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Attenuation of Autoimmune Disease in Fas-Deficient Mice by Treatment With a Cytotoxic Benzodiazepine

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Objective. **Elimination of autoreactive cells relies on Fas-dependent activation-induced cell death mechanisms, an important component of peripheral tolerance. Defects in Fas or its cognate ligand lead to inefficient activation-induced cell death and are specific causes of lymphoproliferative and autoimmune diseases. The present study was undertaken to investigate a novel 1,4-benzodiazepine (Bz-423) that induces apoptosis and limits autoimmune disease in NZB/NZW mice, to determine its activity against lupus-like disease associated with defective Fas expression. We investigated the Fas-dependence of its cytotoxic actions, its therapeutic potential in mice deficient in Fas, and its therapeutic mechanism of action.**

Methods. **Primary lymphocytes isolated from Fas-deficient MRL/*MpJ-Fas^{lpr}* (MRL-*lpr*) mice were tested for sensitivity to Bz-423. Bz-423 was administered to MRL-*lpr* mice for short (1-week) or long (14-week) periods, and its effects on cell survival were determined along with measures of nephritis, arthritis, antibody titers, and Th subpopulations. BALB/c mice were similarly treated to determine if Bz-423 alters normal immune functions in vivo.**

Results. **Administration of Bz-423 to MRL-*lpr* mice significantly reduced autoimmune disease including glomerulonephritis and arthritis. Treatment was associated with decreases in CD4+ T cells and an alteration in the Th1/Th2 balance. At the therapeutic dosage, Bz-423 did not interfere with normal T and B cell responses in BALB/c mice, suggesting that this agent is not globally immunosuppressive.**

Conclusion. **Bz-423 is a novel immunomodulatory agent that is active against disease even in the context of defective Fas signaling. It is a leading compound for further investigation into the development of selective therapies for lupus.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a spectrum of antibodies that recognize self antigens (1–3). Autoantibody–antigen immune complexes deposit in the tissues of the heart, brain, lungs, and kidney, incite tissue destruction, and impair organ function by activating complement and recruiting inflammatory cells (3). The kidney is particularly vulnerable to disease: at least 40% of all SLE patients have renal involvement, and the renal disease associated with lupus has a high mortality rate (4,5). Lymphocytes are primary targets for pharmacologic therapy used to treat organ-threatening SLE, and effective immunosuppressive drugs include corticosteroids, azathioprine, and cyclophosphamide (6–9). Although the mechanisms by which these agents exert their effects on SLE are not fully understood, cytotoxicity against B and T lymphocytes is strongly implicated (10). Advances have been made in devising effective treatment regimens; however, use of these agents is still accompanied by serious complications that limit their administration and overall clinical benefit (11).

Acquisition of agents that more selectively target lupus-determining lymphoid cells would significantly advance SLE treatment. In pursuit of this goal, we previously identified a novel 1,4-benzodiazepine (designated

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Bz-423) with significant cytotoxicity toward B cell lines in culture (12). Incubation of cells with Bz-423 provokes a rapid increase in intracellular superoxide (O_2^-), and this reactive oxygen species (ROS) is an upstream signal that initiates an apoptotic process characterized by cytochrome c release, mitochondrial depolarization, and caspase activation. Cell fractionation experiments reveal that the O_2^- response results from the interaction of Bz-423 with a target in mitochondria. The possibility that Bz-423–induced responses could be tapped for therapeutic uses (such as cytoreduction) was first tested in the (NZB × NZW)F_1 (NZB/NZW) mouse model of lupus, in which germinal center (GC) B cells are pathologically expanded, behave as neoplastic clones, and function as a significant determinant of autoimmune disease (13–16). Administration of Bz-423 limited GC hyperplasia and the development of glomerulonephritis in these mice (12).

We have now extended our studies on this compound and shown that Bz-423 preserves renal function and reduces nephritis when administered to MRL/*MpJ-Fas*^{lpr} (MRL-*lpr*) mice, a strain in which peripheral autoreactive T cells drive a lupus-like disease process (17–20). Treatment is associated with a change in the Th1/Th2 cell balance and small decreases in specific T cell subsets. In addition, we found that treating normal immune mice with Bz-423 did not affect lymphocyte activation, T cell–dependent delayed-type hypersensitivity (DTH), or T cell–dependent antibody production. We derived a mechanistic hypothesis to explain the activity of Bz-423 in autoimmune mice and the lack of an immunosuppressive effect in normal animals.

MATERIALS AND METHODS

Animals and Bz-423 treatment. These studies were approved by the University of Michigan Committee on the Use and Care of Animals. Female MRL-*lpr* mice were purchased from Jackson Laboratories (Bar Harbor, ME). Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Bz-423 was synthesized as previously described (21) and dissolved in DMSO as the vehicle. For the longitudinal study, control mice received vehicle (50 μl DMSO; n = 11), and treatment mice received Bz-423 (60 mg/kg; n = 10) injected intraperitoneally. Peripheral blood was collected from the tail vein for complete blood cell counts and preparation of serum. Mice were killed by CO_2 asphyxiation. Kidneys were bisected and fragments fixed in 10% buffered formalin or snap-frozen in OCT. Spleen fragments were processed in buffered formalin, frozen in OCT, or used to prepare single cell suspensions.

