluble anti-IgM Fab2 dose-dependently killed Ramos cells (Fig. 3A). When used at limiting concentrations (<1 μg/mL), anti-IgM Fab2 had very little effect upon their survival. When anti-IgM Fab2 (1 μg/mL) was applied together with increasing concentrations of Bz-423, the death response of Ramos cells to Bz-423 increased. Compared with cells treated with Bz-423 alone or Bz-423 plus a control antibody, the dose-response of Ramos cells to soluble anti-IgM Fab2 resulted in 50% killing were plotted as axial points, and the straight line connecting these points was drawn to represent the dose pairs that would produce this effect additively (Fig. 3C). When actual combinations of Bz-423 and anti-IgM Fab2 producing 50 ± 5% killing were plotted, the points fell below this line, confirming that BCR stimulation plus Bz-423 have a super-additive or synergistic effect upon cell death in Ramos cells. Together, these results indicate that Bz-423 significantly increased activity in activated B cells, which may account for the therapeutic response observed in lupus mice.

**Bz-423 Augments Fab2-induced Apoptotic Signaling**—To understand the basis for the synergistic death response, we probed the mechanism of cell death engaged in Ramos cells by a synergistic combination of Bz-423 (5 μmol/L) and anti-IgM Fab2 (1 μg/mL). To confirm that apoptosis was being induced, we treated Ramos cells with doses of reagents equipotent at inducing cell death (i.e., 10 μmol/L Bz-423 alone, 10 μg/mL anti-IgM Fab2 alone, or 5 μM Bz-423 plus 1 μg/mL anti-IgM Fab2) and examined the cellular morphology. Each treatment resulted in cytoplasmic vacuolization, nuclear condensation, and plasma membrane blebbing, which is consistent with apoptosis (Fig. 4A, and data not shown). Next, we examined other signaling events that have been shown to be involved in cell death induced by Bz-423 or anti-IgM. O$_2^\bullet$ is rapidly induced by Bz-423 and is necessary for apoptosis and cell killing by this agent. However, inducible gene and protein expression, and specific elements of the apoptotic machinery, including caspase activity and the mitochondria permeability transition (MPT) are not required for Bz-423 to kill cells (3). In contrast, BCR-coupled AICD requires caspase activity, the MPT, and inducible protein synthesis, and is critically mediated by an early rise in intracellular calcium (21). Based on these differences, we treated cells with a range of specific inhibitors to distinguish which signaling events were necessary to induce the synergistic death response.

We first tested whether ROS are required for synergistic killing and whether co-treatment with anti-IgM increases the Bz-423-induced O$_2^\bullet$ response. The antioxidants vitamin E and MnTBAP each protected against killing by Bz-423 and the Bz-423-anti-IgM Fab2 combination, without significantly re-

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**Fig. 3. Bz-423 and anti-IgM Fab2 act cooperatively to increase death in Ramos cells.** A, dose-response of Ramos cells to soluble anti-IgM Fab2. Data are expressed as percent of cells PI-positive at 48 h. B, effect of BCR stimulation with soluble anti-IgM Fab2 (C), 1 μg/mL compared with either nonspecific Fab2 ( ), 1 μg/mL or no antibody ( ) on the dose response to Bz-423. C, an isobologram was constructed to determine the synergistic cytotoxic effects between Bz-423 and anti-IgM Fab2. Points showing the actual dose pairs of Bz-423 and anti-IgM Fab2 resulting in 50% cell death are plotted ( ). A straight line was drawn between the EC$_{50}$ value of Bz-423 in the absence of anti-IgM Fab2, and the EC$_{50}$ value of anti-IgM in the absence of Bz-423. The actual values of the agents in combination fall below this line, confirming a synergistic effect.
Bz-423-induced ROS is necessary for synergy, a further increase of O$_2^-$ did not seem to account for the supra-additive death response.

Cycloheximide, cyclosporin A, Z-VAD-fmk, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were used in similar experiments to determine whether protein synthesis, the MPT, caspases, and the influx of extracellular calcium, respectively, were necessary for synergistic killing. As expected, each of these agents protected against killing by anti-IgM Fab$_2$ and none protected against killing by Bz-423 (see Fig. 4B). Importantly, each agent abolished the synergistic effects of the Bz-423 and Fab$_2$ combination. These data indicate calcium influx, the MPT, and inducible protein synthesis in synergistic killing. Because each of these is a component of the apoptotic AICD pathway, these results suggest that Bz-423-induced O$_2^-$ modulates the death mechanism normally coupled to BCR-stimulation in such a way as to produce a supra-additive death response.

