

Bax Channel Inhibitors Prevent Mitochondrion-mediated Apoptosis and Protect Neurons in a Model of Global Brain Ischemia*

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Ischemic injuries are associated with several pathological conditions, including stroke and myocardial infarction. Several studies have indicated extensive apoptotic cell death in the infarcted area as well as in the penumbra region of the infarcted tissue. Studies with transgenic animals suggest that the mitochondrion-mediated apoptosis pathway is involved in ischemia-related cell death. This pathway is triggered by activation of pro-apoptotic Bcl-2 family members such as Bax. Here, we have identified and synthesized two low molecular weight compounds that block Bax channel activity. The Bax channel inhibitors prevented cytochrome *c* release from mitochondria, inhibited the decrease in the mitochondrial membrane potential, and protected cells against apoptosis. The Bax channel inhibitors did not affect the conformational activation of Bax or its translocation and insertion into the mitochondrial membrane in cells undergoing apoptosis. Furthermore, the compounds protected neurons in an animal model of global brain ischemia. The protective effect in the animal model correlated with decreased cytochrome *c* release in the infarcted area. This is the first demonstration that Bax channel activity is required in apoptosis.

Apoptosis is a conserved cell death mechanism essential for normal development and tissue homeostasis in multicellular organisms. Although apoptosis presumably participates in the development of all cell lineages, aberrations in the expression of pro- or anti-apoptotic proteins have been implicated in the initiation of a variety of human diseases, including arteriosclerosis, heart failure, infertility, autoimmunity, immunodeficiency, and cancer, in addition to diseases affecting the nervous system such as neurodegeneration and ischemia (1–3). Several intracellular apoptosis signaling pathways have been identified, including the death receptor pathway and the mitochondrial pathway (4–6). The induction of apoptosis ultimately converges upon the activation of cysteine proteases of the caspase family. The Bcl-2 family proteins are located upstream at organelle membranes and control the activation of downstream caspases, representing a critical proximal intracellular

checkpoint in the mitochondrial apoptosis pathway. The Bcl-2 family is composed of pro- and anti-apoptotic members. Anti-apoptotic Bcl-2 family members display sequence homology in four α -helical domains called BH1–BH4.³ Pro-apoptotic Bcl-2 members can be further subdivided into more fully conserved, “multidomain” members with homology in the BH1–BH3 domains (e.g. Bax and Bak) or the “BH3-only” members (e.g. Bid, Bad, and Bim). Genetic and biochemical analyses indicate that the multidomain proteins Bax and Bak function as the essential gateway to the intrinsic cell death pathway operating at the mitochondria. The upstream BH3-only members respond to particular apoptotic signals and subsequently, either directly or indirectly, trigger the conformational activation of Bax and/or Bak. Overexpression of the anti-apoptotic protein Bcl-2 or deletion of the pro-apoptotic protein Bax increases resistance to ischemic insults, indicating that the mitochondrial pathway is involved in ischemia-related apoptosis (7, 8). Further evidence for the crucial role of Bax in neuronal cell death was provided by a recent study showing that cerebellum granule neurons are protected against prion-induced apoptosis in Bax^{-/-} mice (9). In normal cells, Bax is present as a soluble monomeric protein in the cytosol. When cells are exposed to various apoptotic stimuli, including hypoxia, the protein translocates specifically to the mitochondria (10–12). Bid activation has been shown to be important in hypoxia-induced apoptosis (13). At the mitochondria, Bax forms oligomers, which are inserted into the outer mitochondrial membrane, permeabilizing the membrane and triggering the release of proteins, including cytochrome *c*, from the mitochondrial intermembrane space (11, 14). In the cytosol, cytochrome *c* forms a complex with the cytosolic proteins Apaf-1 and procaspase-9 (15). Upon complex formation, caspase-9 is proteolytically modified, leading to its activation. The active caspase-9 activates downstream executor caspases, which subsequently cleave several substrates, ultimately leading to apoptotic cell death.

Recombinant oligomeric Bax possesses channel-forming activity in artificial lipid membranes and triggers cytochrome *c* release from liposomes and purified mitochondria (16–18). Although it is clear that Bax has a central function in the regulation of the mitochondrial apoptosis pathway, it remains unclear how the protein executes its pro-apoptotic activity at the molecular level and whether its channel-forming activity is required. To elucidate the function of Bax, we have identified low molecular weight compounds that function as Bax channel blockers and

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† Deceased.

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³ The abbreviations used are: BH, Bcl-2 homology; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; VDAC, voltage-dependent anion channel; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Bci1, Bax channel inhibitor-1; Bci2, Bax channel inhibitor-2; HEK, human embryonic kidney; FACS, fluorescence-activated cell sorter; MEF, murine embryonic fibroblast.

that do not alter Bax homo-oligomerization. Using this pharmacological tool, we show that Bax channel activity is required for cytochrome *c* release from mitochondria and that blocking Bax channel activity inhibits mitochondrion-mediated apoptosis in cells *in vitro*. In addition, the compounds protected neurons against apoptotic cell death in an animal model of global brain ischemia. The effect *in vitro* is Bax-specific because the channel inhibitors did not prevent apoptosis in Bax-deficient cells, whereas they did in Bak-deficient and wild-type cells. These findings strongly suggest that the mechanism by which these compounds inhibit apoptosis *in vivo* is through blockage of Bax channel activity and thus, for the first time, demonstrate involvement of Bax channel activity in the regulation of apoptotic cell death.

MATERIALS AND METHODS

Recombinant Proteins—C-terminally truncated oligomeric Bax was expressed as a glutathione *S*-transferase fusion protein in *Escherichia coli* and purified as described previously (19). Full-length oligomeric Bax with an N-terminal His tag was expressed in and purified from *E. coli* (20). Bid with an N-terminal His tag was expressed in *E. coli* and purified on nickel-nitrilotriacetic acid-agarose as described (21). t^c Bid was generated through cleavage of purified Bid with caspase-8.

Liposome Channel Assay—Liposomes containing 20 mM 5,6-carboxyfluorescein in phosphate-buffered saline (PBS) were prepared as described (18). The liposomes were diluted in PBS to give a suitable fluorescence value. The channel activity assay was performed in 96-well plates on a fluorescence plate reader (FLIPRTM, Novel Tech Systems Inc.). For the assay, 70 ml of PBS, 15 ml of oligomeric Bax (1 μ M) in PBS, and 10 ml of compound in eight dilutions (final concentrations of 22 nM to 3.8 μ M) in 10% Me₂SO in PBS were mixed in a 96-well plate and incubated at room temperature for 1 min. At the end of the incubation, 20 μ l of liposomes in PBS was added, and the fluorescence was monitored in the FLIPRTM every 3 s for 3 min. The fluorescence values at 120 s were used to calculate the IC₅₀ values.

Electrophysiological Recordings—Planar lipid membranes were formed from monolayers made from 1% (w/v) lipids in hexane on 70–80- μ m diameter orifices in a 15- μ m-thick Teflon partition that separated the two chambers as described (22, 23). The lipid-forming solutions contained 90% asolectin (soybean phospholipids) and 10% cholesterol or 42% asolectin, 42% diphytanoylphosphatidylcholine, 8% cardiolipin, and 8% cholesterol. Asolectin, diphytanoylphosphatidylcholine, and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL), and cholesterol was purchased from Sigma. The membranes were made in aqueous solutions containing 250 mM KCl and 1 mM MgCl₂ or CaCl₂ buffered with 5 mM HEPES or MES at pH 7.0 or 5.5, correspondingly. VDAC channels were isolated from rat liver mitochondrial outer membranes and purified according to the standard method (24, 25). Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage clamp mode. Data were filtered by a low pass 8-pole Butterworth filter (Model 9002, Frequency Devices, Inc., Haverhill, MA) at 15 kHz, recorded on a chart recorder, and directly saved in the computer memory with a sampling frequency of 50 kHz. The inhibitory effect of the compounds was determined as follows. After 20 min of continuous recording of Bax-induced channel activity, the inhibitors (50–60 nM) were added either to the *trans*-side or to both sides of the membrane under constant stirring for 2 min. The currents were recorded for 10 min, followed by a series of additions of the inhibitor until a significant decrease or complete inhibition of the conductance was obtained.