Cell culture. Single cell suspensions from freshly isolated spleens of untreated animals were prepared by disaggregating the tissue with forceps and lysing red blood cells. Cells (10^6/ml) were maintained on ice in RPMI (Mediatech, Herndon, VA) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (290 μg/ml), 2-mercaptoethanol (50 μg/ml), and heat-inactivated fetal bovine serum (FBS) (2%; Summit Biotechnology, Santa Ana, CA). Viability experiments were conducted in this same media at 37°C in a 5% CO_2 atmosphere. Bz-423 and other drugs were dissolved in DMSO that was present at a final concentration of 0.5% (volume/volume). After treatment, cells were incubated for 15 minutes with propidium iodide (PI) (1 μg/ml), and viability assessed by flow cytometry based on PI exclusion (22). Data were collected and analyzed using a FACSCalibur (BD Biosciences, San Diego, CA). For each sample, 10,000 events were recorded and the data were analyzed excluding aggregates.

Fluorescence analysis of lymphocyte populations. Surface markers were detected by incubating (20 minutes, 4°C) single cell suspensions with fluorescein isothiocyanate–conjugated (2.5 μg/10^6 cells) or phycoerythrin-conjugated (1 μg/10^6 cells) rat anti-mouse antibodies (anti-Thy 1.2, clone 53-2.1; anti-CD4, clone H129.19; anti-CD8a, clone 53-6.7; anti-B220, clone RA3-6B2; anti-CD69, clone H1-2F3) (PharMingen, San Diego, CA). Viability was simultaneously measured with PI. Data were collected and analyzed by flow cytometry.

Antibody titers, blood urea nitrogen, and proteinuria. Autoantibody (anti-DNA, antihistone, anti-Ro, anti-La) and total immunoglobulin (IgG, IgM) titers were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (23). Serum blood urea nitrogen was measured at the clinical pathology laboratory, University of Michigan Hospital. Proteinuria was measured using ChemStrip 6 (Boehringer Mannheim, Indianapolis, IN).

Histologic analysis. Formalin-fixed sections were stained with hematoxylin and eosin (H&E), periodic acid–Schiff, or Masson's trichrome as previously described (24). Kidney sections were examined by a renal pathologist (who was blinded to the treatment group) and were scored for glomerulonephritis on a 0–4+ scale based on the degree of cellularity, scarring, and crescent formation. Glomerular antibody deposition was assessed by immunofluorescence staining of frozen sections of kidneys (25). Formalin-fixed limbs were processed, sectioned, and stained with H&E. The intercarpal, metacarpophalangeal, and interphalangeal joints were examined. Under blinded conditions, synovitis was graded on a scale of 0–3+ based on the degree of synovial proliferation, transformation of the synoviocytes, and pannus development. Erosions (both cartilage and bone) were noted as being present or absent.

Cytokine enzyme-linked immunospot assays. Assays were conducted in 96-well Multiscreen-IP plates (Millipore, Bedford, MA), and pairs of cytokines were assayed together: interleukin-2 (IL-2) and IL-4, IL-10 and interferon-γ (IFNγ). Plates were coated (18 hours, 4°C) with capture antibodies (5 μg/ml in 100 m*M* carbonate, pH 8.2) specific for each cytokine (rat anti-mouse IL-2 [antibody 702], IL-4 [antibody 404], IL-10 [antibody 417], and IFNγ [antibody 786]) (R&D Systems, Minneapolis, MN). Single cell suspensions of splenocytes from treatment and control MRL-*lpr* mice were prepared as described above. Cells (10^3–10^6/well) were added to the plates and cultured (18 hours, 37°C, 5% CO_2) in Dulbecco's modified

Eagle's medium (Mediatech) containing FBS (10%), penicillin (100 units/ml), streptomycin (10 μg/ml), L-glutamine (2 m*M*), and concanavalin A (2 μg/ml) (all from Sigma, St. Louis, MO). After washing, the wells were incubated with primary antibodies specific for each cytokine: 2 μg/ml of goat anti-mouse IL-2 (AB-402-NA; R&D Systems), rat anti-mouse IL-4 (biotinylated) (18042D; PharMingen), or hamster anti-mouse IFNγ (1222-00; R&D Systems), or 188 ng/ml of goat anti-mouse IL-10 (biotinylated) (BAF417; R&D Systems). After further washing, appropriate species-specific enzyme-conjugated secondary antibodies and substrates were used for detection.

