

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.

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) F_1 (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 NZB/W mice. These activated B cells drive autoantibody production and pathogenicity that ultimately results in lupus nephritis (7, 8). Administering Bz-423 to NZB/W 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 NZB/W 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 NZB/W mice (10). These observations suggested that BCR stimulation might be

Systemic lupus erythematosus (SLE)¹ 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,

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¹ The abbreviations used are: SLE, systemic lupus erythematosus; GC, germinal center; NZB/W, (NZB x NZW) F_1 ; BCR, B cell receptor; AICD, activation-induced cell death; MnTBAP, manganese(III)meso-tetrakis(4-benzoic acid)porphyrin; PI, propidium iodide; MPT, mitochondria permeability transition; ROS, reactive oxygen species; Z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; EC₅₀, concentration of agent at which 50% of the cells are killed; ER, endoplasmic reticulum; InsP₃, inositol-1,4,5-trisphosphate; O_2^- , superoxide; PH, peak height.

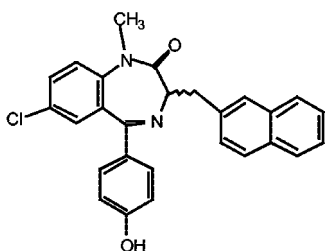


FIG. 1. Chemical structure of Bz-423.

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 Fab₂ 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 L-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 with fluorescent-conjugated anti-Thy 1.2 (1 µg/ml, PharMingen) and/or anti-B220 (1 µg/ml, PharMingen). Samples were analyzed on a FACS-Calibur flow cytometer (BD Biosciences).

B Cell Stimulation—Cell lines were stimulated with soluble goat Fab₂ 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 isobologram 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 isobolograms.

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⁺]_i Using Fura 2—The cell-permeable acetoxymethyl ester form of Fura 2 (2.5 µM, Fura 2-AM) was added to Ramos cells (10⁷/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⁺]_i, 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 10⁶/ml; 200 µl (2 × 10⁵ 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

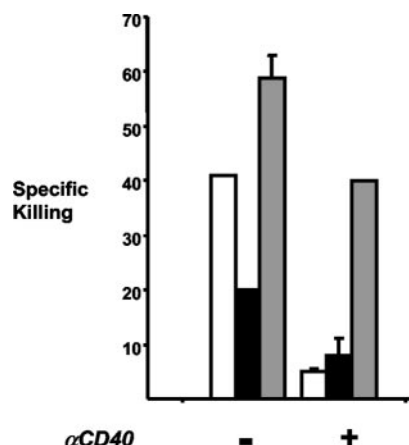


FIG. 2. Effect of BCR and CD40 stimulation on Bz-423-induced apoptosis of primary B cells. Specific killing of primary B cells after treatment with Bz-423 (4 µM) in the presence of indicated stimuli (goat anti-mouse IgM immobilized in culture wells, soluble-purified anti-mouse CD40, 2.5 µg/ml) after 18 h. Mean and standard deviation of specific killing (to account for spontaneous death of primary cells in culture) was calculated by dividing the difference in percentage of dead cells in Bz-423 and control conditions by the percentage of live cells under control conditions. White bars, anti-IgM; black bars, Bz-423; and gray bars, anti-IgM plus Bz-423.

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 Br-A23187-treated cells were used to calculate [Ca²⁺]_i as described (14).

Detection of [Ca²⁺]_i within Single Cells using Flow Cytometry—Ramos cells (10⁷/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 10⁶/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-survival 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 Bcl-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 $\mu\text{g}/\text{ml}$), anti-IgM Fab₂ had very little effect upon their survival. When anti-IgM Fab₂ (1 $\mu\text{g}/\text{ml}$) 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 Bz-423 in the presence of anti-IgM Fab₂ shifted to lower concentrations (Fig. 3B). Anti-CD40 did not change the response of these cells to either Bz-423 alone or the Bz-423-anti-IgM Fab₂ combination (data not shown).