Mitochondrial Cytochrome *c* Release Assay—Mitochondria were isolated from HeLa cells as described (14). In summary, the cells were

suspended in buffer A (10 mM HEPES-NaOH, 210 mM mannitol, 70 mM sucrose, and 1 mM EDTA, pH 7.4) and disrupted by passage through a 25 G1 0.5 \times 25 needle, and the mitochondria were isolated by differential centrifugation. The mitochondria were diluted to 0.4 mg/ml in buffer B (10 mM HEPES-NaOH, 125 mM KCl, 4 mM MgCl₂, 5 mM NaH₂PO₄, 0.5 mM EGTA, and 5 mM succinate, pH 7.5). The compound was added to 1 ml of mitochondrial suspension, and the samples were incubated for 5 min at room temperature. t^c Bid was subsequently added to a final concentration of 10 nM, and the samples were incubated at 30 °C for 15 min and centrifuged twice at 12,000 \times *g* for 10 min. The supernatant was removed and analyzed by Western blotting with an in-house-raised rabbit anti-cytochrome *c* polyclonal antibody. The blots were developed with an ECL detection kit (Amersham Biosciences), and the intensity of the cytochrome *c* bands was determined by densitometry. Mitochondria incubated without addition of t^c Bid were taken as the blank value, and 100% cytochrome *c* release corresponds to mitochondria treated with t^c Bid in the absence of inhibitor.

Quaternary Structure of Purified Bax—Purified recombinant monomeric Bax at a concentration of 0.4 mg/ml was incubated in the presence and absence of the compounds (10 μ M) for 1 h at 37 °C. Alternatively, Bax was preincubated as described above; at the end of the incubation 2% octyl glucoside was added to induce Bax oligomerization; and the samples were further incubated for 2 h. At the end of the incubation periods, the samples were analyzed by gel filtration on a Superdex 200 column (PC 3.2/30) equilibrated in 25 mM HEPES-NaOH, 300 mM NaCl, 0.2 mM dithiothreitol, and 2% (w/v) CHAPS, pH 7.5, in the SMART system (Amersham Biosciences). 50 μ l of the sample was loaded and eluted at 50 μ l/min; 50- μ l fractions were collected; and the eluate was monitored at 280 nm.

Quaternary Structure of Bax in Cells—HeLa cells were seeded and grown to confluence in 15-cm plates. The cells were then treated with 1 μ M staurosporine in the presence and absence of 2 μ M Bax channel inhibitor-1 (Bci1) and Bax channel inhibitor-2 (Bci2) for 16 h. In addition, cells were also treated with the compounds in the absence of staurosporine. Untreated cells were used as a negative control. At the end of the incubation, all cells were collected and washed, and the mitochondria were isolated as described above. The mitochondria isolated from the staurosporine-treated cells were washed with 0.1 M Na₂CO₃, pH 12, to remove non-membrane integrated proteins. Untreated cells and cells treated with the compounds alone were not because this would have removed non-activated Bax completely (14). The washed mitochondria were solubilized in 25 mM HEPES-NaOH, 300 mM NaCl, 0.2 mM dithiothreitol, and 2% (w/v) CHAPS, pH 7.5. After a 1-h incubation at 4 °C, the samples were centrifuged at 100,000 \times *g* for 30 min. The supernatant containing the solubilized mitochondrial proteins was analyzed by gel filtration as described above. The fractions eluted from the column were analyzed by Western blotting with anti-Bax antibodies (anti-Bax NT, catalog no. 06-499, Upstate Biotechnology, Inc.). Alternatively, Bax activation was monitored by immunofluorescence in cells treated with staurosporine using anti-Bax NT antibodies (which do not react with non-activated Bax) following the protocol described previously (26).

Induction of Apoptosis in Cell Lines—HeLa cells or SV40 immortalized murine embryonic fibroblasts from wild-type or knockout cells were seeded in 6-well plates; and after 24 h, the cells were treated with 2 μ M staurosporine for 3 h with and without a 30-min pretreatment with the inhibitors (10 μ M). At the end of the incubation, the mitochondrial membrane potential and cell volume were analyzed by flow cytometry (FACSCalibur, BD Biosciences) after staining the cells with 3,3'-dihexyloxycarbocyanine iodide (Molecular Probes) as described (27). Analysis was performed using the CellQuest program. In parallel, cells were

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treated with 2 μM staurosporine for 6 h with and without pretreatment with the inhibitors (2 μM), and nuclear morphology was analyzed by staining with Hoechst 33342 (Molecular Probes). Alternatively, cell viability was quantified using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate (CellTiter 96[®] AQ_{ueous}, Promega Corp., Madison, WI) according to the recommendations of the supplier. For Bax or Bak overexpression, human embryonic kidney (HEK) cells were transfected using SuperFect reagent (Qiagen Inc.) following the manufacturer's instructions. After 16 h in the presence or absence of the compounds (2 μM), the cells were collected, and hypodiploid cells were quantified by fluorescence-activated cell sorter (FACS) analysis after propidium iodide staining as described previously (27). Alternatively, apoptosis was induced by overexpression of t^cBid in wild-type, Bax^{-/-}, and Bak^{-/-} murine embryonic fibroblast (MEF) cells. t^cBid was expressed by retroviral delivery as described previously, and cell viability was determined after 24 h by FACS analysis after propidium iodide staining (28).

Global Brain Ischemia in Gerbils—Adult male Mongolian gerbils (Elevage Janvier, Le Genest St. Isle, France) weighing 60–80 g were kept in a temperature-controlled (23 \pm 1 $^{\circ}\text{C}$) and light/dark cycle-controlled animal room (lights on at 7 a.m. and off at 7 p.m.). All experiments (eight animals/group) were performed under spontaneous respiration. Anesthesia was induced with 4% isoflurane in a gas mixture of medical air administered via facemask and maintained at 2% isoflurane in the same gas mixture. Bilateral common carotid arteries were dissected and occluded with bulldog clamps for 5 min.

To examine the effect of the Bax channel inhibitor on brain ischemia, Bci1 (3 and 30 mg/kg intraperitoneally) and Bci2 (30 mg/kg intraperitoneally) solubilized in saline were injected 15 min, 24 h, and 48 h after reperfusion. Orotic acid (300 mg/kg intraperitoneally) was used as the reference compound. 7 days after the onset of occlusion, the gerbils were killed by decapitation. The brains were frozen in isopentane (-20°C) and cut into 20- μm -thick sections in a microtome. The sections were stained with cresyl violet, and infarcts in the right and left parts of the hippocampus were scored with a 4-point scale. Pyramidal cells showing atrophy, shrinkage, nuclear pyknosis, dark cytoplasmic coloration, and vacuolation and the disappearance of the radial striated zone indicated cell degeneration: 0, no loss of CA1 neurons; 1, weak damage of CA1 (CA1/subiculum or CA1/CA3 border); 2, loss of CA1 neurons (less than half); 3, loss of CA1 neurons (more than half); and 4, total loss of CA1 neurons and expansion into other areas (CA3, dentate gyrus, and cortex). The total score is the sum of the scores for the left and right hemispheres (29, 30).

Global Brain Ischemia-induced Cytochrome *c* Release in Gerbil Hippocampus—Gerbils were submitted to a 5-min bilateral occlusion of the common carotid arteries as described above. The ischemic gerbils ($n = 2-6$) were killed 2, 4, 24, 48, 96, 120, or 168 h after the onset of ischemia. To evaluate the effects of the Bax inhibitor on the global ischemia-induced cytochrome *c* release in gerbil hippocampus, the animals were treated with Bci1 (30 mg/kg intraperitoneally) 15 min, 24 h, and 48 h after reperfusion. They were killed 5 days after the onset of ischemia.

The hippocampi were excised and kept on ice. They were cut into small pieces and subsequently suspended in 10 mM HEPES-NaOH, 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM EGTA, and protease inhibitor mixture (Roche Applied Science), pH 7.4. The cells were disrupted by passage through a 25-gauge 1-inch needle, and the mitochondria were removed by differential centrifugation as described above for isolation of mitochondria from HeLa cells. Three breaking cycles through the needle were performed, and the supernatant fraction from

each one was treated and analyzed separately. The supernatant fractions corresponding to the cytosolic cell fraction were analyzed by Western blotting with anti-cytochrome *c* antibody. Most cytochrome *c* was detected in the first and second fractions. The Western blots were scanned with a densitometer, and the intensity of the band corresponding to cytochrome *c* was determined. All cytochrome *c* values were normalized to the cytochrome *c* content in the cytosolic fraction from non-ischemic animals, which corresponded to a value of 1.

Statistical Analysis—All data are expressed as means \pm S.E. Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's *t* test. Significance was considered as $p < 0.05$ versus the control group.