In vivo T cell activation. BALB/c mice (11 weeks old) were administered 2 doses of Bz-423 (60 mg/kg; n = 5) or vehicle (n = 5) at 24-hour intervals. Anti-CD3ϵ (clone F500; PharMingen) was given intraperitoneally (25 μg in 100 μl of phosphate buffered saline [PBS]) 2 hours after the last injection of Bz-423. Mice were killed 24 hours after treatment, and the spleens were removed. Single cell suspensions were prepared and analyzed by flow cytometry for surface marker expression.

DTH testing. BALB/c mice (10 weeks old) were injected every other day with either Bz-423 (60 mg/kg; n = 10) or vehicle (n = 10) for 1 week. The dorsal flanks were injected subcutaneously with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (10 m*M* in PBS, pH 7.4; Sigma). A subset of mice from both the vehicle group (n = 5) and the Bz-423 group (n = 5) did not receive this initial TNBS (nonsensitized). Administration of Bz-423 or vehicle continued, and the mice were challenged with TNBS in the left hind footpad 7 days after the first immunization with TNBS. The nonsensitized mice also received this challenge to measure inflammation resulting from the injection process. Footpad thickness was measured 4 hours and 24 hours later, using micrometer calipers. Induration was calculated by subtracting the right hind footpad thickness from that of the left.

T cell–dependent antibody response. BALB/c mice (10 weeks old) were injected with Bz-423 (60 mg/kg; n = 4) or vehicle (n = 4) every other day for 1 week. All mice were immunized intraperitoneally with keyhole limpet hemocyanin (KLH) (50 μg; Pierce, Rockford, IL) in Freund's complete adjuvant (total volume 0.5 ml; Sigma). Administration of Bz-423 (or vehicle) was continued, and 28 days after the first immunization, mice were boosted with KLH in Freund's incomplete adjuvant (Sigma). Serum samples were obtained on day 49 (post–first immunization) and analyzed by ELISA for anti-KLH titers. For the ELISA, microtiter plates were coated with KLH (0.1 mg/ml in PBS) and anti-KLH concentration was quantified by comparison with a standard curve generated with rabbit anti-KLH (ICN Biomedicals, Costa Mesa, CA).

Statistical analysis. Statistical significance was assessed using the Mann-Whitney U test (1-tailed) unless otherwise noted. *P* values are 1-tailed, and data are presented as the mean ± SEM.

RESULTS

Fas-independent killing of B and T cells by Bz-423. The *lpr* mutation blocks death receptor–initiated apoptosis and also prevents the apoptotic response to certain small molecules (e.g., doxorubicin) (26). To determine if Fas is necessary for the activity of Bz-423, we examined the cytotoxic activity of Bz-423 against spleen cells from autoimmune MRL-*lpr* mice, a murine model of SLE in which defective Fas signaling, particularly within the T cell compartment, plays a dominant role in mediating disease (19,20). Freshly isolated splenocytes cultured with Bz-423 were rapidly killed by Bz-423 in a concentration- and time-dependent manner (data not shown). This effect was specific to Bz-423: other benzodiazepines, i.e., diazepam and clonazepam, and controls, i.e., 1-napthol and 1-(2-chlorophenyl-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide, were not cytotoxic.

To determine if cell lineage is associated with differing sensitivity to Bz-423, mixed splenocytes were treated under conditions that afford 80% overall cell death (for Bz-423, 10 μM, 12 hours). The relative proportion of B, T, and CD4+ T and CD8+ T cells remaining viable after culture remained nearly identical to their proportions in untreated controls (data not shown). These results indicate that, although Bz-423 is an effective cytotoxic agent against cells with defective Fas signaling, there is no in vitro selectivity between MRL-*lpr* splenic lymphocyte lineages and subsets.

Selective reduction of splenic T cells with brief administration of Bz-423 to MRL-*lpr* mice. Previous experiments have shown that administering Bz-423 to NZB/NZW mice for 1 week killed splenic B cells, and extended dosing reduced GC B cells and improved autoimmune disease (12). Based on these data and the results showing activity against MRL-*lpr* cells in vitro, we investigated whether Bz-423 is cytoreductive in MRL-*lpr* mice. MRL-*lpr* mice received either Bz-423 (60 mg/kg) or vehicle control daily for 1 week. Splenocytes from mice treated with Bz-423 had lower viability (mean ± SEM 82 ± 1% in controls versus 75 ± 3% in Bz-423–treated mice; $P = 0.008$) and significantly fewer T cells (Thy1.2+), CD4+ T cells, and double-negative T cells (Thy1.2+, B220+, CD4−, CD8−) (Table 1). The selective reduction of MRL-*lpr* T cells by Bz-423 indicates that its effects in vivo are modulated by factors that are not represented by the in vitro model of lymphotoxicity, and differences between NZB/NZW and MRL-*lpr* mice confer differential susceptibility to this benzodiazepine.