Bz-423-induced ROS Affects BCR-coupled Calcium Signaling—Because increased [Ca$^{2+}$], responses are associated with B cell developmental arrest and deletion (23). To measure the [Ca$^{2+}$], in anti-IgM-stimulated cells in the presence of Bz-423, Ramos cells were pre-loaded with Fura-2, treated with Bz-423 or solvent control, and then stimulated with anti-IgM. Fura-2 is a calcium indicator that undergoes a shift in maximal absorption upon binding Ca$^{2+}$, allowing ratiometric determination of [Ca$^{2+}$]. Fluorescence emission at 510 nm was monitored in response to excitation at 340 and 380 nm as a function of time on a microtiter plate reader. The ratio of fluorescence (340 nm/380 nm) was used to calculate the [Ca$^{2+}$]. Brief exposure to Bz-423 (5 μM) prior to treatment with anti-IgM Fab$_2$ resulted in a higher peak [Ca$^{2+}$] that reached a maximum of 200 nM (as compared with 120 nM with anti-Fab$_2$ alone), and the normally transient response was prolonged (Fig. 5). Even after 10 min following BCR stimulation, [Ca$^{2+}$] was ~180 nM in the Bz-423-treated cells, whereas in control cells, the level returned to baseline (~50 nM) within 2 min after the addition of anti-IgM Fab$_2$. These results suggest that Bz-423 is indeed able to modulate calcium signaling induced by BCR activation.

Next, we sought to evaluate whether Bz-423-induced ROS was involved in modulating [Ca$^{2+}$]. In these experiments, [Ca$^{2+}$] was measured at the single cell level, which allowed us to determine both the fraction of cells responding and the magnitude of the response within individual cells. Fluo-3 was
used to make these [Ca^{2+}] measurements because it is a calcium-sensitive fluorophore with properties suitable for flow cytometry. To confirm that the findings obtained with Fura-2 on the plate reader could be repeated with Fluo-3, Ramos cells were loaded with Fluo-3-AM and incubated with Bz-423 or solvent control for 5 min. Fluo-3 fluorescence was measured as a function of time after the addition of anti-IgM, during which samples were maintained at 37 °C. Consistent with the results above, Bz-423 caused the magnitude and duration of the calcium response to Fab2, to increase in the majority of cells. 50% of cells reach a peak calcium level of > 8-fold over baseline, compared with 10% of control cells, and the elevation is prolonged. After 10 min, 64% of cells continued to have a fluorescence intensity above the baseline (Fig. 6). In the control cells, a rapid increase in calcium was induced by Fab2 and resolved within 3 min, after which time, >90% of cells had fluorescence at or about the median fluorescence intensity of unstimulated cells. Thus, by contrast, the calcium response in cells treated with Bz-423 is both amplified and prolonged.

BAPTA, which chelates extracellular calcium and blocks synergistic killing, was used in these experiments to determine whether the increased [Ca^{2+}], resulting from Bz-423 depends upon extracellular Ca^{2+}. Fluo-3-loaded cells were pre-incubated (30 min) with BAPTA prior to Bz-423 or vehicle control. When cells pre-treated with either BAPTA were treated with Bz-423 and then anti-IgM Fab2, the increase in [Ca^{2+}], associated with Bz-423 was significantly blunted (Fig. 6). As well, BAPTA reduced the calcium response to anti-IgM Fab2, in the absence of Bz-423. Because vitamin E reduces Bz-423-induced O_2^-, it was used to determine the functional importance of ROS in modulating [Ca^{2+}]. Vitamin E also significantly reversed the effects that Bz-423 had upon anti-IgM-induced [Ca^{2+}], (Fig. 6). In contrast, vitamin E had no effect upon the calcium response to anti-IgM in the absence of Bz-423.

Taken together, our data show that Bz-423 dramatically increases BCR-coupled calcium signaling and that Bz-423-induced ROS is critical for affecting the increase. Thus, Bz-423 plus anti-IgM produce a synergistic death response because their individual signaling pathways intersect to coordinately affect [Ca^{2+}].
B-cell receptor signaling

Bz-423 Modulates B Cell Receptor Signaling

Fig. 6. Sustained increase in [Ca\(^{2+}\)], depends on ROS and extracellular calcium. Fluo-3-loaded Ramos cells in the absence or presence of BAPTA (500 µM) or MnTBAP (100 µM) were incubated with Bz-423 (10 µM) or vehicle (Me\(_2\)SO), brought to 37 °C, and then analyzed by flow cytometry. Anti-IgM Fab2 (1 µg/ml) was added 30 s after the collection was started (break in collection). [Ca\(^{2+}\)], correlates with Fluo-3 fluorescence. Channels are colored according to cell density. Yellow cell number ≥ 50% of peak height (PH) > dark blue ≥ 25% PH > orange ≥ 12% PH > light blue ≥ 6% PH > pink ≥ 3% PH > green ≥ 1% PH. Numbers indicate the percentage of cells at the end of the collection period (black arrow) with elevated [Ca\(^{2+}\)].

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