



Original Contribution

Bz-423 superoxide signals apoptosis via selective activation of JNK, Bak, and Bax[☆]Neal B. Blatt^a, Anthony E. Boitano^{b,1}, Costas A. Lyssiotis^{b,1}, Anthony W. Opipari Jr.^{c,*}, Gary D. Glick^{b,*}^a Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109, USA^b Department of Chemistry, University of Michigan, Ann Arbor, MI 48109, USA^c Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI 48109, USA

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ABSTRACT

Bz-423 is a proapoptotic 1,4-benzodiazepine with potent therapeutic properties in murine models of lupus and psoriasis. Bz-423 modulates the F₁F₀-ATPase, inducing the formation of superoxide within the mitochondrial respiratory chain, which then functions as a second messenger initiating apoptosis. Herein, we report the signaling pathway activated by Bz-423 in mouse embryonic fibroblasts containing knockouts of key apoptotic proteins. Bz-423-induced superoxide activates cytosolic ASK1 and its release from thioredoxin. A mitogen-activated protein kinase cascade follows, leading to the specific phosphorylation of JNK. JNK signals activation of Bax and Bak which then induces mitochondrial outer membrane permeabilization to cause the release of cytochrome c and a commitment to apoptosis. The response of these cells to Bz-423 is critically dependent on both superoxide and JNK activation as antioxidants and the JNK inhibitor SP600125 prevents Bax translocation, cytochrome c release, and cell death. These results demonstrate that superoxide generated from the mitochondrial respiratory chain as a consequence of a respiratory transition can signal a sequential and specific apoptotic response. Collectively, these data suggest that the selectivity of Bz-423 observed in vivo results from cell-type specific differences in redox balance and signaling by ASK1 and Bcl-2 proteins.

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Introduction

Bz-423 is a proapoptotic 1,4-benzodiazepine with potent therapeutic properties against murine lupus and psoriasis [1–3]. The absence of either general toxicities or significant effects on normal immune responses in treated mice indicates that Bz-423 has selective effects on pathogenic cells. Affinity-based screening of a phage-

display human cDNA expression library identified the oligomycin-36 sensitivity conferring protein (OSCP), a component of the mitochon-37 drial F₁F₀-ATPase, as the molecular target of Bz-423 [4]. Binding of 38 Bz-423 to the OSCP modulates the enzyme and induces a state 3 to 39 state 4 respiratory transition, leading to the formation of superoxide 40 by the mitochondrial respiratory chain (MRC). 41

To gain an understanding of the cellular response to Bz-423, we 42 previously characterized the general features of apoptosis in a Burkitt 43 lymphoma cell line (Ramos) [1]. In these cells, Bz-423-induced 44 increase in superoxide is followed by caspase activation, mitochon-45 drial electrochemical gradient ($\Delta\Psi_m$) collapse, and the release of 46 cytochrome c into the cytoplasm nearly simultaneously, consistent 47 with mitochondrial outer membrane permeabilization (MOMP) and 48 the release of cytochrome c from the mitochondrial intermembrane 49 space [5]. Following these events, morphological and biochemical 50 evidence of apoptosis is detected. In rat liver isolated mitochondria, 51 Bz-423 induces reactive oxygen species (ROS), but does not cause 52 gradient collapse or swelling. These data show that Bz-423-induced 53 superoxide does not directly trigger opening of the permeability 54 transition pore, and implicates extramitochondrial factors in the 55 mechanism coupling Bz-423-induced ROS to apoptosis. 56

To identify factors that couple Bz-423-generated superoxide to 57 apoptosis, the response to Bz-423 was studied in detail in mouse 58 embryonic fibroblasts (MEFs) [6]. Although MEFs are significantly less 59 sensitive to Bz-423-induced killing than either primary B cells or B 60 cell lymphoma-derived cell lines (i.e., longer incubation times and 61

Abbreviations: JNK, c-Jun N-terminal kinase; ASK1, apoptosis signal-regulating kinase 1; OSCP, oligomycin-sensitivity conferring protein; MRC, mitochondrial respiratory chain; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial electrochemical gradient/transmembrane potential; MOMP, mitochondrial outer membrane permeabilization; MEF, mouse embryonic fibroblast; MAP, mitogen-activated protein; DHE, dihydroethidium; CM-H₂DCFDA, 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; DCF, dichlorodihydrofluorescein; MnTBAP, manganese (III) tetrakis (4-benzoic acid)porphyrin; DKO, Bax/Bak double knockout; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cox IV, cytochrome c oxidase; V- β , the β -subunit of complex V; MPT, mitochondrial permeability transition; GSH, glutathione; BSO, L-buthionine sulfoximine; MKK, MAP kinase kinase; CHX, cycloheximide; SOD, superoxide dismutase; MFI, median fluorescence intensity; PEG-CAT, polyethylene glycol-catalase conjugate.

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higher Bz-423 concentrations are required), and in vivo data have yet to identify cytotoxic effects on nonlymphoid cells, MEFs were selected to exploit the use of well-characterized single gene knock-outs [6], to identify signaling molecules that are part of the Bz-423 response, and to identify factors that potentially explain the relative resistance of fibroblasts. Using these cells, we show that cytosolic factors, including proapoptotic Bcl-2-family proteins and mitogen-activated protein (MAP) kinases, couple Bz-423-induced ROS to an apoptotic cascade that is reflected back to the mitochondria to release cytochrome *c*. The release of cytochrome *c* commits the cell to apoptosis. The events described following Bz-423 treatment of MEFs demonstrate how ROS generated by modulation of the mitochondrial F_1F_0 -ATPase can induce a sequential and specific apoptotic signal transduction pathway.

Materials and methods

Reagents

Bz-423 was synthesized as previously described [7]. Dihydroethidium (DHE) and 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) were obtained from Invitrogen Corp. (Carlsbad, CA). Manganese (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP) was purchased from Alexis Biochemicals (Lausen, Switzerland). Unless otherwise specified, all additional reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines and culture

SV40-transformed WT, *Bax*^{-/-}, *Bak*^{-/-}, and DKO (*Bax*^{-/-}*Bak*^{-/-}) MEFs (a gift from S. Korsmeyer) were maintained in DMEM supplemented with heat-inactivated FBS (10%), 1X nonessential amino acids (Invitrogen), 2-mercaptoethanol (100 M), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (290 µg/mL). In

vitro experiments were conducted in media containing 2% FBS unless otherwise noted. Organic compounds were dissolved in media containing 0.5% DMSO.

Immunofluorescence

Cells were cultured on glass chamber slides (Nalge Nunc International, Rochester, NY). Cells were fixed (0.25 h, RT) with PBS containing paraformaldehyde (2%). To remove the fixative, cells were washed five times with PBS containing saponin (10% w/v) and heat-inactivated FBS (5%). Cells were incubated (overnight, 4°C, 1 µg/mL) with antibodies for detection of activated Bax (Catalog No. 06-499, Millipore, Charlottesville, VA) and Bak (06-536, Millipore). Following six washes, the cells were incubated (0.5 h, RT, 5 µg/mL) with biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories, Burlingame, CA). Following six washes, the cells were incubated (0.5 h, RT, 5 µg/mL) with fluorescein-conjugated avidin D (A-2001, Vector Laboratories). Samples were examined by microscopy using a Leica DM-LB microscope. Images (630×) were captured using a SPOT RS slider digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) interfaced to a Macintosh PC. Fluorescence microscopy of isolated mitochondria was performed as previously described [1].