Amelioration of disease in Bz-423–treated MRL-*lpr* mice. Since CD4+ T cells are required for autoantibody production and fulminant nephritis and arthritis in MRL-*lpr* mice (27–29), extended dosing with Bz-423 might improve disease in this strain. To test this hypothesis, we designed an experiment in which mice were

Table 1. Lymphocyte subsets present in the spleen after 1 week of treatment*

	Control mice	Bz-423–treated mice	*P*
T cells	23.5 ± 1.9	18.1 ± 1.0	0.016
B cells	19.3 ± 1.5	17.6 ± 0.6	0.15
Double-negative T cells	7.8 ± 0.7	5.3 ± 1.1	0.005
CD4+ T cells	10.9 ± 1.0	8.5 ± 0.3	0.031
CD8+ T cells	5.8 ± 0.3	6.7 ± 0.4	0.06

* Ten-week old MRL-*lpr* mice were treated daily with Bz-423 (60 mg/kg; n = 10) or vehicle control (n = 10) for 7 days. Cells were quantified based on the total number of splenocytes recovered and flow cytometric determination of surface markers. T cells were defined by the expression of Thy1.2, and subsets were differentiated based on coexpression of CD4 or CD8. Double-negative T cells were identified by the presence of Thy1.2 and B220, and B cells by B220 without Thy1.2. *P* values were determined by Student's *t*-test. Values are the mean ± SEM total cells × 10^6.

injected with either Bz-423 or vehicle control every other day beginning at 8 weeks of age as described in Materials and Methods. The study continued until mice were 22 weeks old (14 weeks of treatment), when mortality from renal failure typically is near 50% (30). Each animal was monitored weekly for renal function (proteinuria) and analyzed at the end of the study to determine effects on autoimmune nephritis, arthritis, lymphoproliferative disease, and autoantibody titers.

Mice treated with Bz-423 developed significantly less proteinuria (Figure 1) and had lower serum blood urea nitrogen levels measured after 14 weeks of treatment (mean ± SEM 27 ± 5 mg/dl, versus 44 ± 14 mg/dl in controls; $P = 0.0024$). The reduction in azotemia was associated with less histopathologic evidence of nephritis

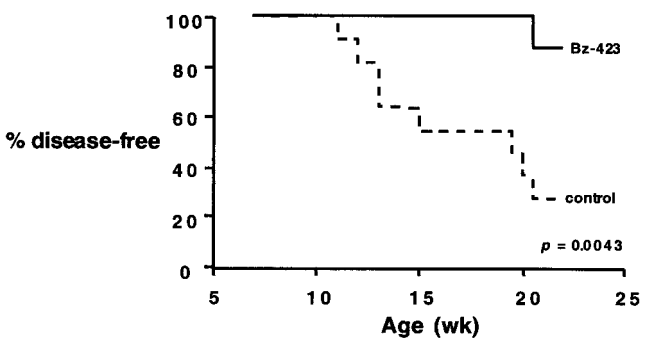

Figure 1. Protection of renal function with Bz-423 treatment. MRL-*lpr* mice were treated with Bz-423 (60 mg/kg every other day) (solid line) or vehicle (dashed line) for 14 weeks. Urine was tested weekly and disease defined as an elevated urine protein level (>2+; >100 mg/dl) observed in 2 consecutive weeks. *P* value was determined by Kaplan-Meier analysis.

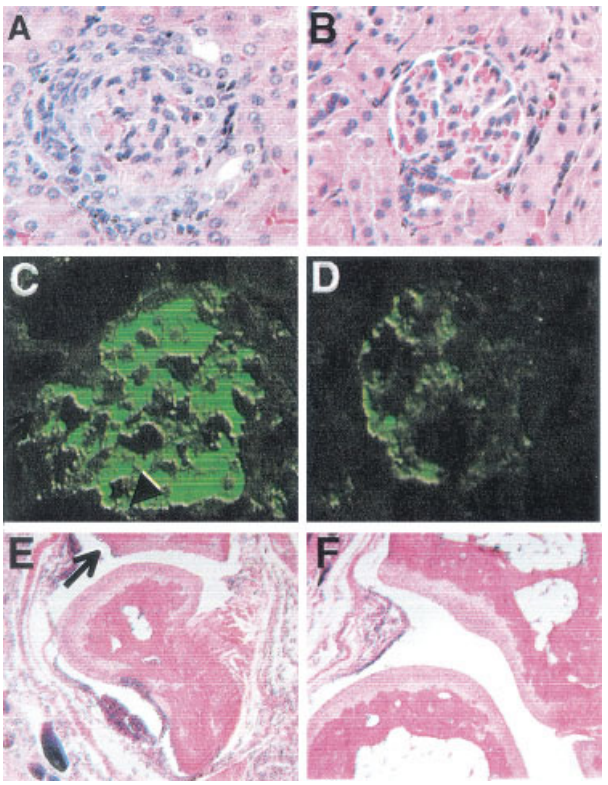

Figure 2. Reduction of kidney and joint disease with Bz-423 treatment. MRL-*lpr* mice were treated for 14 weeks with Bz-423 or vehicle (control) as described in Figure 1. Glomeruli from control mice (**A** and **C**) and Bz-423–treated mice (**B** and **D**) were stained with hematoxylin and eosin (H&E) (**A** and **B**) or with immunofluorescence to detect C3 deposition (**C** and **D**). Joints from the hind paws of control mice (**E**) and Bz-423–treated mice (**F**) were stained with H&E. **Arrows** indicate synovial thickening; **arrowhead** indicates bony erosion. (Magnification ×400 in **A–D** and **F**; ×200 in **E**.)