Isobolograms were used to confirm that Bz-423 in combination with anti-IgM Fab₂ produce an effect whose magnitude is significantly increased relative to each individual agent. The concentrations of Bz-423 and anti-IgM Fab₂ that each result in 50% death 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 $50 \pm 5\%$ 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 μM) and anti-IgM Fab₂ (1 $\mu\text{g}/\text{ml}$). To confirm that apoptosis was being induced, we treated Ramos cells with doses of reagents equipotent at inducing cell death (*i.e.* 10 μM Bz-423 alone, 10 $\mu\text{g}/\text{ml}$ anti-IgM Fab₂ alone, or 5 μM Bz-423 plus 1 $\mu\text{g}/\text{ml}$ 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^- 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^- 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-

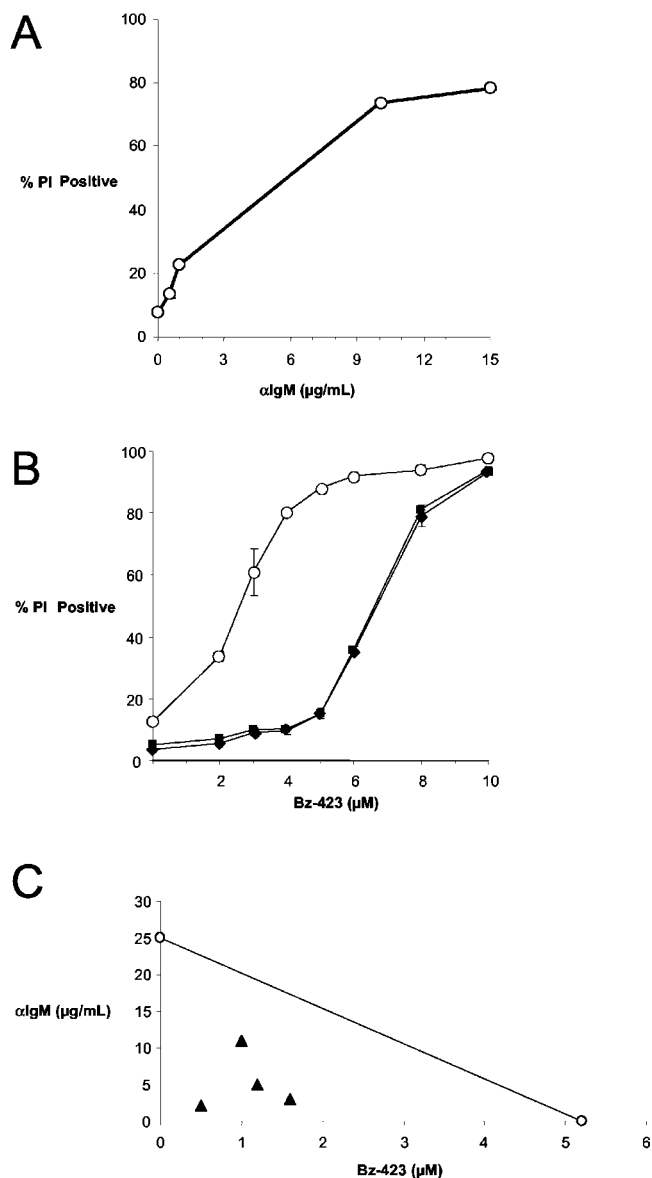


FIG. 3. Bz-423 and anti-IgM Fab₂ act cooperatively to increase death in Ramos cells. A, dose-response of Ramos cells to soluble anti-IgM Fab₂. Data are expressed as percent of cells PI-positive at 48 h. B, effect of BCR stimulation with soluble anti-IgM Fab₂ (○, 1 $\mu\text{g}/\text{ml}$) compared with either nonspecific Fab₂ (■, 1 $\mu\text{g}/\text{ml}$) or no antibody (◆) on the dose response to Bz-423. Data are expressed as percent of cells PI-positive at 48 h. At this concentration, anti-IgM Fab₂ was not significantly cytotoxic as a single agent. C, an isobologram was constructed to determine the synergistic cytotoxic effects between Bz-423 and anti-IgM Fab₂ resulting in 50% cell death are plotted (▲). A straight line was drawn between the EC₅₀ value of Bz-423 in the absence of anti-IgM Fab₂ and the EC₅₀ 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.