Detection of intracellular superoxide, $\Delta\Psi_m$, cell death, and hypodiploid DNA

Detection of intracellular superoxide formation was performed monitoring the oxidation of DHE to oxyethidium by flow cytometry using the FL2 channel (585 nm) [8]. DHE (4 µM) was added to cells 30 min prior to flow cytometric analysis. Measurement of intracellular peroxide formation by the oxidation of CM-H₂DCFDA to dichlorodihydrofluorescein (DCF) was performed by flow cytometry in the FL1 channel (530 nm). Cells were preloaded (30 min) with CM-H₂DCFDA (3 µM) prior to media exchange and Bz-423 treatment. Measurement of $\Delta\Psi_m$ with DiOC₆(3) was performed by flow cytometry as previously

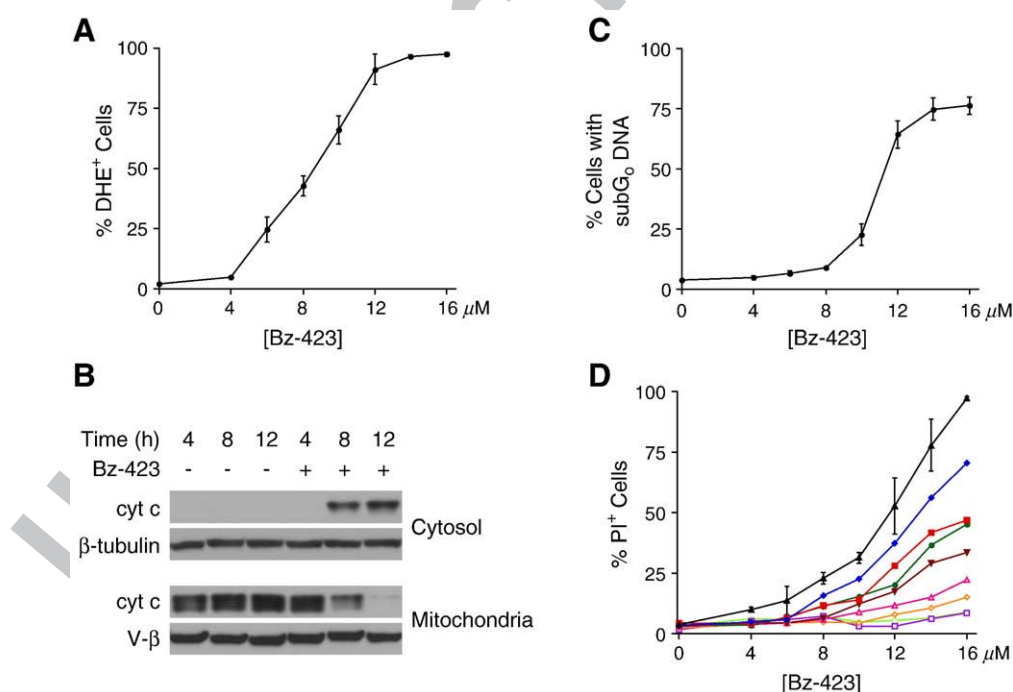


Fig. 1. Bz-423 induces apoptosis in MEFs. (A) Superoxide production (1 h) was measured using dihydroethidium (DHE). (B) Cytochrome *c* release into the cytosol was determined by Western blot following Bz-423 treatment (12 µM) for the indicated times. (C) Apoptosis (24 h) was measured by identifying cells with sub-G₀ DNA content. (D) MEFs were incubated with the indicated concentrations of Bz-423 and then Bz-423 was washed out of the media at various time points (1 h, light green open circles; 3 h, purple open squares; 5 h, orange open diamonds; 7 h, pink open triangles; 8 h, brown inverted triangles; 10 h, red squares; 12 h, blue diamonds; no wash, black triangles). Cell viability was determined by propidium iodide (PI) exclusion after 24 h of total culture.

121 described [1]. Cell viability and hypodiploid diploid DNA content was
122 assessed by staining with propidium iodide (PI) using flow cytometry
123 as previously described [1].

124 Preparation of whole cell extracts

125 Cells (20×10^6) were pelleted and washed with PBS prior to lysis
126 with WCE lysis buffer (25 mM HEPES, pH 7.7, 150 mM NaCl, 2.5 mM
127 $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β -glycerophosphate,
128 0.5 mM DTT containing 1 mM phenylmethylsulfonyl fluoride (PMSF),
129 complete protease inhibitor cocktail tablet (Roche), 3.3 mM NaF, and
130 0.1 mM sodium orthovanadate). Following incubation on ice (30 min),
131 the lysate was centrifuged (16,000 g, 0.5 h, 4°C) to pellet insoluble
132 cellular debris. Total protein content in the supernatant was quantified
133 by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

134 Mitochondria isolation

135 Cells (10^7) were harvested and washed with ice-cold PBS. Cells
136 were resuspended in ice-cold buffer A (200 μ L, 20 mM HEPES-KOH, pH
137 7.5, 10 mM KCl, 10 mM β -glycerophosphate, 5 mM NaF, 1.5 mM $MgCl_2$,
138 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 1 mM sodium
139 orthovanadate, 250 mM sucrose, complete protease inhibitor cocktail
140 tablet, and 0.1 mM PMSF). The cell suspension was allowed to sit on ice
141 (20 min) and then was disrupted by 10 strokes through a 28.5-G

142 needle. The homogenate was centrifuged (1000 g, 10 min, 4°C) to pellet
143 nuclei. The resulting supernatant was centrifuged (10,000 g, 30 min,
144 4°C) to obtain the mitochondrial fraction. The supernatant from this
145 centrifugation was harvested as the cytosolic fraction. The purity of
146 fractions was tested by immunoblotting with antibodies specific for
147 the cytosolic proteins β -tubulin or glyceraldehyde-3-phosphate dehy-
148 drogenase (GAPDH), or the mitochondrial proteins cytochrome c
149 oxidase (Complex IV, Cox IV) or the β -subunit of complex V (V- β). 149

150 Immunoblot analysis

151 Cell lysates were separated by SDS-PAGE, and transferred to
152 polyvinylidene difluoride membranes as previously described [1]. The
153 membranes were incubated with primary antibodies for proteins of
154 interest, including cytochrome c (556433, BD Biosciences, Franklin
155 Lakes, NJ), Bax (06-499, Millipore), Bak (06-536, Millipore), Bad
156 (610392, BD Biosciences), ASK1 (sc-7931, Santa Cruz Biotechnology),
157 thioredoxin (sc-20146, Santa Cruz Biotechnology), MKK7 (4172, Cell
158 Signaling Technology, Danvers, MA), phospho-MKK7
159 Ser²⁷¹/Thr²⁷⁵ (4171, Cell Signaling Technology), MKK4 (9152, Cell
160 Signaling Technology), phospho-MKK4 Ser²⁵⁷/Thr²⁶¹ (9156, Cell
161 Signaling Technology), JNK (9252, Cell Signaling Technology), phos-
162 pho-JNK Thr¹⁸³/Tyr¹⁸⁵ (9251, Cell Signaling Technology), p38 (9212,
163 Cell Signaling Technology), phospho-p38 Thr¹⁸⁰/Tyr¹⁸² (9215, Cell
164 Signaling Technology), c-Jun (9162, Cell Signaling Technology), 164