at the end of the study (Figures 2 and 3). Treatment was also associated with preservation of normal glomerular architecture: mice administered Bz-423 had less cellular infiltration, fibrosis, and crescent formation compared with controls (average histopathologic score after treatment 2+ in Bz-423–treated mice versus 4+ in control mice; $P < 0.02$). Similarly, complement deposition in the glomeruli was also decreased (mean score 1–2+ and 3–4+ in Bz-423–treated and control mice, respectively; $P < 0.007$). By the end of the study, 40% of controls and none of the Bz-423–treated mice had died with evidence of significant renal disease (Figure 3). Histopathologic examination of joints revealed marked synovitis, synovial cell proliferation, pannus formation, and erosion of articular cartilage and subchondral bone in control animals. In contrast, synovitis was significantly reduced in mice that received Bz-423, and fewer treated mice had

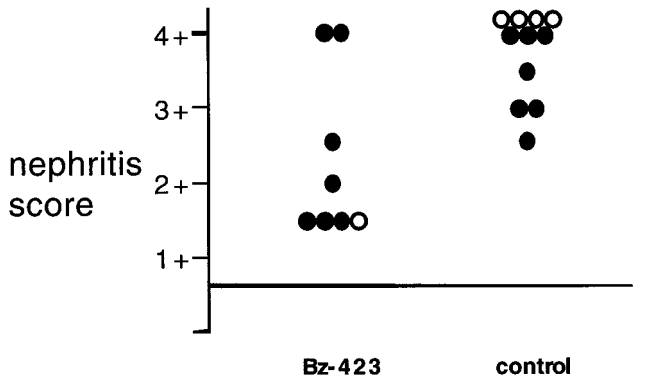

Figure 3. Decrease in glomerulonephritis with Bz-423 treatment. MRL-*lpr* mice were treated for 14 weeks with Bz-423 or vehicle (control) as described in Figure 1. At the termination of the treatment period, glomerulonephritis was scored using criteria described in Materials and Methods. Open circles represent mice that died during the treatment period. Histologic data were available for only 1 of the 3 Bz-423–treated mice that died during the study; however, all 3 mice had proteinuria measurements of <2+ prior to death, indicative of minimal glomerular disease. These animals died as a result of intraperitoneal adhesions causing bowel obstruction; the adhesions were caused by the injections.

evidence of articular erosion or pannus formation (Table 2 and Figure 2).

Reductions in spleen weight or lymphoproliferative disease (scored histopathologically) were not observed (data not shown), providing evidence against a lymphoablative effect. Moreover, at the end of the study, differences in blood counts (hematocrit, platelets, total white blood cells) were not detected between treatment and control animals (data not shown), which suggests that at the dosage used, Bz-423 does not interfere with hematopoiesis. In contrast, serum levels of autoantibodies (anti-DNA, antihistone, and anti-La) were slightly reduced in Bz-423–treated animals (Table 3), whereas

Table 2. Effect of Bz-423 on arthritis*

	Control mice	Bz-423–treated mice	P†
Mean synovitis score	2+–3+‡	1+	0.001
No. of mice with ≥2+ synovitis	7	0	0.01
No. of mice with erosions	4	2	0.3
No. of mice with pannus formation	4	1	0.13

* Hind paws were evaluated histopathologically and scored for synovitis on a 0–3+ scale (n = 7 in each group). Erosions (bony and cartilaginous) and pannus formation were noted as present or absent.
† Determined by Mann-Whitney U test for synovitis score and by cross-tabulation with chi-square analysis for number of mice with ≥2+ synovitis, erosions, and pannus formation.
‡ Scores of 2+ in 4 mice and 3+ in 3 mice.

Table 3. Mean serum antibody titers measured by ELISA*

	Control mice	Bz-423–treated mice	P
Anti-ssDNA, units/ml	887 ± 124	508 ± 73	0.02
Anti-dsDNA, units/ml	650 ± 172	247 ± 38	0.08
Antihistone, OD_{405}	1.4 ± 0.2	0.6 ± 0.2	0.006
Anti-La, units/ml	529 ± 174	226 ± 61	0.03
Anti-Ro, units/ml	456 ± 124	304 ± 97	0.16

* Values are the mean ± SEM. ELISA = enzyme-linked immunosorbent assay; anti-ssDNA = anti–single-stranded DNA; anti-dsDNA = anti–double-stranded DNA; OD_{405} = optical density at 405 nm.