ducing death caused by a high concentration of anti-IgM Fab₂ alone (Fig. 4B). Because these results demonstrated that Bz-423 induced O_2^- is necessary for synergistic killing, we measured O_2^- during combined treatment to determine whether a greater ROS response correlated with the increased killing observed under synergistic conditions. Surprisingly, staining for O_2^- increased equivalently in response to Bz-423 alone or together with anti-IgM Fab₂ (Fig. 4C). Hence, the increase in ROS was strictly proportional to the concentration of Bz-423, and BCR stimulation did not affect the magnitude or duration of this response (Fig. 4C, and data not shown). Thus, although

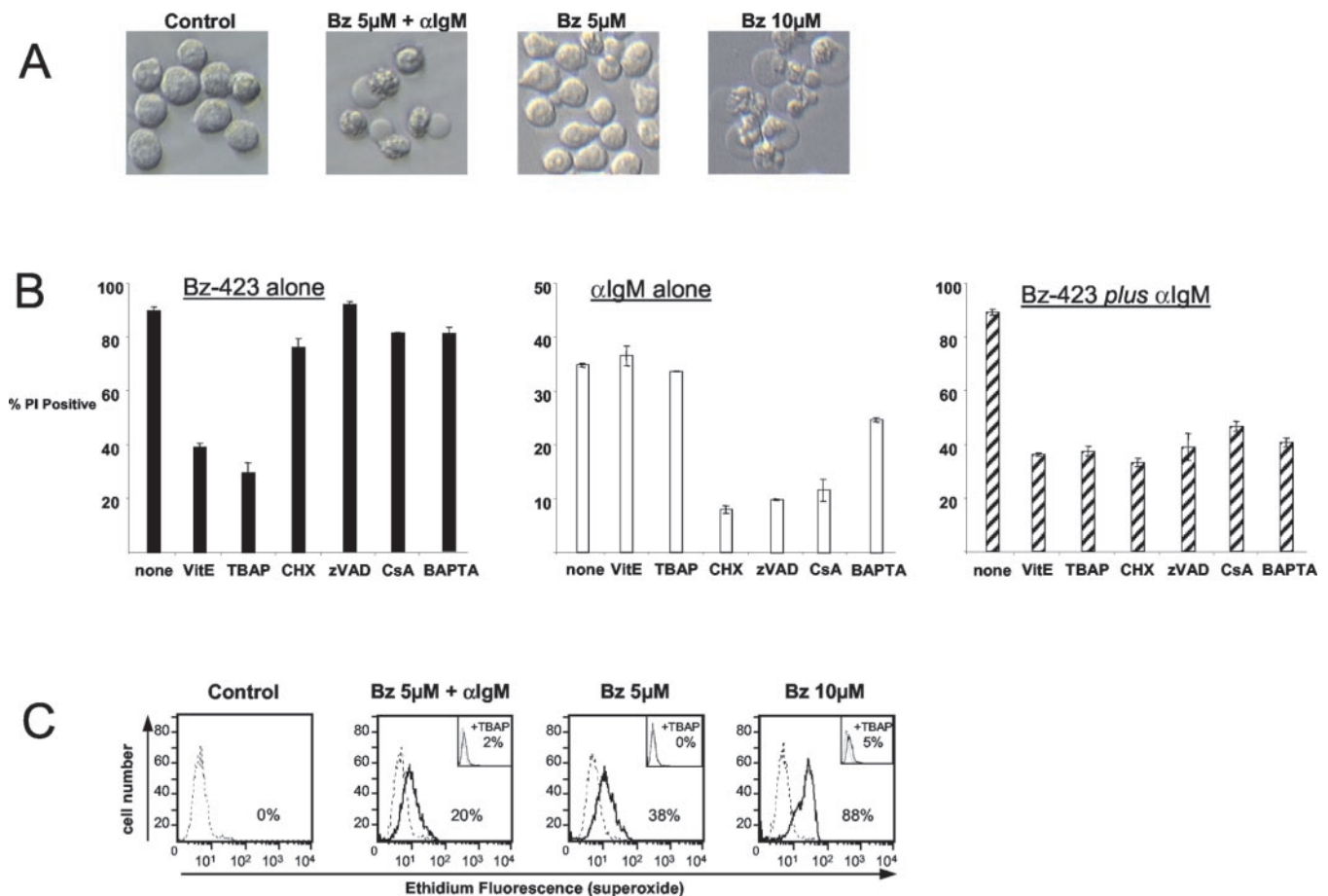


FIG. 4. Characterizing cell death under synergistic conditions. *A*, interference contrast microscopy (400 \times magnification) demonstrated a similar apoptotic appearance of cells treated with 5 μ M Bz-423 plus anti-IgM Fab₂ or cells treated only with 10 μ M Bz-423. *B*, cytotoxicity as a function of key mediators of apoptosis. Ramos cells were pre-treated with inhibitors as follows: vitamin E (100 μ M); MnTBAP (100 μ M); CHX (1 μ g/ml); z-VAD (100 μ M); CsA (0.5 μ M); and BAPTA (500 μ M). Cell death was induced by treating cells with either Fab₂ alone (white bars, 25 μ g/ml), Bz-423 alone (black bars, 10 μ M), or (striped bars) anti-IgM Fab₂ (1 μ g/ml) plus Bz-423 (5 μ M). *C*, O₂⁻ response to Bz-423 was not affected by anti-IgM Fab₂. Ramos cells were treated with anti-IgM Fab₂ alone (dashed lines, control), Bz-423, or the combination of these agents at the indicated concentrations (solid lines). After 1 h, the O₂⁻ response was measured using DHE by flow cytometry. *Insets*, MnTBAP (100 μ M) limited the O₂⁻ response.