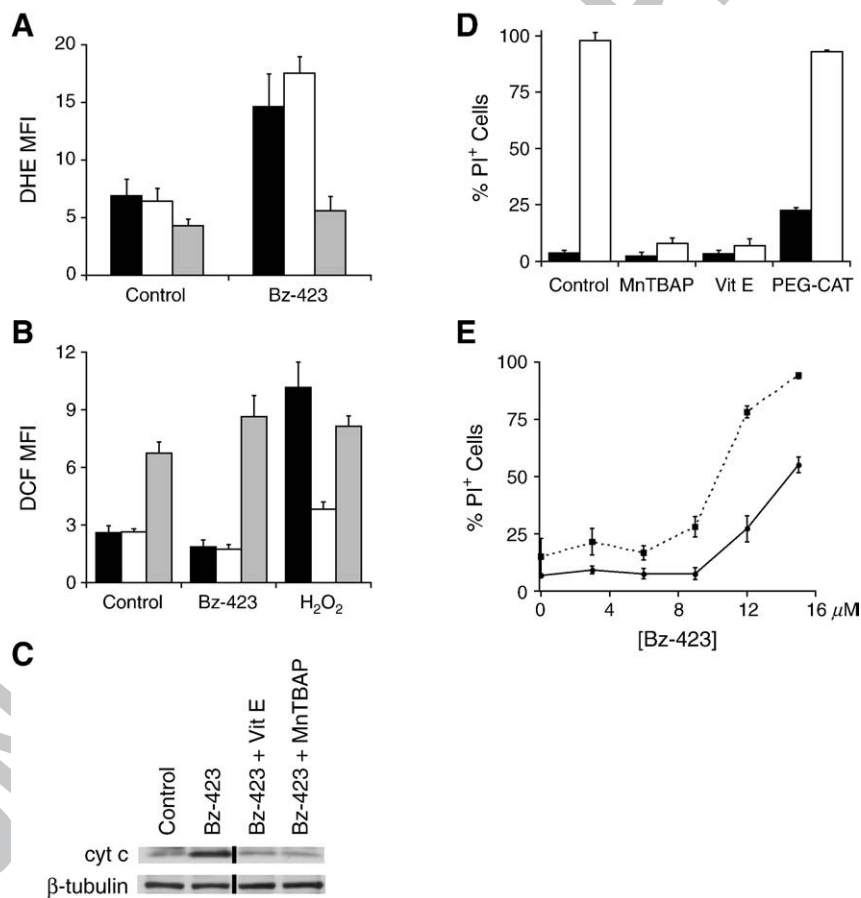


Fig. 2. Apoptosis by Bz-423 requires superoxide. (A,B) Cells were pretreated with MntBAP (30 min, 100 μ M, gray bars), PEG-CAT (overnight, 200 U/mL, white bars), or vehicle (black bars), prior to the addition of Bz-423 (12 μ M). Superoxide production (1 h, panel A) was determined by recording the FL2 channel median fluorescence intensity (MFI) as a marker of the oxidation of DHE to oxyethidium. Peroxide formation (1 h, panel B) was determined by recording the FL1 channel MFI as a marker of the oxidation of DCF. (C) Cytochrome c release into the cytosol was measured in MEFs following Bz-423 treatment (12 μ M) with or without pretreatment (30 min) with MntBAP (100 μ M) or vitamin E (100 μ M). The black vertical line indicates that nonadjacent lanes from the same gel were used to compile this figure. (D) MEFs were pretreated with vitamin E (30 min, 100 μ M), MntBAP (30 min, 100 μ M), PEG-CAT (overnight, 300 U/mL), or vehicle (black bars), prior to the addition of Bz-423 (15 μ M, white bars). Cell viability (24 h) was determined by PI exclusion. $P < 0.001$ for vehicle vs MntBAP, $P < 0.001$ for vehicle vs vitamin E, $P = 0.45$ for vehicle vs PEG-CAT. (E) After 24 h of pretreatment with vehicle (solid line) or BSO (1 mM, dashed line), MEFs were incubated with Bz-423 and cell viability determined by PI exclusion at 24 h. $P < 0.05$ for vehicle vs BSO at all [Bz-423] ≥ 3 μ M.

phospho-c-Jun Ser⁶³ (9261, Cell Signaling Technology), ATF2 (9226, Cell Signaling Technology), phospho-ATF2 Thr⁶⁹/Thr⁷¹ (9225, Cell Signaling Technology), GAPDH (MAB374, Millipore), -tubulin (T4026, Sigma-Aldrich), cytochrome *c* oxidase (A6403, Invitrogen), and -subunit of complex V (A21351, Invitrogen). Blots were then incubated with horseradish peroxidase conjugated secondary antibodies (NA931 or NA934, GE Healthcare Biosciences, Piscataway, NJ) and reacted with chemiluminescence reagents (GE Healthcare Biosciences).

173 Immunoprecipitations

174 Cytoplasmic protein (500 µg) was incubated (2 h, 4°C) with agarose
175 conjugated anti-ASK1 (15 µg, sc-7931-AC, Santa Cruz Biotechnology)
176 or anti-thioredoxin (15 µg, sc-20146-AC, Santa Cruz Biotechnology)
177 while rotating. The immunocomplexes were isolated by centrifugation
178 (1000 g, 1 min). The immunoprecipitate was washed three times with
179 buffer A and once with buffer B (50 mM Tris, pH 7.5, 10 mM MgCl₂,
180 0.02% BSA) prior to analysis via immunoblot.

181 Statistical analysis

182 Where indicated, statistical significance was assessed by a
183 Student's *t* test. *P* values are two-tailed, and all data are presented as
184 mean ± one standard deviation (SD), unless otherwise noted.
185 Figures contain representative data of experiments performed in
186 triplicate.

187 Results

188 Isolated mitochondria respond to Bz-423

189 Incubating mitochondria isolated and purified from MEFs with Bz-
190 423 under conditions supporting state 3 respiration results in
191 increased superoxide within the mitochondria (Fig. S1A). This
192 response is consistent with inhibition of the F₁F₀-ATPase, and
193 demonstrates that mitochondria respond to Bz-423 independent of
194 other components of the cell. In this cell-free system, however, Bz-423
195 does not cause ΔΨ_m collapse or trigger cytochrome *c* release (Figs. S1A
196 and B). Together, these data show that Bz-423 does not directly induce
197 opening of the mitochondrial permeability transition (MPT) pore and
198 reveals that extramitochondrial factors couple mitochondrial-generated
199 superoxide to eventual cytochrome *c* release (and MOMP) at
200 which point the cell is irreversibly committed to die [9].

201 Bz-423 induces apoptosis in MEFs

202 As with isolated mitochondria, Bz-423 rapidly increases super-
203 oxide levels in MEFs within 1 h and the magnitude of the increase is
204 concentration dependent (Fig. 1A). Consistent with the activation of an
205 intrinsic apoptotic pathway [5], release of cytochrome *c* into the
206 cytosol is detected at 8 h (Fig. 1B). By 12 h, mitochondria are depleted
207 of cytochrome *c* (Fig. 1B) and ΔΨ_m has collapsed (data not shown).
208 Activation of caspases-9 and 3 between 8 and 12 h is observed
209 consistent with activation of the apoptosome by cytochrome *c* (data
210 not shown). These events are followed by apoptotic DNA fragmenta-
211 tion and cell death (24 h, Figs. 1C and D). Of note, the EC₅₀ values for
212 apoptotic DNA changes are similar to the EC₅₀ for changes in plasma
213 membrane permeability indicating that Bz-423-induced cell death
214 results from apoptosis.

215 To investigate the kinetics of cell death, Bz-423 was washed out of
216 the culture media at multiple time points, and cell viability measured
217 at 24 h (Fig. 1D). While incubation of MEFs with Bz-423 for 1 h is
218 sufficient to generate superoxide, it is insufficient, irrespective of Bz-
219 423 concentration, to cause cell death. Treatment of MEFs with Bz-423
220 for 8 h, the point at which cytochrome *c* release is first observed,
221 causes modest cell death. The maximal death response requires

222 exposure of MEFs to Bz-423 for at least 12 h, the point at which we
223 observe ΔΨ_m collapse and near complete release of cytochrome *c*
224 from the mitochondria. By comparison, lymphocytes show apoptotic
225 DNA changes by 5 h and require less drug to induce apoptosis [1].

Bz-423-induced apoptosis dependent on superoxide

226 To examine the nature of the ROS signal generated by Bz-423, MEFs
227 were pretreated with various antioxidants prior to the detection of
228 ROS with DHE and DCF. As seen in Fig. 2A, pretreatment with MnTBAP
229 dramatically decreases the DHE response induced by Bz-423
230 (*P*=0.001). While Bz-423 does not induce a DCF response on its
231 own, pretreatment with MnTBAP significantly augments this signal
232 (*P*<0.001, Fig. 2B). These results are consistent with the actions of
233 MnTBAP as a superoxide dismutase mimetic (see review by Patel and
234 Day [10]). In contrast, pretreatment with a cell permeant form of
235 catalase conjugated to polyethylene glycol (PEG-CAT, [11]) does not
236 inhibit the Bz-423 DHE or DCF responses (*P*=0.12 and *P*=0.62,
237 respectively), despite reducing the DCF response induced by hydrogen
238 peroxide (*P*=0.001, Figs. 2A and B). Taken together, these results are
239 consistent with the specific production of superoxide by Bz-423 via its
240 ability to modulate the F₁F₀-ATPase.