total IgG and total IgM were not altered (mean ± SEM IgG level 26 ± 3.9 mg/ml in control mice versus 23 ± 2.5 mg/ml in treated mice [$P = 0.33$]; IgM level 0.33 ± 0.06 mg/ml in control mice versus 0.34 ± 0.1 mg/ml in treated mice [$P = 0.23$]). Treatment of MRL-*lpr* mice with Bz-423 for 14 weeks reduced splenic CD4+ T cells, while changes in CD8+ T cells, double-negative T cells, or B cells were not observed (Table 4). Analysis of T cell subsets by enzyme-linked immunospot assay demonstrated a particular reduction in Th2 cells after dosing with Bz-423 (fewer IL-4– and IL-10–producing cells) with an increase in Th1 (IFNγ)–secreting cells, although the numbers of cells secreting IL-2 were similar (Table 5).

Bz-423 does not interfere with normal immune function. Our findings with both NZB/NZW and MRL-*lpr* mice demonstrate that Bz-423 has a therapeutic effect against murine lupus. To investigate the general immunomodulatory properties of Bz-423, we designed a series of experiments to determine if the therapeutic dosage interferes with normal immune responses. Since normal immune functions are perturbed in the NZB/NZW and MRL-*lpr* strains (31,32), BALB/c mice were used in these studies. In the first group of experiments, BALB/c mice were given Bz-423 (60 mg/kg) or vehicle

Table 4. Lymphocyte subsets present in the spleen after 14 weeks of treatment*

	Control mice	Bz-423–treated mice	P
T cells	76 ± 2	73 ± 4	0.24
B cells	7 ± 1	7 ± 2	0.44
Double-negative T cells	32 ± 4	31 ± 4	0.50
CD4+ T cells	38 ± 3	33 ± 2	0.05
CD8+ T cells	16 ± 1	17 ± 2	0.30

* Percentage representation of each population was determined by surface marker staining as described in Table 1. Values are the mean ± SEM.

Table 5. Effect of Bz-423 on cytokine-producing splenocytes measured by enzyme-linked immunospot assay*

	Control mice	Bz-423–treated mice	*P*
No. of cells ($\times 10^5$) secreting IL-2	99 ± 18	79 ± 19	0.21
No. of cells ($\times 10^5$) secreting IL-4	102 ± 27	15 ± 12	<0.001
No. of cells ($\times 10^5$) secreting IL-10	14 ± 5	1.1 ± 0.3	<0.001
No. of cells ($\times 10^5$) secreting IFNγ	203 ± 29	422 ± 36	<0.001

* Values are the mean ± SEM. IL-2 = interleukin-2; IFNγ = interferon-γ.

for 7 days and examined for evidence of lymphotoxicity. After treatment, the total number of splenic lymphocytes (Bz-423–treated mice mean ± SEM 47 ± 6 × 10^6 cells, control mice 49 ± 3 × 10^6 cells; $P = 0.37$), T cells (Bz-423–treated mice 20 ± 3 × 10^6 cells, control mice 21 ± 1 × 10^6 cells; $P = 0.42$), and B cells (Bz-423–treated 27 ± 4 × 10^6 cells, control mice 28 ± 2 × 10^6 cells; $P = 0.35$) in BALB/c mice were not altered by Bz-423. In comparison, a single therapeutic dose of cyclophosphamide (100 mg/kg) (33) reduced lymphocyte numbers by 30%, which was primarily accounted for by a reduction in B cells (data not shown). The absence of a cytoreductive effect of Bz-423 in BALB/c mice indicates that at the dose tested, Bz-423 may have selectivity for lymphocytes and/or processes associated with autoimmunity.

We next sought to address the possibility that the therapeutic activity of Bz-423 in MRL-*lpr* mice principally involves modulation of a *functional* immune response rather than direct cytotoxic action against autoimmune cells. Such an alternative might account for the apparent discrepancy between the relatively small overall decrease in lymphocytes and the much larger effects of 14 weeks of treatment on disease end points and the Th1/Th2 balance. Experiments were designed to specifically determine if Bz-423 affects 1) lymphocyte activation, 2) T cell–dependent DTH responses, and 3) T cell–dependent antibody production.

To study activation, BALB/c mice were challenged with a single dose of anti-CD3 (25 μg) to polyclonally stimulate T cells (34). Mice received 2 doses of Bz-423 (60 mg/kg) or vehicle 24 hours and 2 hours prior to the injection of anti-CD3. Mice were killed 24 hours after anti-CD3 administration, and splenic T cell populations were enumerated for CD69 expression (an early activation marker) (35) by flow cytometry. Stimulation with anti-CD3 resulted in a 30-fold increase in the percentage of activated T cells (CD69+, Thy1.2+) (unstimulated 0.3 ± 0.1%, anti-CD3–stimulated 9 ± 1% [mean ± SEM]). This response was not diminished by treatment with Bz-423 (CD69+, Thy1.2+ 12 ± 1%). Indeed, the data even suggest a small increase in the fraction of CD69+ T cells in Bz-423–treated mice. On this basis, we conclude that Bz-423 does not prevent T cell activation in vivo.