RESULTS

Bax Channel Inhibitors in the Liposome Channel Assay—To elucidate the molecular mechanism by which Bax exerts its pro-apoptotic activity, a large collection of low molecular weight compounds was screened for Bax channel inhibitors. The results enabled us to identify and synthesize compounds possessing specific Bax channel inhibitory activity. Here, we describe the mechanism of two of these inhibitors, Bci1 and Bci2 (Fig. 1A). Bax channel-forming activity was first studied using liposomes charged with the fluorescent dye 5,6-carboxyfluorescein. When the liposomes were incubated with either C-terminally truncated or full-length oligomeric Bax, its channel-forming activity induced the release of 5,6-carboxyfluorescein from the liposomes, which was measured as an increase in fluorescence over time. Addition of Bci1 or Bci2 to the assay solution inhibited Bax channel-forming activity in a concentration-dependent manner (Fig. 1, B and D). The IC₅₀ values were determined to be 0.81 ± 0.22 and 0.89 ± 0.29 μM , respectively (Fig. 1, C and E). At 2.4 μM , the activity was completely inhibited in both cases.

Electrophysiological Characterization of the Bax Inhibitors—Because the liposome assay cannot distinguish between various possible mechanisms of inhibition such as inhibition of Bax insertion into the lipid membrane, the prevention of Bax channel assembly, or a genuine blockage of the Bax channels, we characterized the compounds electrophysiologically. Two typical experiments are illustrated in Fig. 2. Fig. 2A shows an example of Bax channel activity 15 min after addition of 20 nM full-length oligomeric Bax. Addition of 0.15 μM Bci1 caused a gradual decrease in Bax-induced conductance from ~ 9 to 0.7 nanosiemens over 10 min (Fig. 2B). The conductance was completely inhibited after addition of 0.20 μM Bci1 (Fig. 2C). The inhibitory effect of Bci2 is illustrated in Fig. 2 (D–F). To avoid any possible interaction between the inhibitor and Bax other than on the Bax-formed channels, the protein and the compound were added to the opposite sides of the membrane. Fig. 2D shows Bax-induced channels 5 min after addition of 30 nM full-length oligomeric Bax to the *cis*-compartment. Addition of 0.15 μM Bci2 to the *trans*-compartment caused a gradual decrease in Bax-induced conductance (Fig. 2E), with complete inhibition after addition of 0.2 μM Bci2 (Fig. 2F). The inhibitory effects of both Bci1 and Bci2 were seen in the 0.10–0.22 μM range.

We previously demonstrated that full-length oligomeric Bax channels have a wide distribution of conductances, ranging from 100 picosiemens to tens of nanosiemens (18, 31). When the Bax-induced conductance was large (more than ~ 20 nanosiemens), addition of Bci1 up to 2.6 μM did not inhibit the channels. Bax channel activity is enhanced at acidic pH. The possibility that acidic pH might influence the interaction between Bax channels and the inhibitor was tested by performing experiments at both neutral (pH 7.0, $n = 4$) and acidic (pH 5.5, $n = 9$) pH. No difference in inhibition was detected (data not shown).

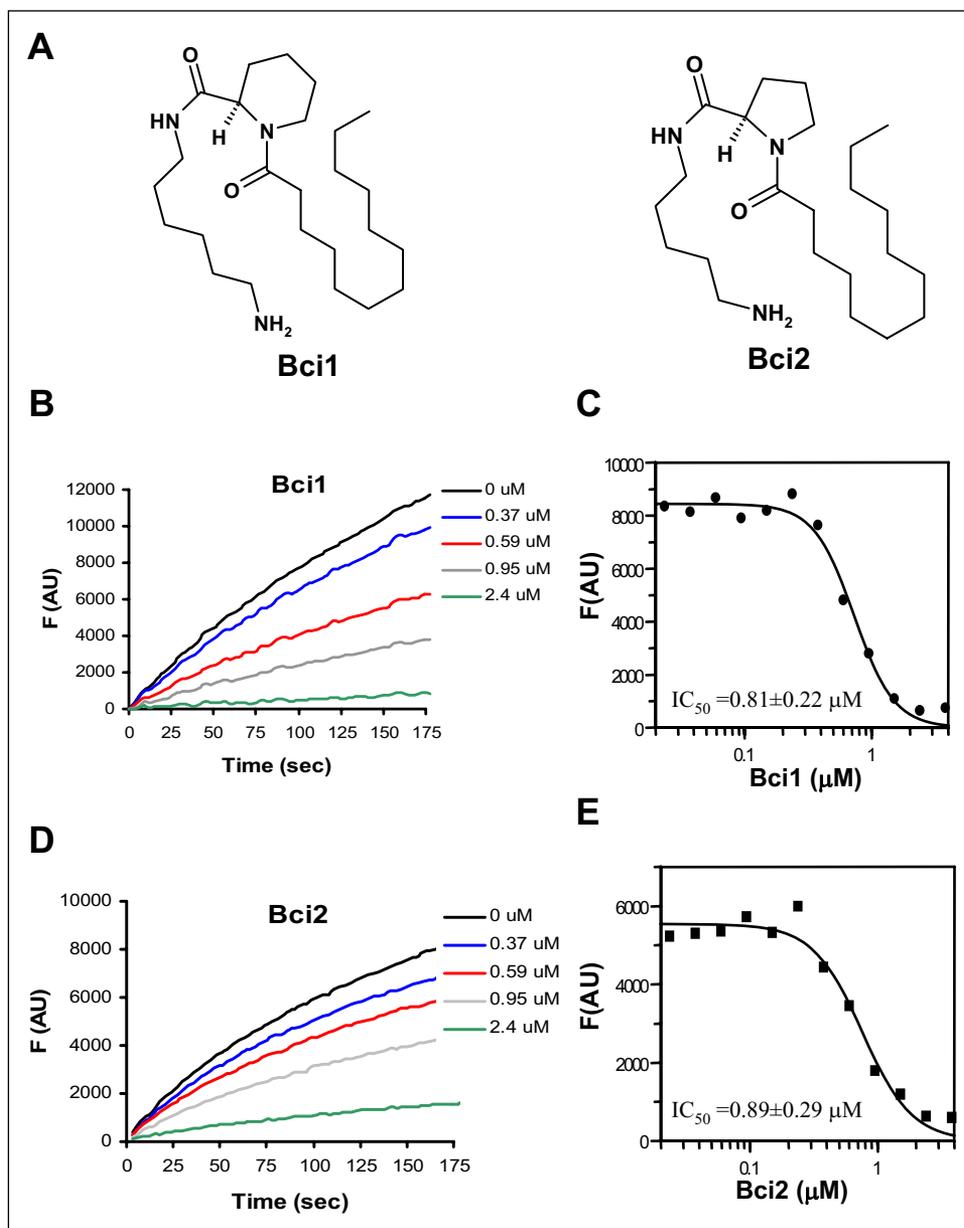


FIGURE 1. Structures of the Bax channel inhibitors and inhibition of Bax channel-forming activity in liposomes. *A*, the structures of Bci1 and Bci2 are shown. *B–E*, liposomes containing 20 mM 5,6-carboxyfluorescein were diluted to 1 ml in PBS. Oligomeric Bax was preincubated with increasing concentrations of Bci1 or Bci2 at room temperature for 1 min. *B* and *D*, at time 0, liposomes were added to the Bax/compound mixture, and the change in fluorescence (excitation at 488 nm and emission at 520 nm) was recorded every 3 s for 3 min. *C* and *E*, the fluorescence values at $t = 120$ s were plotted against the log compound concentration, and the IC_{50} values were determined. The IC_{50} values were determined to be $0.81 \pm 0.22 \mu\text{M}$ ($n = 15$) for Bci1 and $0.89 \pm 0.29 \mu\text{M}$ ($n = 10$) for Bci2. *AU*, arbitrary units.

VDAC is one of the major proteins in the mitochondrial outer membrane. It has been suggested that VDAC could play a role in cytochrome *c* release during apoptosis (32). To test the specificity of the inhibitors, we performed control experiments with VDAC channels. No effect on either VDAC channel conductance or gating properties was observed in the presence of $0.20\text{--}0.75 \mu\text{M}$ Bci1 ($n = 5$) or $0.1\text{--}2.0 \mu\text{M}$ Bci2 ($n = 2$) (data not shown).