241 To determine the importance of Bz-423-induced superoxide in the
242 MEF death response, MEFs were pretreated with the antioxidants
243 vitamin E or MnTBAP. Each of these agents prevents both cytochrome
244 *c* release (Fig. 2C) and Bz-423-induced cell death (*P*<0.001, Fig. 2D). In
245

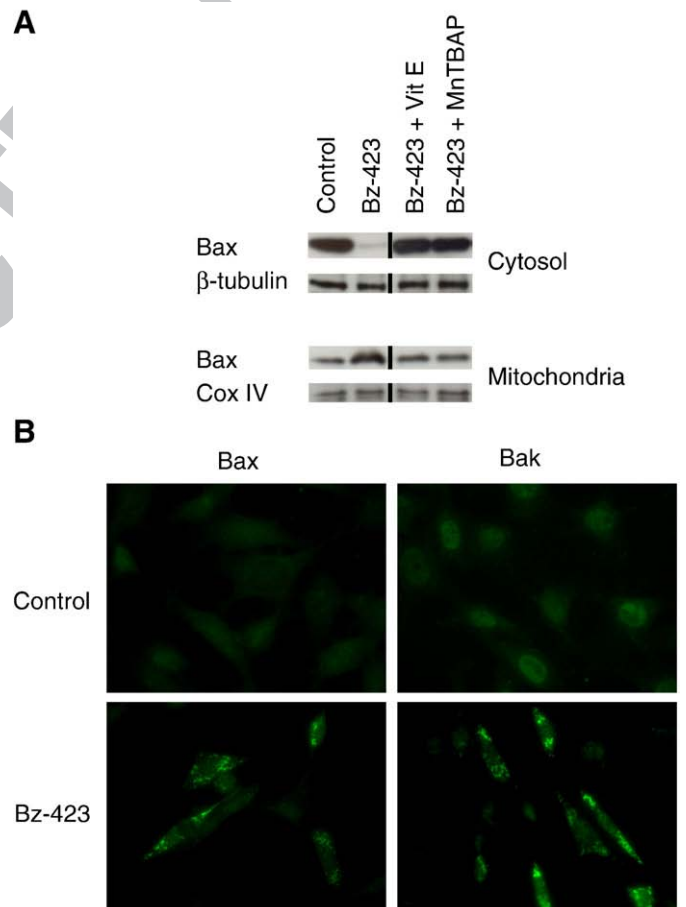


Fig. 3. Bz-423 activates Bak and Bax. (A) MEFs were pretreated with MnTBAP (100 µM) or vitamin E (100 µM) for 30 min prior to treatment with Bz-423 (12 µM, 12 h) and cell fractionation. Cytosolic or mitochondrial Bax was detected by immunoblot. The black vertical line indicates where nonadjacent lanes from the same gel were used to compile this figure. (B) MEFs were treated with vehicle or Bz-423 (12 µM, 12 h) followed by detection of activated N-terminal Bax or Bak by immunofluorescence microscopy.

contrast, pretreatment of MEFs with PEG-CAT, which does not inhibit Bz-423 superoxide production, fails to inhibit Bz-423-induced cell death ($P=0.45$, Fig. 2D). These findings confirm that Bz-423-induced apoptosis requires superoxide. Furthermore, the correlation observed between the amount of inhibition of cytochrome *c* release and inhibition of cell death supports the hypothesis that cytochrome *c* release is a key checkpoint in this response [9].

Glutathione (GSH) is a major component of the cellular defense to oxidants and also functions as a regulator of oxidant-sensitive enzymes [12]. To assess the importance of GSH in determining the cellular response to Bz-423, MEFs were treated with L-buthionine sulfoximine (BSO), an inhibitor of γ -glutamylcysteine synthetase, the rate-limiting step in GSH synthesis [13]. Treatment of MEFs with BSO (1 mM, 24 h) decreases both cytoplasmic and mitochondrial GSH stores by greater than 99% relative to those in untreated cells (data not shown). After pretreatment, MEFs were incubated with Bz-423 for 24 h, and cell viability was determined (Fig. 2E). Glutathione-depleted

cells are more sensitive to Bz-423 demonstrating the importance of endogenous GSH levels in determining cellular sensitivity to Bz-423. The magnitude of this sensitization is similar to what is observed with other prooxidants [14,15].

Bz-423-induced apoptosis depends on Bcl-2 proteins

The next series of experiments focused on determining the signaling mechanism intervening between Bz-423-induced ROS and MOMP, reflected by cytochrome *c* release. MOMP results from either (i) activation and homo-oligomerization of proapoptotic multidomain Bcl-2 proteins Bax and/or Bak or (ii) opening of the MPT pore leading to mitochondrial swelling-induced rupture of the outer membrane [9]. Since we did not observe cytochrome *c* release when isolated mitochondria are exposed to Bz-423 (Fig. S1), we focused on experiments to determine whether Bax and/or Bak is required for Bz-423-induced cytochrome *c* release. In their inactive states, Bax is