A DTH assay in which mice were sensitized to TNBS (36,37) was used to assess T cell–dependent cellular immune function. In the treatment group, mice received Bz-423 (60 mg/kg every other day) for 1 week and then were injected once with TNBS, after which the dosing of Bz-423 was continued. After an additional 7 days, the mice were challenged with TNBS in the hind footpad and the DTH response was judged by measuring induration of the footpad 24 hours later. As seen in Table 6, the responses in mice receiving Bz-423 were statistically indistinguishable from those in control mice.

Lastly, BALB/c mice were challenged with KLH to assess T cell–dependent antibody production (38,39). Bz-423 was administered to BALB/c mice for 1 week (60 mg/kg every other day) prior to immunization with KLH, and dosing with Bz-423 was continued throughout the study. The animals were boosted with KLH 28 days after the initial challenge, and antibody levels were quantified 21 days later. Anti-KLH and total IgG titers were statistically indistinguishable in Bz-423–treated and control mice (Table 6), implying that exposure to Bz-423

Table 6. Effect of Bz-423 on in vivo immune function*

Group	DTH/TNBS footpad thickness, $\times 10^2$ cm	Anti-KLH, % total IgG
Control		
Immunized	4.8 ± 1.0	22 ± 8
Nonimmunized	2.9 ± 0.2	–
Bz-423–treated		
Immunized	4.0 ± 1.5	18 ± 1
Nonimmunized	2.5 ± 0.9	–

* BALB/c mice were treated with Bz-423 (60 mg/kg every other day) or vehicle for 1 week prior to immunization, and dosing was continued throughout the study. For the delayed-type hypersensitivity (DTH) experiment, mice were immunized with 2,4,6-trinitrobenzenesulfonic acid (TNBS) or left untreated. After 7 days, all mice were challenged with TNBS in the left hind footpad, and footpad thickness was measured after 24 hours. Results are reported as left minus right footpad thickness. For the keyhole limpet hemocyanin (KLH) experiment, mice were immunized with KLH and boosted 28 days later. Antibody production in response to KLH was measured 21 days after the boost. Values are the mean ± SEM (n = 5 for TNBS studies; n = 4 for KLH studies). Values determined in Bz-423–treated mice were not statistically significantly different from those in control mice.

does not suppress normal lymphoid responses required for antibody production.

Collectively, these results indicate that Bz-423 does not block normal immune responses or deplete lymphocytes in BALB/c mice. Furthermore, they provide evidence against the possibility that Bz-423 controls lupus by nonspecifically dampening overall immune system function or by interfering with critical elements of the normal immune response.

DISCUSSION

In previous work (12), we characterized the proapoptotic properties of Bz-423 and its efficacy against nephritis in NZB/NZW mice, a murine model of SLE in which B cells play a central role in the development and pathogenesis of disease. The present study tested the effectiveness of Bz-423 in MRL-*lpr* mice, a second model of SLE, in which disease is primarily controlled by T cells that are defective in Fas-mediated activation-induced cell death (AICD) (17–20). Administering Bz-423 to MRL-*lpr* mice reduces autoimmune nephritis and improves survival. The reduction in nephritis, determined histopathologically at the end of the study, was, as expected, accompanied by improved renal function (less proteinuria) throughout the course of treatment. In addition, treated mice had a blunted humoral autoimmune response, with lower serum concentrations of anti–single-stranded DNA, anti–double-stranded DNA, and histone antibodies. These specific immunoglobulin changes, however, occurred in the context of unchanged total IgG and IgM levels and normal blood cell count indices. Thus, Bz-423 diminishes the immune response to autoantigens without altering total immunoglobulin levels and without suppressing bone marrow hematopoiesis.

In contrast to nephritis, arthritis in MRL-*lpr* mice is independent of circulating antibodies (e.g., rheumatoid factor) and is directly mediated by activated CD4+ T cells (40). Hence, the effect of Bz-423 on preventing arthritic changes suggests a mechanism involving CD4+ T cells. Indeed, our observations that 8-week-old animals dosed for 1 week have reduced CD4+ and double-negative T cell subsets and that 22-week-old mice treated for 14 weeks have fewer CD4+ T cells than controls supports the notion of a T cell–specific effect.

In experiments with NZB/NZW mice, GC B cells displayed the greatest sensitivity to Bz-423 (12). Parallels between NZB/NZW GC B cells and MRL-*lpr* T cells suggest a possible mechanism to explain the observed cell-type selectivity in the 2 strains. In MRL-*lpr* mice, the *lpr* mutation prevents proper Fas signaling (19,20). Consequently, AICD of T cells is defective, which results in an expanded population of activated CD4+ T cells, some of which mediate autoimmune disease. In the NZB/NZW model, the B cell population has an activated phenotype and persists abnormally due to a failure of B cell AICD, normally triggered by B cell receptor (BCR) crosslinking (41–44). Of note, although Fas and Fas ligand are intact in the NZB/NZW strain, defective AICD and the development of autoreactive B cells has been linked to resistance against Fas-mediated apoptosis (45).