Bz-423-induced ROS is necessary for synergy, a further increase of O₂⁻ 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 Fab₂ and none protected against killing by Bz-423 (see Fig. 4*B*). Importantly, each agent abolished the synergistic effects of the Bz-423 and Fab₂ combination. These data implicate 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 O₂⁻ 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 [Ca²⁺]_i is an immediate and integral part of BCR-coupled signaling and ROS can modulate calcium release and re-uptake mechanisms, we hypothesized that Bz-423 amplifies BCR-coupled calcium signaling by means of a ROS-dependent mechanism. [Ca²⁺]_i increases rapidly in response to BCR stimulation, and the magnitude and duration of this increase determine the cellular response to stimulation

(22). In some instances, more exaggerated [Ca²⁺]_i responses are associated with B cell developmental arrest and deletion (23). To measure the [Ca²⁺]_i 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 Ca²⁺, allowing ratiometric determination of [Ca²⁺]_i. Fluorescence emission at 510 nm was monitored in response 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 [Ca²⁺]_i. Brief exposure to Bz-423 (5 μ M) prior to treatment with anti-IgM Fab₂ resulted in a higher peak [Ca²⁺]_i that reached a maximum of 200 nM (as compared with 120 nM with anti-Fab₂ alone), and the normally transient response was prolonged (Fig. 5). Even after 10 min following BCR stimulation, [Ca²⁺]_i 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 Fab₂. 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 [Ca²⁺]_i. In these experiments, [Ca²⁺]_i 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