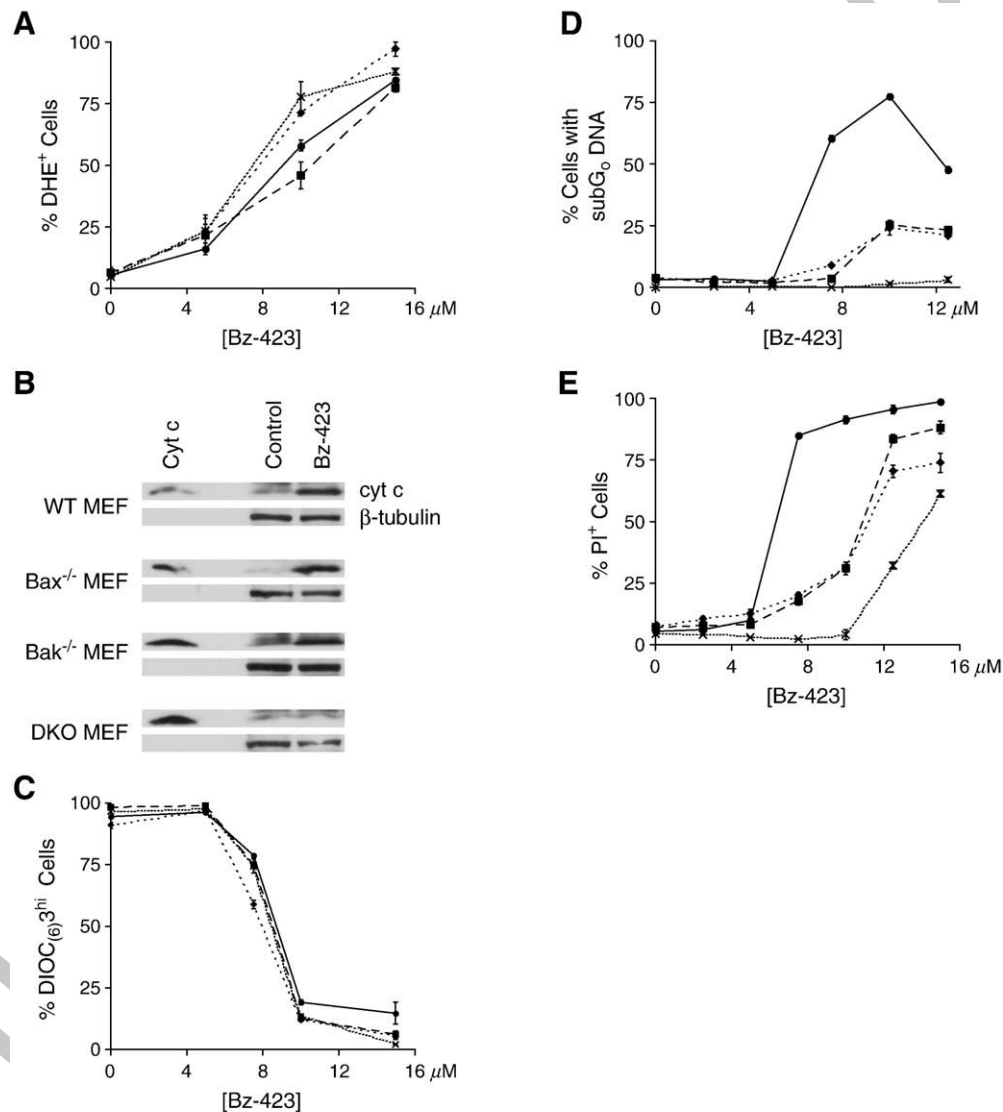


Fig. 4. Bak and Bax knockout block Bz-423-induced death. (A) Superoxide was measured using DHE in WT MEF (circles, solid line), $Bax^{-/-}$ MEF (squares, long dashes), $Bak^{-/-}$ MEF (diamonds, short dashes), and DKO MEF ("X", dotted line) after treatment with Bz-423 (1 h). (B) Cytosolic fractions prepared from WT, $Bax^{-/-}$, $Bak^{-/-}$, and DKO MEF following treatment (12 h) with Bz-423 (12 μ M) or control were analyzed for cytochrome *c*. (C) $\Delta\Psi_m$ was measured using $DIOC_6(3)$ in WT, $Bax^{-/-}$, $Bak^{-/-}$, and DKO MEF following Bz-423 (12 h). (D) Apoptosis was determined by hypodiploid DNA content in WT (circles, solid line), $Bax^{-/-}$ (squares, long dashes), $Bak^{-/-}$ (diamonds, short dashes), and DKO MEFs ("X", dotted line) following Bz-423 treatment (48 h). $P < 0.01$ for WT vs $Bax^{-/-}$, $Bax^{-/-}$, or DKO MEFs at all [Bz-423] $> 5 \mu$ M, $P < 0.01$ for $Bak^{-/-}$ vs DKO MEFs at all [Bz-423] $> 5 \mu$ M, $P < 0.01$ for $Bax^{-/-}$ vs DKO MEFs at all [Bz-423] $> 7.5 \mu$ M. (E) Cell viability was determined by PI exclusion in WT, $Bax^{-/-}$, $Bak^{-/-}$, and DKO MEF following Bz-423 treatment (48 h). $P < 0.05$ for WT vs $Bax^{-/-}$ or $Bax^{-/-}$ MEFs at all [Bz-423] $> 5 \mu$ M, $P < 0.02$ for WT vs DKO MEFs at all [Bz-423] $\geq 2.5 \mu$ M, $P < 0.02$ for $Bak^{-/-}$ vs DKO at [Bz-423] between 2.5 and 12.5 μ M, $P < 0.01$ for $Bax^{-/-}$ vs DKO at all [Bz-423] $> 2.5 \mu$ M.

278 primarily a cytoplasmic protein, while Bak is associated with
 279 mitochondria [16]. On activation, both proteins undergo conforma-
 280 tional changes and, in the case of Bax, translocate to the mitochondria,
 281 causing MOMP and allowing cytochrome *c* release from the
 282 intermembrane space.

283 In control MEFs, Bax is detected almost exclusively in the cytosolic
 284 fraction, whereas treatment with Bz-423 for 12 h increases the
 285 amount of Bax in the mitochondrial fraction (Fig. 3A). These findings
 286 are consistent with Bz-423 treatment causing Bax activation and
 287 translocation. Pretreating cells with MnTBAP or vitamin E inhibits Bax
 288 translocation, indicating that this response depends on Bz-423
 289 triggered superoxide (Fig. 3A). To confirm that Bax undergoes
 290 conformational activation and assess whether Bak is similarly
 291 activated in response to Bz-423, MEFs were incubated with antibodies
 292 to Bax and Bak that recognize an N-terminal epitope that is only
 293 accessible on activation. Using immunofluorescence microscopy, we
 294 find that cells treated with Bz-423 for 12 h display a bright, punctate
 295 staining pattern with each antibody (Fig. 3B). This pattern is not seen
 296 in control cells, and these results are consistent with conformational
 297 activation and mitochondrial localization of these proteins following
 298 treatment with Bz-423.

299 MEFs derived from *Bax*^{-/-}, *Bak*^{-/-}, or double knockout mice (DKO)
 300 were used to determine if these proteins are required for the apoptotic
 301 response to Bz-423. Wild-type MEFs, along with the three knockout
 302 strains, increase superoxide in response to Bz-423, consistent with
 303 these proteins being involved downstream of the initial ROS signal

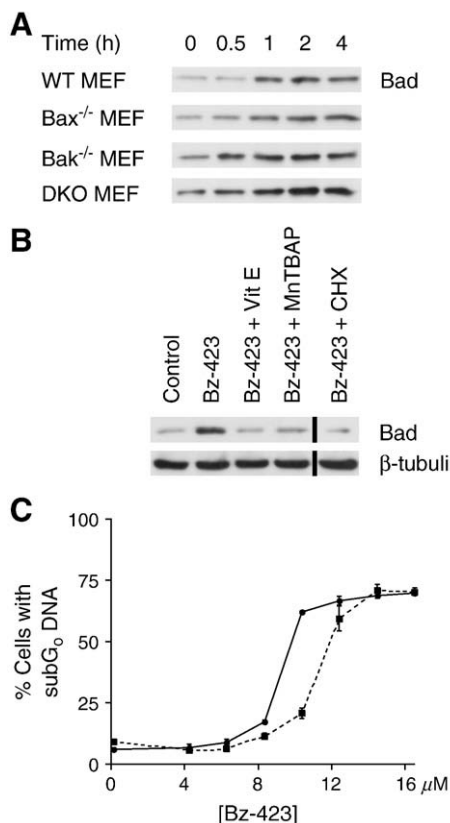


Fig. 5. Bad expression increases in response to Bz-423. (A) After treatment with Bz-423 (10 μ M), lysates were prepared and immunoblotted to detect Bad protein in WT, *Bax*^{-/-}, *Bak*^{-/-}, and DKO MEF. (B) WT MEF were pretreated with MnTBAP (100 μ M), vitamin E (100 μ M), CHX (1 μ g/mL), or vehicle for 30 min prior to treatment with Bz-423 (10 μ M, 2 h) and then immunoblotted for Bad. The black vertical line indicates where nonadjacent lanes from the same gel were used to compile this figure. (C) Following treatment with Bz-423 for 48 h, WT (solid line) and *Bad*^{-/-} (dashed line) MEFs were analyzed to detect cells with sub-G₀ DNA content. $P < 0.05$ for WT vs *Bad*^{-/-} MEFs at 8 and 10 μ M Bz-423.