Thus, persistent activation is a common factor in cells targeted by Bz-423, suggesting that activation, particularly in the absence of functional AICD mechanisms, may enhance sensitivity to Bz-423 killing. In support of this hypothesis, preliminary experiments show that BCR stimulation and secondary activation of T cells significantly increase the apoptotic responsiveness to Bz-423 (Bednarski JJ, et al: unpublished observations). In addition, the molecular mechanism of Bz-423 killing involves O_2^- generated by mitochondria. In this regard, it is also worth noting that activated autoimmune T lymphocytes have reduced levels of antioxidants to detoxify ROS, including O_2^- (46,47). Hence, a molecular signal generated by Bz-423 intersects with a point of inherent vulnerability in activated autoimmune T cells, which may contribute to the selectivity observed in vivo.

At the therapeutic dose, administration of Bz-423 to normal immune mice did not reduce either T or B cells and did not significantly interfere with experimentally induced humoral- or cell-mediated immune responses. Hence, Bz-423 is not a broad-acting immunosuppressive agent like prednisone or cyclophosphamide, which also effectively treat lupus (48). Mechanistically, this information is consistent with the model proposed above, in which activation sensitizes cells to Bz-423. In the normal immune state, AICD mechanisms are intact, limiting the survival and number of activated T cells (49). The cytotoxic action of Bz-423, if concentrated on activated immune cells as proposed above, assumes importance only when normal mechanisms of AICD fail, such as in autoimmune disease. Hence, the proposed activity of Bz-423 is redundant with normal physiologic mechanisms and therefore, unlikely to significantly modulate normal immune behavior. It remains a possibility, however, that selectivity is dose dependent and that higher doses of Bz-423 would be broadly lymphotoxic.

Although disease pathogenesis in MRL-*lpr* mice

requires CD4+ T cells, a clear understanding of the individual roles of Th1 and Th2 subsets in this disease has not emerged (50). IL-4, IL-10, and IFNγ play important, but complex, roles in the pathogenesis of murine and human lupus (51). Analysis of the Th1/Th2 cytokine response in mice treated with Bz-423 reveals a significant change relative to controls: Bz-423 caused a marked decrease in the Th2 cell response (IL-4– and IL-10–secreting spleen cells) and an increase in Th1 (IFNγ)–producing cells. Disease in MRL-*lpr* mice has been associated with increased Th1 cells (IFNγ), as well as increased Th2 cells (IL-4 and IL-10), relative to normal immune mice (52,53). In experimental lupus induced in BALB/c mice by idiotypic manipulation and in the natural disease course in NZB/NZW mice, increased levels of IL-4 and IL-10 have been detected (54,55). Treatment with IL-4 antagonists decreases anti-DNA titers and reduces renal disease (56). Backcrossing MRL-*lpr* mice with IL-4 knockout mice produces a phenotype with less renal disease and diminished adenopathy (57).

Experimental treatment of lupus mice with a new benzoquinone-containing natural product reduces autoantibodies and nephritis, and also affects the Th1/Th2 balance by inhibiting the Th2 response (58). Because Th2 cells support B cell development and the humoral arm of the autoimmune response, the Th2 reduction may underlie the effects on autoantibody production and nephritis in Bz-423–treated mice. This important connection could account for the observed reduction in autoantibody titers, which occurred in the absence of a cytoreductive effect on B cells.

It is possible that the increase in IFNγ-secreting cells associated with Bz-423 treatment also contributes to the observed therapeutic responses. Depending on the specific model and stage of disease, IFNγ has been associated with both autoimmune disease promotion and disease protection (59,60). In accordance with our results, IFNγ has a particularly important role in limiting joint disease in murine arthritis induced by collagen injection (61,62). Conversely, the phenotype of MRL-*lpr* mice in which IFNγ is knocked out is improved with respect to glomerulonephritis (63,64), providing evidence that IFNγ in fact promotes disease. These findings conflict with the results of antibody-blocking experiments that fail to show a significant contribution of IFNγ to MRL disease (65). These differences may be explained by an age-dependence on the effects of IFNγ, which has been observed in the antiidiotype model of lupus as well as in MRL-*lpr* mice (55,60). Given this complexity, additional experiments are necessary to elucidate whether the Th1/Th2 response to Bz-423 is significant with regard to its therapeutic effects.

In conclusion, our data indicate that Bz-423 is a leading compound for studies aimed at development of more selective cytotoxic molecules with properties useful for treating SLE and possibly other related disorders. Investigations in progress are directed at defining the molecular target of Bz-423 as a basis to advance drug discovery and at further testing of the proposed model as an explanation for the observed selectivity in vivo.

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