Bid is another pro-apoptotic member of the Bcl-2 protein family. We have found that Bid forms stable pores in planar lipid membranes containing cardiolipin after cleavage with caspase-8 (t^{Bid}) (33). We tested the inhibitory effect of Bci1 on t^{Bid} -induced conductance. Here again, there was no decrease in the current of t^{Bid} channels in the presence of the inhibitor up to $0.50 \mu\text{M}$ ($n = 3$). Furthermore, the two compounds were tested in three *in vitro* ion channel assays (Na^+ channel, Na^+/K^+ -ATPase and Na^+/H^+ antiporter) and showed no significant inhibitor effects at a concentration of $1 \mu\text{M}$. These data indicate that the inhibitors have a strong Bax specificity and do not function as nonspecific ion channel blockers.

*Bax Channel Inhibitors Prevent Cytochrome *c* Release from Mitochondria*—We further tested whether the Bax channel inhibitors could prevent cytochrome *c* release from isolated HeLa cell mitochondria. Mitochondria isolated from HeLa cells have endogenous non-activated Bax loosely attached to the outer mitochondrial membrane, which can be activated through incubation with t^{Bid} . Bid induces Bax oligomerization and insertion into the mitochondrial outer membrane, with subsequent cytochrome *c* release from the intermembrane space (34). At the end of the incubation period, the mitochondria were removed by centrifugation, and the supernatant was analyzed for cytochrome *c* by Western blotting. As shown in Fig. 3, the compounds inhibited t^{Bid} -induced cytochrome *c* release in a concentration-dependent manner. $20 \mu\text{M}$ Bci2 inhibited the release to $>90\%$ (Fig. 3, *B* and *C*). The fact that a plateau value was reached at $10\text{--}20 \mu\text{M}$ Bci1 is most likely due to the limited solubility of the compound in the buffer required for the mitochondrial experiments (Fig. 3, *A* and *C*). A limited solubility of Bci1 might delay its inhibitory effect, allowing formation of larger Bax channel complexes that are less efficiently inhibited by the

FIGURE 2. Inhibition of Bax channel activity in planar lipid membranes. Shown are continuous current recordings of the Bax-induced channels in planar membranes in the absence of inhibitor (A and D) and after addition of Bci1 (B and C) or Bci2 (E and F) to the aqueous solutions, which contained 250 mM KCl, 1 mM MgCl₂, and 5 mM MES, pH 5.5. A–C, 20 nM full-length oligomeric Bax and the indicated concentrations of Bci1 were added to both sides of the membrane. D–F, 30 nM full-length oligomeric Bax was added to the *cis*-compartment, and the indicated concentrations of Bci2 were added to the opposite *trans*-side of the membrane. The times after addition of Bax and the inhibitors are specified. The *dashed lines* show the zero-current level. The applied voltage was +20 mV. Current recordings were filtered using an averaging time of 30 ms.

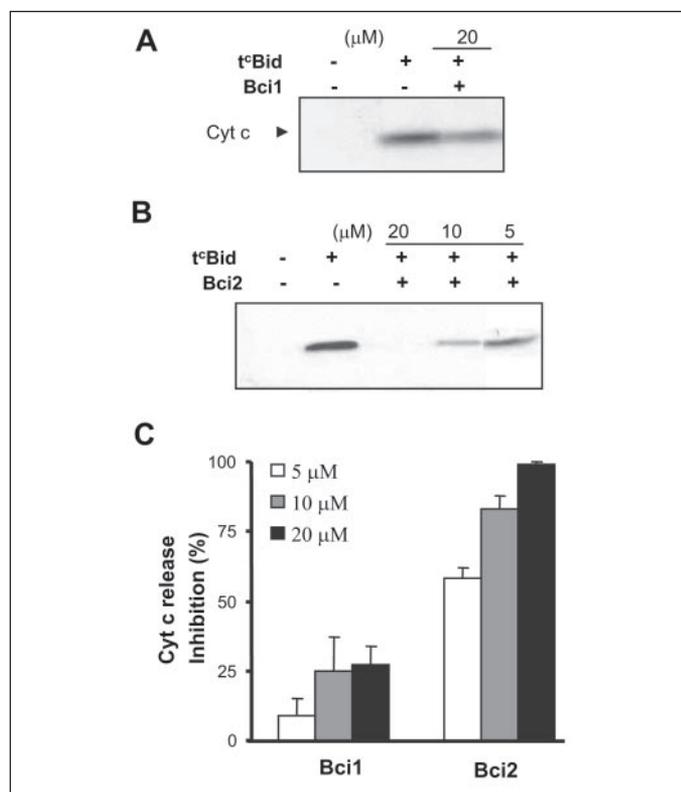
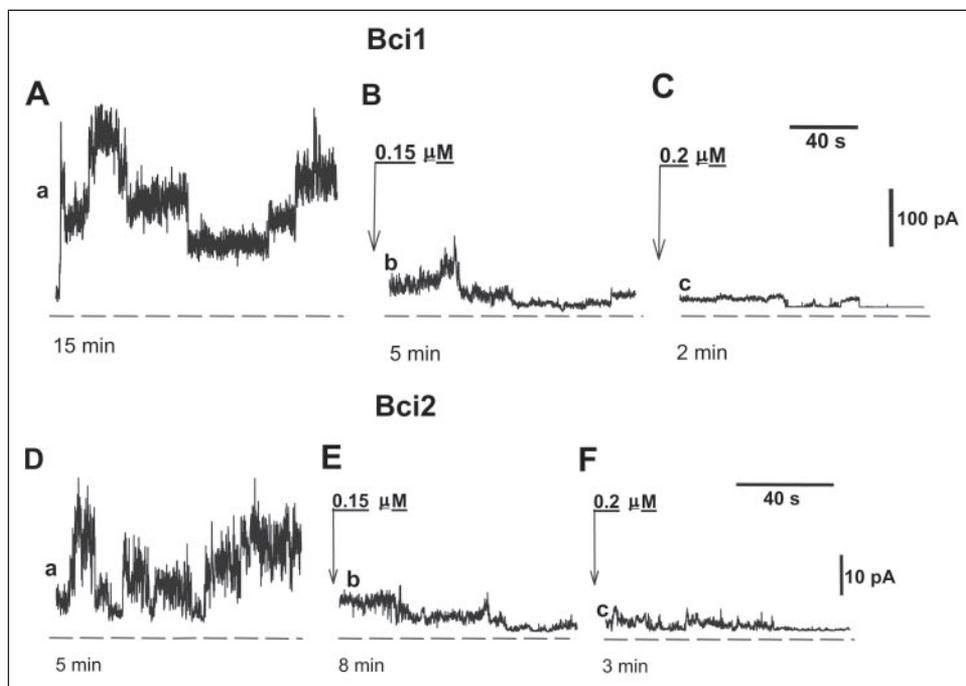


FIGURE 3. Inhibition of Bax-induced cytochrome *c* release from isolated mitochondria. Isolated HeLa cell mitochondria were incubated in buffer B with the indicated concentrations of Bci1 or Bci2 for 5 min at room temperature. Subsequently, Bax activation was induced by addition of 10 nM tBid. The mixtures were incubated at 30 °C for 15 min. At the end of the incubations, the samples were centrifuged at 12,000 × *g* for 10 min. The supernatant was analyzed by Western blotting with anti-cytochrome *c* (Cyt *c*) antibody. All experiments were performed in duplicate. A: *first lane*, untreated; *second lane*, tBid; *third lane*, tBid and Bci1 (20 μM). B: *first lane*, untreated; *second lane*, tBid; *third lane*, tBid and Bci2 (20 μM); *fourth lane*, tBid and Bci2 (10 μM); *fifth lane*, tBid and Bci2 (5 μM). C, the intensity of the cytochrome *c* bands was determined by densitometry. The blank value corresponds to mitochondria without addition of tBid, and 100% release corresponds to mitochondria treated with 10 nM tBid in the absence of inhibitor (*n* = 4).

compound, as seen in the electrophysiological experiments. These results show that inhibition of Bax channel activity is accompanied by inhibition or reduction of cytochrome *c* release from isolated mitochondria.

Bax Channel Inhibitors Do Not Alter the Conformational Activation of Bax—Although the electrophysiological results show that the Bax channel inhibitors were able to inhibit Bax channel activity once the channels had been formed and were active in the lipid membranes, we wanted to exclude that the compounds interfered with and inhibited the conformational changes associated with Bax activation. First, purified monomeric Bax was incubated in the presence of the compounds at 10 μM. As shown in Fig. 4 (A–C), the compounds did not change the quaternary structure of Bax, reflected in the elution of Bax as a monomer. Oligomerization and activation of Bax can be triggered by incubation with octyl glycoside (35), causing a shift in the elution time from the gel filtration column (Fig. 4D). When monomeric Bax was preincubated with the compounds and subsequently treated with 1% octyl glycoside, no change in the oligomerization state of Bax was detected compared with activation by octyl glycoside alone (Fig. 4, E and F). Thus, the compounds do not prevent or interfere with the conformational changes required for activation of Bax channel activity.