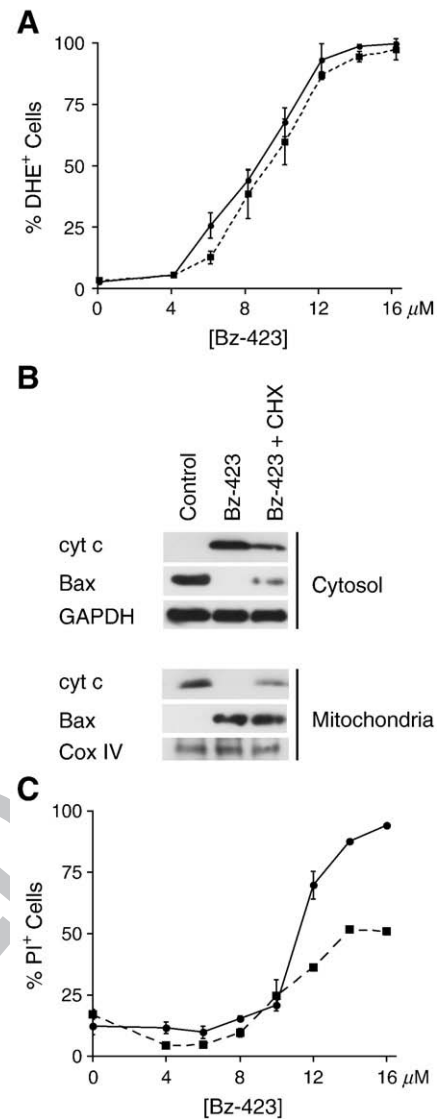


Fig. 6. CHX inhibits Bz-423-induced apoptosis. (A) Following pretreatment (30 min) with CHX (1 μ g/mL, dashed line) or vehicle (solid line), MEFs were incubated with Bz-423 and superoxide production (1 h) was monitored with DHE. (B) MEFs were pretreated with CHX (1 μ g/mL) or vehicle for 30 min prior to incubation with Bz-423 (12 μ M, 12 h), fractionated as indicated, and immunoblotted to detect cytochrome *c* and Bax. (C) Following pretreatment with CHX (1 μ g/mL, dashed line) or vehicle (solid line), MEFs were incubated with Bz-423 and cell viability (24 h) was determined by PI exclusion. $P < 0.02$ for vehicle vs CHX at [Bz-423] > 10 μ M.

(Fig. 4A). The cells were then analyzed for cytochrome *c* release and $\Delta\Psi_m$ after 12 h of treatment, a time point at which Bax and Bak 305 activation and Bax translocation are readily detectable. Knockout of 306 either Bax or Bak has little if any effect on Bz-423-induced cytochrome 307 *c* release, whereas cytochrome *c* release is blocked in DKO MEFs (Fig. 308 4B). In contrast to the results with cytochrome *c* release, $\Delta\Psi_m$ collapse 309 following Bz-423 is not inhibited in any of the knockout cell lines (Fig. 310 4C). These results show that Bz-423-induced MOMP is mediated by 311 Bax and Bak, which have redundant functions in these cells. 312

Differences in Bz-423-induced apoptotic DNA fragmentation are 313 observed among control, single knockout, and DKO cells that are 314 consistent with the differences in cytochrome *c* release (Fig. 4D). In 315 particular, DKO MEFs do not undergo apoptosis as indicated by the 316 absence of hypodiploid DNA content even after 48 h of culture. 317 Supporting the involvement of both Bax and Bak in the mechanism, 318 the single knockout cell lines show an intermediate level of apoptotic 319 DNA changes compared to WT and DKO MEFs. Similar differences are 320

also observed when overall cell viability based on plasma membrane integrity is assessed in these four cell lines (Fig. 4E). Higher concentrations of Bz-423 (>10 μ M) are able to overcome the protection afforded by knockout of Bak and Bax against cell death measured by PI exclusion. This finding suggests that in the absence of effective MOMP, higher concentrations of Bz-423 eventually induce nonapoptotic cell death, likely via secondary necrosis from prolonged alterations in redox balance and $\Delta\Psi_m$ collapse [17].

Bax and Bak activation is regulated by the balance between antiapoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-x_L, Mcl-1) and proapoptotic BH3-only proteins [16]. Because increased expression or, in some cases, posttranslational modification of BH3-only proteins can activate the proapoptotic functions of Bax and Bak [18], we screened cellular lysates to detect changes in selected BH3-only proteins following Bz-423 treatment. We find that Bad levels increase within 1 h of Bz-423 treatment (Fig. 5A), whereas no changes in the expression level of other BH3-only proteins (Puma, Bim, Bid, Bik, Bmf, or Blk) are observed (data not shown). The increase in Bad expression is blocked in cells pretreated with antioxidants (vitamin E or MnTBAP) while knockout of Bax and/or Bak has no effect on Bad expression (Figs. 5A and B). Pretreatment with the protein synthesis inhibitor cycloheximide (CHX) also prevents the increase in Bad levels (Fig. 5B). These results indicate that Bz-423-induced superoxide increases Bad levels

independent of Bak and Bax and also indicates that Bz-423 signals de novo protein synthesis upstream of Bax and Bak activation. To determine the relative importance of Bad in Bz-423-induced apoptosis, we tested MEFs from Bad^{-/-} mice. We find that knockout of this BH3-only protein inhibits, but does not prevent, apoptosis (Fig. 5C). From these results, we conclude that Bad is not the sole signal through which Bax and Bak are activated following Bz-423 treatment.

Because Bz-423 increased Bad expression is blocked by CHX, we next examined the effect of CHX on the overall Bz-423 response mechanism. Consistent with the direct interaction of Bz-423 with the F₁F₀-ATPase, superoxide generation (Fig. 6A) is not inhibited by CHX. CHX partially inhibits Bax translocation, cytochrome c release, and Bz-423-triggered cell death (Figs. 6B and C). These results, when combined with partial protection afforded by knockout of Bad, suggest that the commitment to cell death as indicated by MOMP and mediated by Bax/Bak activation is controlled by two separate signal-response couples, one dependent on and one independent of de novo protein synthesis.

Bz-423 activates MAP kinases

The partial inhibition of Bz-423-induced apoptosis by CHX suggests that in MEFs additional nonprotein synthesis-dependent