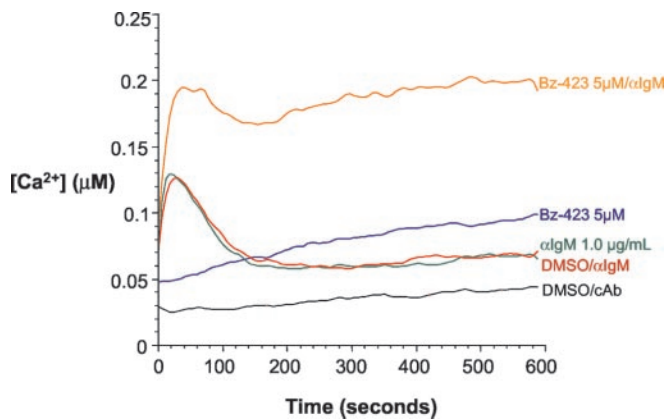


FIG. 5. **Bz-423 alters BCR-coupled calcium signaling.** Cells preloaded with the calcium indicator Fura-2 were incubated with Bz-423 (5 μ M) or vehicle control for 2 min at 37 $^{\circ}$ C; then, as indicated, anti-IgM Fab₂ (1 μ g/ml) was added immediately prior to recording fluorescence of cells (maintained at 37 $^{\circ}$ C) as a function of time. The fluorescence of Fura-2 was monitored ratiometrically at 510 nm after excitation at 340 nm and 380 nm and used to calculate $[Ca^{2+}]_i$ as described under "Experimental Procedures." Data are representative tracings of five or more experiments. In control experiments, the ionophore Br-A23187 was used in the presence of $CaCl_2$ (10 mM) and then in the presence of EGTA (35 mM) to quantify the maximum and minimum fluorescence responses, respectively,

used to make these $[Ca^{2+}]_i$ 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 $^{\circ}$ 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 fluorescent 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 below 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$. Vitamin E also significantly reversed the effects that Bz-423 had upon anti-IgM-induced $[Ca^{2+}]_i$ (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 $[Ca^{2+}]_i$.

DISCUSSION

The selectivity of Bz-423 against GC B cells in autoimmune NZB/W mice prompted these studies, which have identified synergy between the cytotoxic effects of receptor stimulation and Bz-423 in B cells. Functionally, this synergy means that B cells undergo significant apoptosis in response to amounts of Bz-423 and anti-IgM that, as single agents, are not cytotoxic. Although ROS are not necessary for BCR-coupled AICD, the O_2^- generated by Bz-423 is critical for the synergistic response.

Given that O_2^- does not diffuse across membranes (24) and only a small fraction of superoxide is vectorially released into the intermembrane space and enters the cytosol through voltage-dependent anion channels in the outer membrane (25, 26), how does O_2^- induced in mitochondria by Bz-423 affect BCR-coupled calcium responses that are regulated by cytosolic enzymes and other organelles? O_2^- generated within the mitochondrial matrix is converted to H_2O_2 by manganese superoxide dismutase in the matrix. H_2O_2 freely and rapidly diffuses through membranes and enters the cytosol (27). In the reducing intracellular environment, O_2^- , H_2O_2 , and/or $-OH$ (the product of H_2O_2 breakdown in the Fenton reaction) are able to oxidize a variety of protein and lipid moieties throughout the cell, which ultimately results in the modulation of Ca^{2+} signaling. Hence, it is likely that H_2O_2 is the ROS that mediates the observed effects.

Our findings show that the synergistic response depends upon extracellular calcium. However, because calcium influx across the plasma membrane is coupled to depletion of intracellular calcium stores in B cells (28), the present data do not necessarily indicate that Bz-423-induced ROS directly affects calcium channels or pumps in the plasma membrane. Although this is a possibility, extracellular calcium sources may be engaged more indirectly, through capacitative regulation.