Because this is an artificial activation system, we further tested whether Bax activation is affected in cells undergoing apoptosis (Fig. 4G). Exposure of HeLa cells to staurosporine induces mitochondrion-mediated apoptosis, which is accompanied by Bax oligomerization and activation in the cells (14). In untreated cells, only Bax monomers (fractions 19–21) were detected in the mitochondrial preparation (Fig. 4G, NT). After treatment with staurosporine, most of the mitochondrion-associated Bax was found as oligomers that eluted in fractions 8–14 (Fig. 4G, *Stauro*). Cells treated with staurosporine in the presence of the Bax channel inhibitors (Fig. 4G, *Stauro + Bci1* and *Stauro + Bci2*) showed an elution profile similar to that of cells treated with staurosporine alone. The majority of Bax in the mitochondria was detected as oligomers (fractions 8–14). The staurosporine-treated mitochondria were alkali-washed to ensure that the detected Bax oligomer had been inserted into the mitochondrial membrane. When the cells were

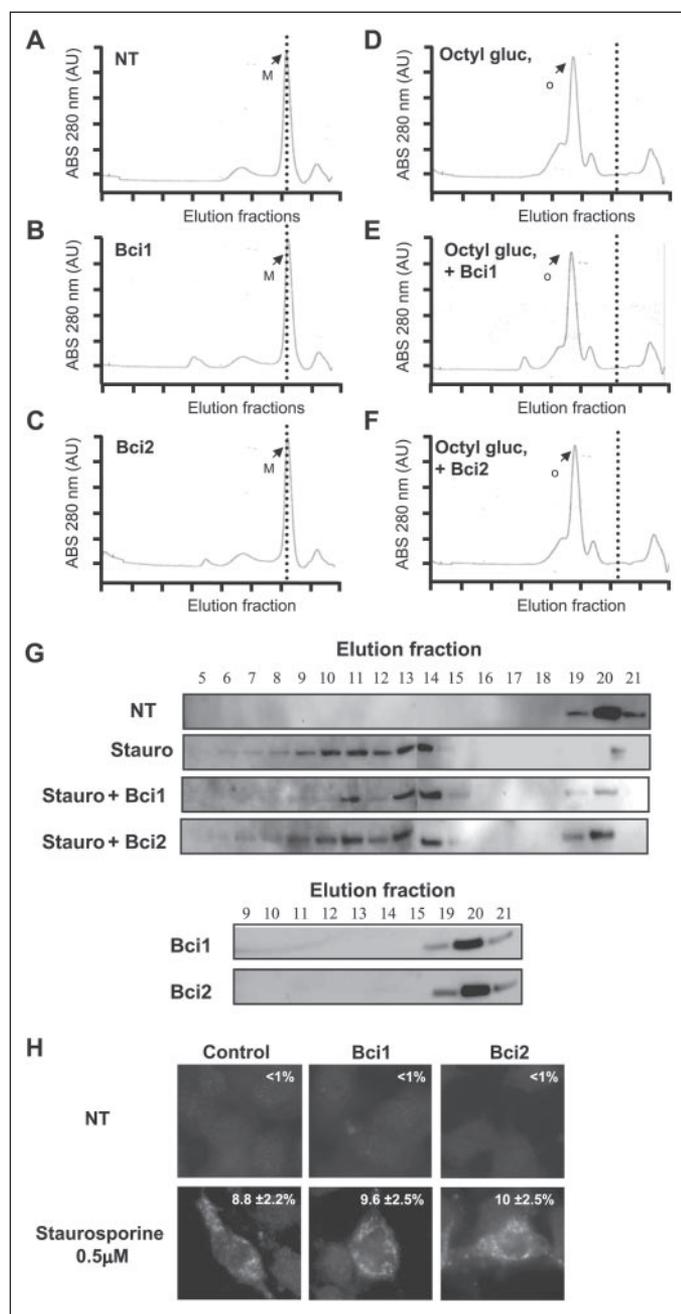


FIGURE 4. Bax channel inhibitors do not affect the conformational activation of Bax.

A–C, recombinant monomeric Bax was incubated in the absence and presence of 10 μM Bci1 or Bci2 for 1 h at 37 $^{\circ}\text{C}$. D–F, recombinant monomeric Bax was incubated with 1% octyl glycoside (*Octyl gluc*) in the absence and presence of 10 μM Bci1 or Bci2 for 1 h at 37 $^{\circ}\text{C}$. At the end of the incubations 50 μl of the samples was analyzed by gel filtration on a Superdex 200 column in the SMART system. The eluate was monitored at 280 nm, and 50- μl fractions were collected. M corresponds to monomeric Bax (22,000 Da) (A–C), and O corresponds to a Bax oligomer of 160,000 Da (D–F). The dotted lines correspond to the elution position of the monomer. ABS, absorbance; AU, arbitrary units. G, mitochondrial extracts from untreated cells (NT) or from cells treated with staurosporine (*Stauro*), staurosporine and Bci1 (*Stauro + Bci1*), staurosporine and Bci2 (*Stauro + Bci2*), Bci1, and Bci2 were analyzed by gel filtration as described above. The elution fractions were analyzed by Western blotting with anti-Bax antibody. Fractions 19–21 correspond to the Bax monomer, and fractions 13–14 correspond to a 160,000-Da Bax oligomer. The samples also contained higher order Bax oligomers (fractions 8–11). H, HeLa cells were treated with 0.5 μM staurosporine for 5 h in the presence or absence of 1 μM Bci1 or Bci2, and Bax activation was analyzed by immunofluorescence using an antibody against the N terminus of Bax. The percentages of active Bax-positive cells are indicated.

exposed to the compounds alone, only Bax monomers were detected in the mitochondria (Fig. 4G, *Bci1* and *Bci2*), demonstrating that the compound themselves did not induce Bax oligomerization. Finally, as an

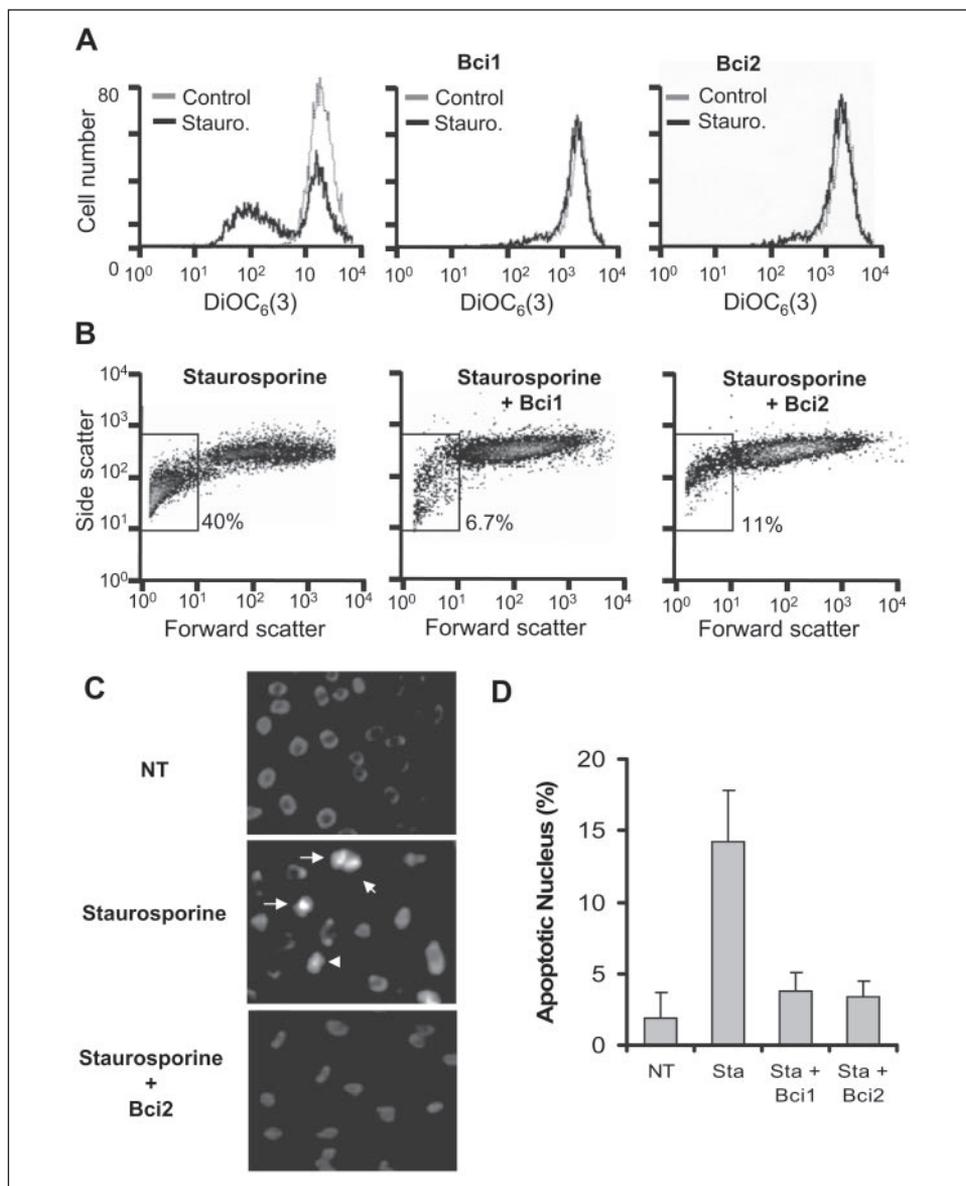
alternative strategy to analyze Bax conformational activation, we assessed the activation of Bax by immunofluorescence in cultured cells. Using an antibody against the N terminus of Bax, which is exposed only when the protein is activated by apoptotic stimuli, we observed no significant differences in the percentage of cells with activated Bax (\sim 10% of the cells) after treatment with staurosporine for 5 h in the presence or absence of Bci1 or Bci2 (Fig. 4H). Thus, our results demonstrate that the compounds do not modify the changes in the quaternary structure of Bax required for its activation or its translocation and insertion into the mitochondrial membrane.