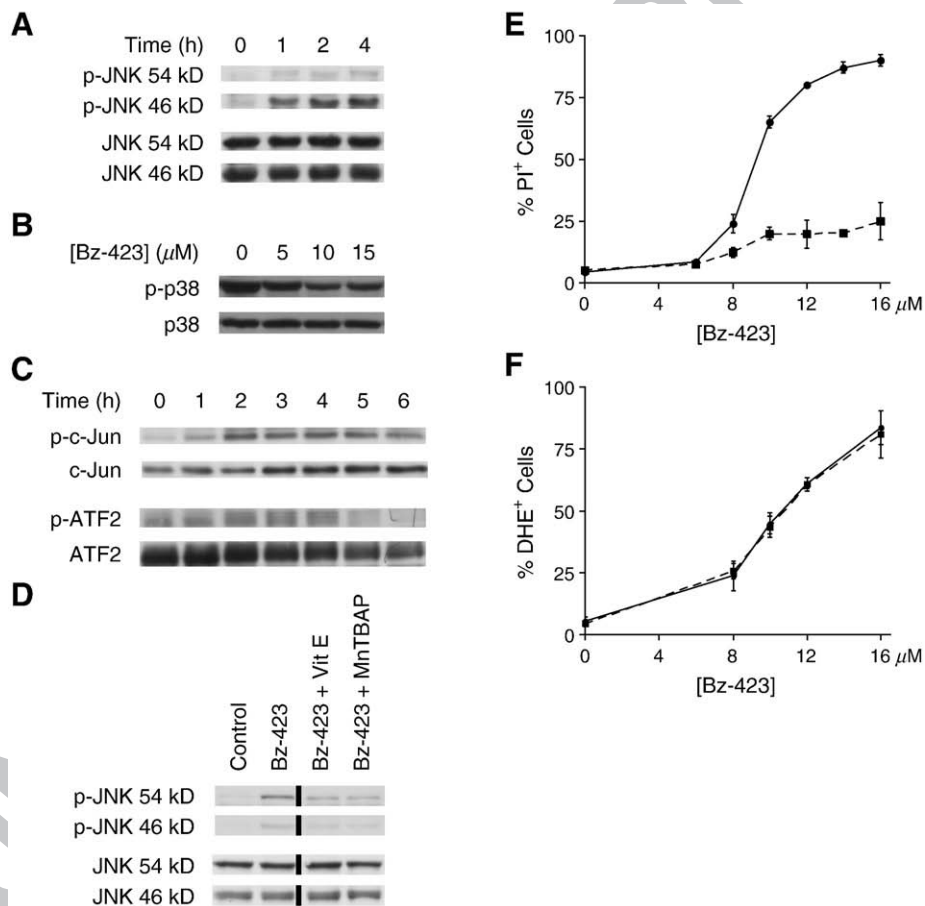


Fig. 7. Bz-423 activates JNK. (A) Lysates prepared from MEFs treated with Bz-423 (10 μ M) were immunoblotted to detect total and phosphorylated JNK. (B) Following treatment (2 h) with the indicated concentrations of Bz-423 (in μ M), total cellular lysates were immunoblotted for total and phospho-p38. (C) Lysates prepared from MEFs treated with Bz-423 (10 μ M) were immunoblotted to detect total and phosphorylated c-Jun and ATF2. (D) MEF were pretreated with MnTBAP (100 μ M), vitamin E (100 μ M), or vehicle for 30 min prior to treatment with Bz-423 (10 μ M, 2 h). Whole cell lysates were immunoblotted for JNK and phospho-JNK expression. The black vertical line indicates where nonadjacent lanes from the same gel were used to compile this figure. (E) Following pretreatment with SP600125 (10 μ M, dashed line) or vehicle (solid line), MEFs were incubated with Bz-423 and viability (24 h) was determined by PI exclusion. $P < 0.01$ for vehicle vs SP600125 at [Bz-423] > 8 μ M. (F) Following pretreatment with SP600125 (10 μ M, dashed line) or vehicle (solid line), MEFs were incubated with Bz-423 and superoxide was detected by DHE (1 h).

cellular signals are activated by Bz-423 downstream of superoxide and upstream of Bax, and Bax. The MAP kinases, JNK and p38, are good candidates to link these responses because activation of JNK and p38 often occurs following changes in redox balance, and both can link cellular stress to activation of Bax and Bak and to changes in gene transcription [18–20]. Treatment of MEFs with Bz-423 induces phosphorylation of JNK (Fig. 7A) but not p38 (Fig. 7B). To determine the functional consequence of JNK activation, we assayed the phosphorylation state of two of its substrates, the transcription factors c-Jun and ATF2 [21]. Treatment with Bz-423 causes sustained phosphorylation of both of these proteins (Fig. 7C), consistent with JNK activation effecting changes in gene transcription [22].

Pretreating MEFs with antioxidants inhibits JNK activation (Fig. 7D), placing JNK downstream of Bz-423-induced superoxide. We used SP600125, a kinase inhibitor selective for JNK [23], to determine if JNK is required for the Bz-423 apoptotic response. As seen in Fig. 7E, pretreatment with SP600125 almost completely prevents Bz-423 killing of MEFs implying that Bz-423-induced JNK activation is central to the death mechanism. Indeed, pretreatment with SP600125 blocks the Bz-423-induced increase in Bad levels, Bax activation, and cytochrome c release (data not shown), but does not inhibit Bz-423-induced superoxide (Fig. 7F). These results indicate that JNK activation is required for Bz-423-induced apoptosis in MEFs, and that this kinase is activated at a proximal point in the signaling cascade triggered by Bz-423, prior to the activation of Bax/Bak.

As part of the MAP kinase signaling cascade, JNK is phosphorylated by the upstream kinases MKK4 and MKK7 [19]. As expected, we find that MKK4 and MKK7 are phosphorylated following treatment with Bz-423 (Fig. 8A). MKK4/7 are in turn substrates of apoptosis signaling-regulating kinase-1 (ASK1) that couples changes in cellular redox balance with activation of JNK [20]. ASK1 is essential for sustained activation of JNK in apoptosis induced by oxidants, including hydrogen peroxide and TNF- α [19,22]. In its inactive state, ASK1 is a cytosolic protein complexed with reduced thioredoxin-1 (Thx1). When Thx1 is oxidized, it dissociates from ASK1, enabling ASK1 to autoactivate [19]. Therefore, we tested whether Bz-423 induces Thx1-ASK1 dissociation and ASK1 phosphorylation. In control cells, an intact Thx1-ASK1 complex coimmunoprecipitates, whereas treatment

with Bz-423 causes a time-dependent dissociation of ASK1 from Thx1 (Fig. 8B). Moreover, within 30 min of treatment, increasing amounts of phosphorylated ASK1 are detected (Fig. 8C). Taken together, these results are consistent with a mechanism in which Bz-423-induced ROS activates a sequential protein kinase cascade that involves ASK1 and JNK that in turn leads to activation of Bak and Bax through protein synthesis-dependent and -independent signals.

Discussion

Inhibition of F_1F_0 -ATPase induces a state 3 to state 4 transition leading to formation of reduced intermediates in mitochondria [4,24,25]. These reactive intermediates (e.g., reduced ubiquinones) can form at both the matrix and the intermembrane sides of the inner membrane and release superoxide into both compartments [26]. Superoxide can also be formed on the matrix side at complex I most likely via the transfer of an electron from a half-reduced flavin mononucleotide to molecular oxygen [27,28]. Superoxide levels in cells are limited by dismutation to hydrogen peroxide through the action of Mn-superoxide dismutase (SOD) in the mitochondrial matrix or Cu,Zn-SOD in the intermembrane space and cytoplasm [26]. As part of cellular antioxidant defenses, reduced glutathione present both in the mitochondrial matrix and in cytoplasm can also react with (and detoxify) ROS including superoxide [29]. Depletion of GSH with BSO sensitizes cells to Bz-423, supporting the hypothesis that ROS generated by Bz-423 is critical for its apoptosis. Moreover, these findings suggest that part of the selectivity of Bz-423 for different cell types may result from variation in cellular antioxidant defenses. Similarly, such differences underlie the selectivity of several redox active anticancer agents [30].

Bz-423 rapidly activates ASK1 in MEFs. This MAP kinase kinase is increasingly recognized as a cytosolic redox sensor that triggers apoptosis [20]. ASK1 is found as a homooligomer and may also complex with several other proteins, including thioredoxin, 14-3-3 proteins, and calcineurin in untreated cells [19,31]. Thioredoxin binds to and inhibits ASK1 by interfering with the homophilic interaction of ASK1 via its N-terminal coiled-coil domain [32]. Oxidation of cysteine residues in thioredoxin to cystine releases thioredoxin from this complex and activates ASK1 through *trans*-autophosphorylation [32]. This activation is triggered by prooxidants including hydrogen peroxide, the heavy metals arsenic, cadmium, and mercury, and the complex I inhibitor rotenone, as well as the oxidative burst that accompanies TNF- α signaling [33–35].

Following exposure to various prooxidants, activation of ASK1 is generally associated with activation of both p38 and JNK [20]. In contrast, we only detect activation of JNK following treatment with Bz-423. The interactions among kinases and substrates in the MAPK cascade are highly regulated. Treatment-specific and cell-type-specific responses are in part mediated by the differential involvement of scaffold proteins in the regulation of ASK1 [36]. One example is JNK/stress-activated protein kinase-associated protein 1 (JSAP1) that is activated by ASK1 in response to oxidants, and in turn leads to the preferential recruitment of MKK7-JNK3 as opposed to p38 as ASK1 substrates [36]. Gemin5 is another recently described scaffolding protein that is required for JNK1 activation following prooxidant treatment with hydrogen peroxide and TNF- α [37]. Gemin5 specifically interacts with ASK1, MKK4, and JNK1. Gemin5 potentiates ASK1 homooligomerization, and does not interact with MKK7, MKK3, MKK6, or p38. Thus, the observed MKK4/7-JNK-specific recruiting function of Bz-423-activated ASK1 may depend on the specific expression and activation of stimulus-specific adaptors. Alternatively, specific MAP kinase activation is regulated by the action of dual specificity phosphatases [38]. For example, oxidative stress-mediated hepatotoxicity following treatment with carbon tetrachloride is associated with activation of the phosphatase MKP-1 that dephosphorylates p38 and ERK, and is activated by JNK [39].