There are multiple mechanisms by which Bz-423-induced ROS might modulate Ca^{2+} signaling. One possibility is that Bz-423-induced ROS ultimately increases signals in the BCR-induced cell death pathway that result in sustained Ca^{2+} levels. Bruton's tyrosine kinase and Syk (two of several protein tyrosine kinases activated by the BCR; Ref. 29) are candidates for points of intersection, because H_2O_2 induces their activation (30, 31) and each has a critical role in determining calcium signaling by phosphorylating phospholipase C γ (32). The BCR initially phosphorylates and activates Syk as well as phosphatidylinositol 3-kinase. Phosphatidylinositol 3-kinase generates the second messenger phosphatidylinositol 3,4,5-trisphosphate that mediates membrane recruitment of Bruton's tyrosine kinase which, along with Syk, phosphorylates and activates phospholipase C γ (29). Inositol-1,4,5-trisphosphate (InsP3) generated by phosphorylating phospholipase C γ raises $[Ca^{2+}]_i$ by binding to InsP3 receptors in the endoplasmic reticulum (ER) membrane, depleting stores within the ER and triggering influx through the plasma membrane (33). Bruton's tyrosine kinase is a particularly appealing candidate, because when Bruton's tyrosine kinase is persistently activated, for example, by point mutation in its pleckstrin-homology domain, BCR cross-linking generates a sustained increase in $[Ca^{2+}]_i$ identical to the response observed in our experiments (34).

Alternatively, because the ER lies close to the mitochondria, ROS emanating from the mitochondria might directly regulate Ca^{2+} release channels in the ER (ryanodine receptors and InsP3 receptors) or alter mechanisms that normally lower $[Ca^{2+}]_i$ such as re-uptake by the Ca^{2+} -ATPase in the ER (35). Calcium-specific channels and pumps are sensitive to modulation by ROS either through direct oxidation of sulfhydryl groups located on the ion transport proteins (36, 37), peroxida-