Bax Channel Inhibitors Protect Cells in Vitro against Bax-mediated Apoptosis—To analyze the efficacy of the Bax inhibitors in living cells, we pretreated HeLa cells with 15 μM Bci1 or Bci2 for 30 min, and apoptosis was subsequently induced with 2 μM staurosporine. After a 3-h incubation, the mitochondrial membrane potential was analyzed by FACS. Cells treated with staurosporine alone showed a significant cell population with a decreased mitochondrial membrane potential. Both Bax inhibitors were highly efficient in blocking the decrease in the mitochondrial membrane potential, with no significant decrease observed after staurosporine treatment compared with untreated controls (Fig. 5A). A reduction in cell volume is generally an early sign of apoptosis. Thus, this parameter was employed to discriminate between apoptotic and viable cells. After staurosporine treatment, 40% of the cell population presented decreased forward side scatter by FACS analysis, indicating a decrease in cell volume (Fig. 5B). In contrast, after pretreatment of the cells with Bci1 or Bci2, only 6.7 and 11% of the cells showed reduced forward side scattering. Thus, the compounds efficiently blocked cellular shrinkage induced by staurosporine. Furthermore, analysis of the nuclear morphology by Hoechst 33342 staining revealed that inhibition of Bax channel activity resulted in a reduction in the appearance of apoptosis-related nuclear morphology, reflected in chromatin condensation and nuclear fragmentation (Fig. 5C, *arrows*). The results are summarized in Fig. 5D. In the untreated cultures, 1.8% showed condensed nuclei, whereas after staurosporine treatment, this increased to 14.2%. However, after cotreatment with Bci1 or Bci2, the percentages decreased to 3.7 and 3.4, respectively. Taken together, these results indicate that the compounds are cell-permeable and efficiently block proapoptotic activity.

As an alternative approach to specifically induce Bax-mediated apoptosis, HEK cells were transfected with a Bax expression vector under the control of the cytomegalovirus promoter. As shown in Fig. 6A, Bax overexpression promoted apoptotic cell death 16 h after transfection, reflected in an increase in the hypodiploid cell populations from 4% in mock-transfected cells to 36%. Treatment of the Bax-transfected cells with Bci1 or Bci2 reduced the hypodiploid cell fraction to 10 and 11%, respectively. As an additional control, the pro-apoptotic multidomain protein Bak was overexpressed in HEK cells. In contrast to Bax, Bak expression did not show a strong effect on apoptotic HEK cell death, with only 12% hypodiploid cells. Treatment with the Bax channel inhibitors showed no significant effect on Bak-mediated apoptosis (data not shown).

To exclude that the compounds work through a mechanism other than Bax channel inhibition, they were tested in MEFs from wild-type and Bax knockout animals. To evaluate the effect of the compounds, the MEF cells were treated with 0.2 and 3 μM staurosporine for 16 h in the presence or absence of Bci1 or Bci2, and cell viability was measured (Fig. 6B). After treatment of the wild-type cells with 0.2 or 3 μM staurosporine, 36 and 5% were still viable after 16 h, respectively. Cotreatment with 0.2 μM staurosporine and Bci1 or Bci2 increased cell viability to 62 and 65%, respectively. At 3 μM staurosporine, the compounds showed

FIGURE 5. Bax channel inhibitors block the decrease in the mitochondrial membrane potential and protect against apoptosis induced by staurosporine treatment of cells. *A*, HeLa cells were pretreated with the Bax inhibitors (10 μ M), and after 30 min, staurosporine (*Stauro.*; 2 μ M) was added to the culture medium. After 3 h, the mitochondrial membrane potential was measured by FACS analysis after 3,3'-dihexyloxacarbocyanine iodide (*DiOC₆(3)*) staining. *B*, cell volume was analyzed in HeLa cells treated as described for *A*, *C*, after a 6-h incubation in the absence (*NT*) or presence of 2 μ M staurosporine with and without pretreatment with the Bax inhibitors Bci1 (not shown) and Bci2, the effects of the Bax inhibitor on nuclear morphology were determined by nuclear staining with Hoechst 33342. Cells with condensed chromatin are indicated by arrows. *D*, the percentage of cells with condensed chromatin and fragmented nuclei was determined by cell counting in the different groups. *Sta.*, staurosporine.



no protective effect. At the low staurosporine concentration, the Bax^{-/-} cells were more resistant, and 70% of the cells were viable after 16 h; cotreatment with the Bax channel inhibitors did not significantly increase viability. At the high staurosporine concentration, the results were identical to those obtained for the wild-type cells; cell viability was <10%; and the inhibitors showed no protective effect. Similar results were obtained when the mitochondrial membrane potential was analyzed (data not shown). These results show that Bax-dependent cell death is efficiently prevented by the inhibitors, whereas the deleterious effects of staurosporine unrelated to Bax are not prevented.

Finally, to establish in more detail the specificity of the compounds, the protective effect was examined in wild-type, Bax^{-/-}, and Bak^{-/-} MEF cells overexpressing t^cBid. In MEF cells, both Bax and Bak have been shown to be absolutely required for t^cBid-induced apoptosis; only deletion of both protects the cells against t^cBid-mediated apoptosis (28). As shown in Fig. 6C, retrovirus-mediated expression of t^cBid induced cell death in all three cell lines, with between 44 and 25% cells dead, consistent with previously published data (28). When the cells were grown in the presence of 1 μ M Bci1, no protective effect was detected in the wild-type cells or in the Bax^{-/-} cells, in which cell death depends on

Bak activation. In the Bak^{-/-} cells, in which t^cBid-induced cell death relies on Bax activity, the compound showed a pronounced protective effect, decreasing the dead cell population from 44 to 6%. The compound itself was shown to have no deleterious effects on the cell lines (Fig. 6C, upper panels). In summary, the protective effect observed after treatment of cells with the Bax channel inhibitors correlated very well with the expression of Bax, strongly supporting that their mechanism of action in the cells is through inhibition of Bax channel activity.