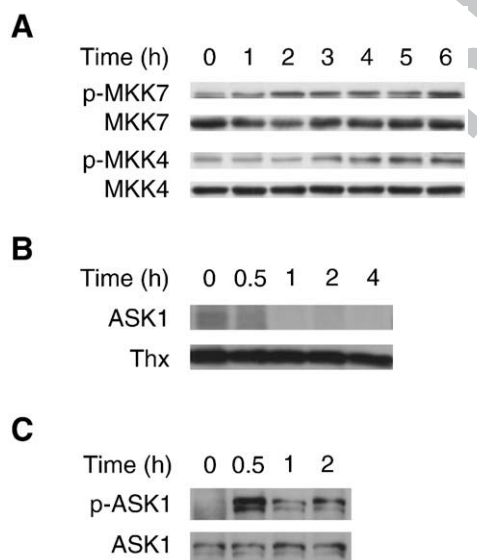


Fig. 8. Bz-423 activates upstream MAP kinases. (A) Lysates prepared from MEFs treated with Bz-423 (10 μ M) were immunoblotted to detect total and phosphorylated MKK4 and MKK7. (B) Coimmunoprecipitation of Thx and ASK1 after treatment with Bz-423 (12 μ M) shown by immunoprecipitating Thx and then blotting the immune complexes for ASK1 and Thx. (C) Lysates prepared from cells treated with Bz-423 (12 μ M) were immunoprecipitated for ASK1 and then blotted to detect total ASK1 and phospho-ASK1.

Treatment with Bz-423 may also activate this phosphatase, as we observe dephosphorylation of p38 (Fig. 7B) and ERK (unpublished observations).

The identity of the signal that leaves the mitochondria to trigger extramitochondrial apoptotic pathways following Bz-423 treatment is unclear. The ability of the superoxide dismutase mimetic MnTBAP to prevent Bz-423-induced apoptosis (and not PEG-CAT) implies that superoxide, and not hydrogen peroxide, is the relevant ROS. However, recent reports have questioned the dismutase functions of commercial lots of this compound, and suggested that MnTBAP may instead be acting as a xanthine oxidase inhibitor [40]. In support of its role as a superoxide dismutase mimetic, we find that MnTBAP inhibits DHE oxidation and induces a prominent DCF response in cells treated with Bz-423. In addition, MnTBAP does not significantly inhibit the DCF response in cells treated with hydrogen peroxide (Fig. 2B). We have also previously shown that allopurinol, a specific inhibitor of xanthine oxidase, does not inhibit Bz-423-induced DHE oxidation [1]. Taken together, these findings indicate that MnTBAP is acting as a superoxide dismutase mimetic and support our hypothesis that treatment with Bz-423 specifically generates superoxide. Recent studies indicate that this reaction occurs via a sulfenic acid intermediate that does not involve hydrogen peroxide [41,42]. Collectively, these findings suggest the possibility that superoxide is the direct link between the initial mitochondrial response to Bz-423 and the activation of ASK1. Indeed, ASK1 is activated by other superoxide sources including methylglyoxal and NADPH oxidase [43–45]. For this reaction with thioredoxin to occur, superoxide must be exported from mitochondria into the cytosol. Although it does not readily diffuse through lipid membranes, superoxide can be transported from the mitochondria through via the voltage-dependent anion channel located in the mitochondrial outer membrane [46].

The precise mechanism by which JNK activates Bax and Bak activation following Bz-423 treatment in MEFs is not yet clear. Both SP600125 and CHX prevent increased levels of Bad, suggesting that the observed increase results from JNK-regulated de novo protein synthesis. Because knockout of Bad or pretreatment with CHX only provides modest protection against Bz-423-induced apoptosis, other nonprotein synthesis-dependent mechanisms by which Bax and Bak are activated must be involved. Indeed, JNK can activate Bax or Bak by mechanisms independent of protein synthesis that include the direct phosphorylation of Bax, Bcl-2, and various BH3-only proteins [18,47,48]. Based on our results showing limited protection by CHX and greater protection by SP600125, we hypothesize that JNK orchestrates both protein synthesis-dependent and -independent effects.

Cytochrome c release

A key checkpoint in Bz-423-induced apoptosis is the release of cytochrome c from mitochondria. The mechanisms through which this molecule is released are still debated [9]. One long-standing hypothesis proposes that opening of the MPT pore leads to swelling of the inner mitochondrial membrane that ruptures the outer mitochondrial membrane, releasing proapoptotic proteins from the intermembrane space [9]. Oxidizing agents induce MPT pore opening, suggesting that Bz-423 may be acting along these lines [49–51]. However, experiments with isolated mitochondria derived from MEFs or rat liver confirm that Bz-423 does not directly induce the release of cytochrome c from the intermembrane space nor does it cause large amplitude mitochondrial swelling seen following MPT [1]. These findings argue that Bz-423-generated superoxide does not directly induce the MPT. An alternative hypothesis is that cytochrome c is selectively released by MOMP triggered by activation of proapoptotic multidomain proapoptotic Bcl-2 proteins [9,16]. Our data are consistent with this hypothesis-Bax and Bak are essential for cytochrome c release and apoptosis following treat-

ment with Bz-423. Therefore, Bax and Bak represent the apoptotic signal that returns to the mitochondria to cause the release of this key mediator.

Comparison to other MRC inhibitors

ROS-mediated apoptosis is caused by other agents that modulate the MRC including antimycin A, rotenone, thenoyltrifluoroacetone and troglitazone [52,53]. Cyanoaziridines like imexon also induce oxidant-dependent apoptosis via the direct reaction with and depletion of glutathione [54–56]. Oligomycin, the best characterized F_1F_0 -ATPase inhibitor, can give rise to a respiratory transition and generate ROS [4,25]. However, the binding site for this compound is located within the membrane-spanning F_0 component [57]. In addition to the mitochondrial F_1F_0 -ATPase, oligomycin inhibits the Na^+/K^+ - and H^+/K^+ -ATPases [58,59], suggesting that these enzymes contain similar binding pockets. In contrast to Bz-423, oligomycin induces time-dependent inhibition of the F_1F_0 -ATPase [60], and is associated with decreases in total cellular ATP [52]. Because apoptosis requires sufficient levels of ATP to allow caspase activation through apoptosome formation [61], ATP synthesis inhibitors that result in a large decrease in intracellular ATP levels would not be able to engage apoptosis process like Bz-423, and instead induce necrosis [62]. Hence, Bz-423 is not directly comparable to oligomycin. However, some other agents that inhibit the F_1F_0 -ATPase seem to have similar properties to Bz-423. For example, 3,3'-diindolylmethane induces redox-regulated apoptosis via inhibition of the F_1F_0 -ATPase [63,64]. This compound has antitumor properties in animal models of cancer [65,66], and, taken with our data, suggests that modulation of the F_1F_0 -ATPase may be a valuable approach for new drug development [67].

Conclusions

Our data show that redox balance modulates ASK1-JNK-Bax/Bak signaling in response to Bz-423. Because antioxidants inhibit all downstream signals, superoxide is positioned at a proximal point in the response mechanism engaged following modulation of F_1F_0 -ATPase activity by Bz-423. However, the reduction in cellular glutathione levels with BSO only modestly sensitizes MEFs to this compound, which suggests that the variation in glutathione existing across cell types may only be a minor factor in determining the sensitivity of cells to Bz-423, and is consistent with data showing that the apoptotic response of cells to other prooxidants does not strictly correlate with total cellular glutathione [14,68–71].

In contrast to the limited role for glutathione, inhibition of JNK signaling profoundly inhibits the cellular response to Bz-423 by preventing activation of the proapoptotic Bcl-2, as well as inhibiting apoptosis and overall cell death. Since members of the MAP kinase family are differentially regulated across cell types (and stages of development) [72], the ability of a cell to activate these kinases in response to Bz-423 may be a key factor in modulating the selective response of Bz-423. Furthermore, the partial requirement for de novo protein synthesis (which follows MAP kinase activation) in the apoptotic response in MEFs helps to explain why these cells require prolonged exposure to Bz-423 relative to lymphoid cell types where Bz-423-induced death is independent of de novo protein synthesis [73]. These findings suggest that a different signal transduction pathway might link Bz-423-triggered superoxide to MOMP and apoptosis in lymphocytes. Such differences likely are key elements that underlie the favorable selectivity observed in vivo.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2008.07.022.

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