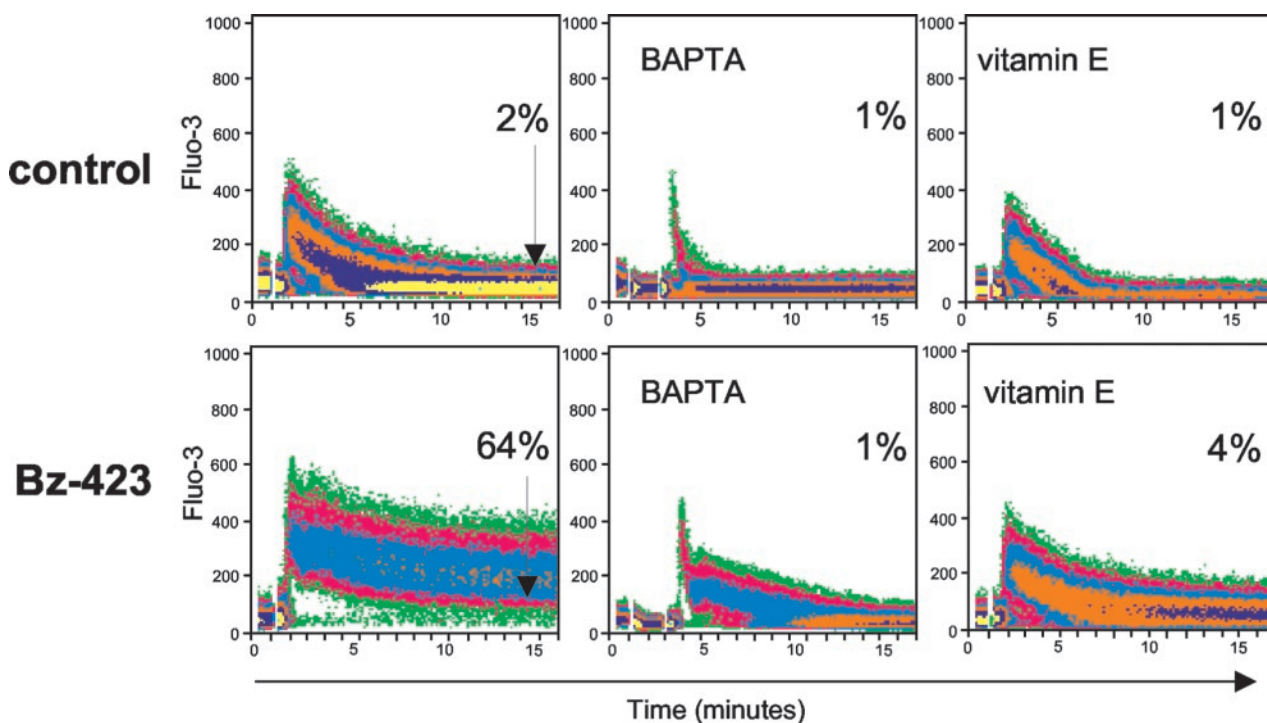


FIG. 6. Sustained increase in $[Ca^{2+}]_i$ depends on ROS and extracellular calcium. Fluo-3-loaded Ramos cells in the absence or presence of BAPTA ($500 \mu M$) or MnTBAP ($100 \mu M$) were incubated with Bz-423 ($10 \mu M$) or vehicle (Me_2SO), brought to $37^\circ C$, and then analyzed by flow cytometry. Anti-IgM Fab₂ ($1 \mu g/ml$) was added 30 s after the collection was started (break in collection). $[Ca^{2+}]_i$ correlates with Fluo-3 fluorescence. Channels are colored according to cell density. Yellow cell number $\geq 50\%$ of peak height (PH) > dark blue $\geq 25\%$ PH > orange $\geq 12\%$ PH > light blue $\geq 6\%$ PH > pink $\geq 3\%$ PH > green $\geq 1\%$ PH. Numbers indicate the percentage of cells at the end of the collection period (black arrow) with elevated $[Ca^{2+}]_i$.

tion of membrane phospholipids (38), or inhibition by lower ATP levels (39).

Bz-423-induced ROS could also modulate ER channels in a more indirect manner, using cytochrome *c* as a messenger to the ER. A recent description (40) of the interplay between calcium and cytochrome *c* coordinating mitochondrial-ER interactions and driving apoptosis provides another explanation for the exaggerated calcium response and insight into how increased calcium may trigger apoptosis. Boehning *et al.* (40) identified small-scale release of cytochrome *c* from mitochondria in response to agents including staurosporine and ceramide. Because Bz-423 acts directly on mitochondria, it is conceivable that it also causes a similar cytochrome *c* release. Boehning *et al.* then showed that the small amount of cytochrome *c* released is sufficient to bind to and promote calcium conductance through InsP₃ receptors in the ER. The released calcium feeds back to the mitochondria, triggering a coordinate permeability transition and massive cytochrome *c* release from all mitochondria in the cell, ultimately engaging the caspase-dependent death machinery. It is possible that in an analogous fashion, the direct actions of Bz-423 on the mitochondria lead to a similar early, small-scale cytochrome *c* response that contributes to the increased BCR-induced calcium release by sensitizing InsP₃ receptors.

Because $[Ca^{2+}]_i$ is fundamental for B cell activation, proliferation, and apoptosis, it is not surprising that the changes in calcium signaling effected by Bz-423 are functionally important. Among the parameters that influence BCR-driven apoptosis, the magnitude of the rise in $[Ca^{2+}]_i$ induced by receptor ligands is directly correlated with the apoptotic outcome induced by BCR stimulation (41). Furthermore, the magnitude and duration of the intracellular calcium response to BCR ligation precisely determines the differential triggering of the transcriptional regulators nuclear factor- κB , c-Jun NH₂-terminal kinase, nuclear factor of activated T cells, and extracellular

signal-regulated kinase that ultimately dictate the characteristics of a cellular response (42). Thus, in addition to influencing BCR-coupled calcium signals in AICD, Bz-423 may modulate other B cell responses to antigen receptor stimulation.

In summary, we have demonstrated that the cytotoxic, immunomodulatory agent Bz-423 synergizes with BCR stimulation, and it is likely that this effect accounts (at least in part) for the selectivity observed *in vivo*. Considering that other receptors on B cells, which are effectively targeted by therapeutic antibodies presently in clinical use, are also coupled to calcium signals (for example the CD20 B cell-specific surface molecule targeted by Rituxan; Ref. 43), it may be possible to exploit Bz-423 for cancer chemotherapy. Our findings suggest how the properties of Bz-423 could provide therapeutic utility for autoimmunity, and current efforts are aimed at evaluating these possibilities.

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