Bax Channel Inhibitors Protect Neurons in an Animal Model of Global Brain Ischemia—We went on to test the compounds *in vivo* in an animal brain ischemia model. Brain ischemia has been shown to be associated with increased apoptotic cell death in and around infarcted brain areas (36). Transgenic experiments have suggested that mitochondrion-dependent apoptosis is involved in this type of brain injury (7). To test whether the Bax inhibitors have a protective effect, we used a model of global brain ischemia in the gerbil. Ischemia was induced by a 5-min bilateral occlusion of the common carotid arteries. Bci1 was administered intraperitoneally at 3 and 30 mg/kg and Bci2 at 30 mg/kg 15 min, 24 h, and 48 h after reperfusion. At day 7, the hippocampal damage was analyzed. Ischemic animals (Fig. 7, B and F) had a signifi-

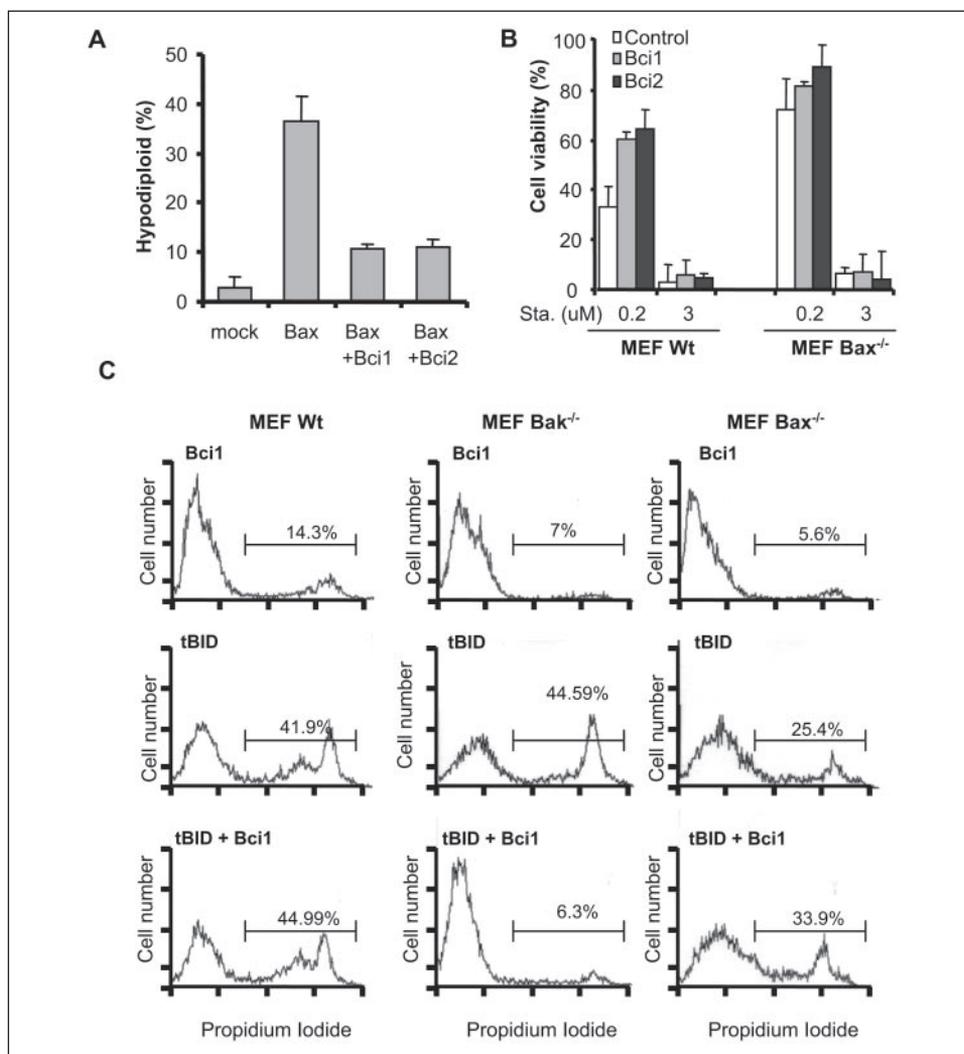


FIGURE 6. Protective effect of the Bax channel inhibitors correlates with Bax expression. *A*, HEK cells were transfected with an expression vector for Bax in the presence or absence of Bax channel inhibitors. 16 h after transfection, apoptosis was quantified by FACS analysis through determination of the hypodiploid cell population. *B*, after a 30-min pretreatment period with and without 2 μ M Bci1 or Bci2, staurosporine (Sta.) was added to final concentrations of 0.2 and 3 μ M, respectively. Cell viability was determined after 16 h by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. *C*, wild-type (Wt), Bax^{-/-}, and Bak^{-/-} MEF cells were retrovirally transfected with a tBid expression vector in the absence or presence of 1 μ M Bci1, and cell viability was determined after 24 h by propidium iodide staining and FACS analysis.

cant loss of neurons in the CA1 region (arrow) compared with sham-operated animals (Fig. 7, *A* and *E*). Animals treated with Bci1 (Fig. 7, *C* and *G*) and Bci2 (Fig. 7, *D* and *H*) showed significant protection. The hippocampal damage in the various animal groups was scored by histological analysis as described under "Materials and Methods." As shown in Fig. 7*I*, the hippocampal damage score was significantly decreased by 17% ($p < 0.05$) and 45% ($p < 0.001$) when the gerbils were treated with 3 and 30 mg/kg Bci1, respectively. Treatment with 30 mg/kg Bci2 decreased the damage by 55% ($p < 0.001$). The positive control (orotic acid) gave a reduction of 26% at 300 mg/kg. Thus, in the gerbil brain ischemia model, the Bax channel inhibitor showed a clear protective effect.

Bax Channel Inhibitors Prevent Cytochrome *c* Release from Neurons in the Animal Model—Redistribution of cytochrome *c* from mitochondria to the cytosol has been reported following ischemic injury in animal models (37, 38). To determine whether the protective effect of the Bax channel inhibitors was linked to reduced cytochrome *c* release, we measured the cytochrome *c* levels in the hippocampus, which contained the infarcted brain areas. Because the damaged tissue constituted only a minor part of the hippocampus, no decrease in mitochondrion-associated cytochrome *c* could be detected (data not shown); however, the cytosolic cytochrome *c* levels were significantly increased 4, 5, and 7 days after ischemia by 25, 77, and 32%, respectively ($p < 0.001$) (Fig. 8, *A* and *C*). After treatment with Bci1 administered intraperitoneally at 30

mg/kg, cytochrome *c* release was significantly reduced by 88% ($p < 0.001$) compared with untreated ischemic animals 5 days after reperfusion (Fig. 8, *B* and *D*). Thus, the protective effect of Bci1 in brain ischemia is associated with a clear decrease in cytosolic cytochrome *c* levels, indicating that the compounds block Bax activity at the mitochondria *in vivo*.

DISCUSSION

Several studies have indicated that apoptotic cell death contributes to tissue damage in pathologies associated with ischemia, including stroke and myocardial infarction (39, 40). The best evidence that the mitochondrial pathway is involved in ischemia-related apoptotic cell death comes from studies with transgenic animals. Mice overexpressing the anti-apoptotic protein Bcl-2 are less vulnerable to ischemic insults (7). Consistent with these findings, animals lacking the pro-apoptotic protein Bax are also more resistant (8). In addition, increased cytochrome *c* release has been detected in ischemic tissues in animal models (38). In a previous study, we showed that global transient ischemia in the gerbil model induces two waves of apoptosis; however, only the second one is associated with significant cytochrome *c* release (41). The first wave is associated with caspase-8 activation, whereas activation of caspase-3 is detected in both waves. In this study, we wanted to examine whether inhibition of Bax channel activity could prevent activation of mitochon-

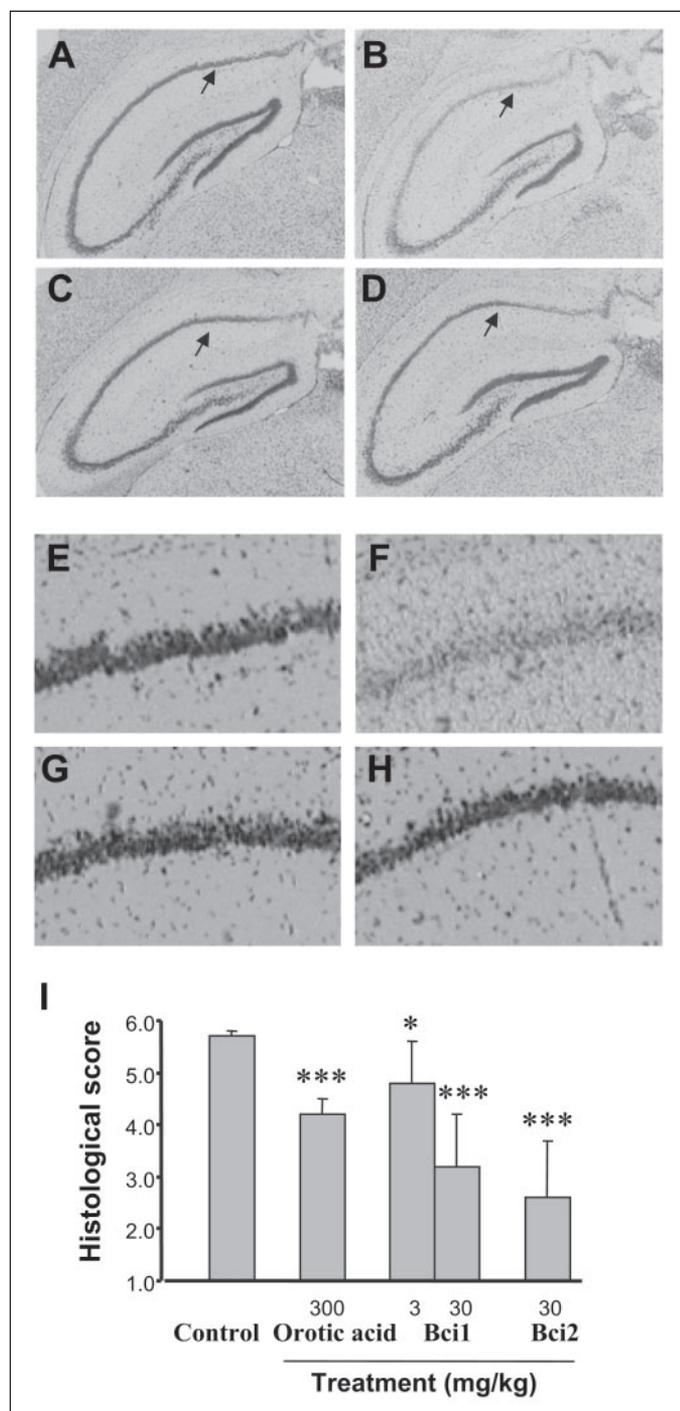


FIGURE 7. Effect of Bax channel inhibitors on hippocampal damage 7 days after global brain ischemia in gerbils. Gerbils were submitted to a 5-min bilateral occlusion of the common carotid arteries under isoflurane anesthesia to induce transient global ischemia. Bci1, Bci2, and the reference compound orotic acid were injected intraperitoneally at the doses indicated 15 min, 24 h, and 48 h after reperfusion. 7 days after the onset of occlusion, the gerbils were killed; brains were cut in 20- μ m-thick sections in a microtome; and the sections were stained with cresyl violet. *A*, sham-operated gerbil; *B*, ischemic gerbil treated with vehicle; *C*, ischemic gerbil treated with Bci1 (30 mg/kg); *D*, ischemic gerbil treated with Bci2 (30 mg/kg); *E–H*, magnifications of the areas indicated by arrows in *A–D*, respectively; *I*, hippocampal infarct damages scored according to the scale described under "Material and Methods." *, $p < 0.05$; ***, $p < 0.001$.

dron-induced apoptosis, reduce apoptotic cell death, and protect against ischemia-induced tissue damage.

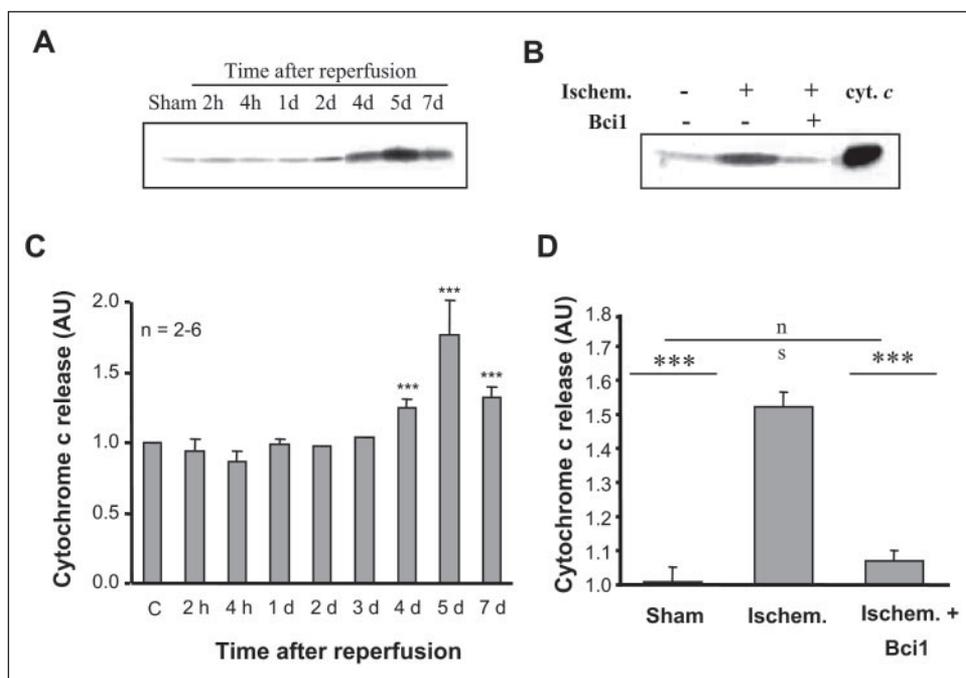
Although Bax possesses clear channel-forming activity *in vitro*, the central question is still whether the channel-forming activity is required

for its pro-apoptotic activity *in vivo*. Bax oligomers (corresponding to tetramers) have been shown to trigger cytochrome *c* release from liposomes, showing that Bax channels alone have the intrinsic capacity to form channels in lipid membranes large enough to release cytochrome *c* without the presence of any other proteins (16). To define the contribution of Bax channel activity in the apoptosis program, we decided to screen for molecules that block this activity, offering a pharmacological tool to study the relevance of Bax channel properties *in vitro* and *in vivo*. Here, we have shown that low molecular weight compounds that block Bax channel activity in liposomes and in planar lipid membranes also block cytochrome *c* release from isolated mitochondria. In addition, we have shown that the compounds do not affect Bax conformational changes required for Bax oligomerization and activation. These results strongly suggest that the mechanism by which Bax triggers cytochrome *c* release from mitochondria is through formation of channels in the outer mitochondrial membrane and suggest a two-step model for the activation of Bax-mediated apoptosis: first, the conformational activation and insertion into the mitochondrial membrane; and second, the formation of channels that trigger (directly or indirectly) the release of apoptogenic factors from the mitochondria. The electrophysiological results demonstrate that the compounds function as genuine channel blockers, although the molecular mechanism remains unknown. Bax channel activity up to ~ 20 nanosiemens was efficiently inhibited by the compounds, and once the low conductance channels were inhibited, no large channel activity was subsequently observed. On the contrary, once larger channel activity had formed in the lipid membrane, the compounds did not inhibit this activity. It is perceivable that low conductance Bax channels have a different mode of action compared with large Bax conductance channels, which appear to form only once low conductance Bax channels are present. Because inhibition of the low conductance Bax channels prevents cytochrome *c* release and protects cells from apoptosis, this might indicate that the physiologically relevant Bax channels involved in cytochrome *c* release are the low conductance Bax channels. This is supported by results showing that cytochrome *c* release occurs before a massive translocation of Bax to the mitochondria can be detected (42).

To characterize the Bax channel inhibitors further, we treated cells with staurosporine. Staurosporine is known to activate the mitochondrial apoptosis pathway, inducing cytochrome *c* release in the cells (43). The compounds prevented the characteristic morphological changes associated with apoptosis in HeLa cells, including the decrease in the mitochondrial membrane potential, the reduction in cell volume, and the appearance of chromatin condensation and nuclear fragmentation. Thus, the compounds are entering the cells and show a clear protective effect. The protective effect closely correlated with the presence of Bax in the cells. Although the compounds showed no protective effect in Bax^{-/-} MEFs, a strong protective effect was seen in Bak^{-/-} MEFs and in Bax-overexpressing HEK cells.

To evaluate the compounds *in vivo*, we used a transient global brain ischemia model in the gerbil. In this model, an extensive death of neurons is seen in the hippocampus after the ischemic insult (44). The compounds showed a pronounced protective effect, reducing the tissue damage by up to $>50\%$. It is worth pointing out that the animals were not pretreated with the compounds. We used a post-ischemia treatment protocol, administering the compound first 15 min after reperfusion. The results indicate that inhibition of Bax channel activity by low molecular weight compounds could be an efficient target for treatment of ischemia-induced tissue damage in various pathologies. To link the protective effect in the animals to mitochondrion-induced apoptosis, we determined cytosolic cytochrome *c* levels in the infarcted brain area.

FIGURE 8. Cytochrome c release during global brain ischemia in gerbils. A and C, gerbils were submitted to a 5-min bilateral occlusion of the common carotid arteries to induce transient global ischemia as described in the legend to Fig. 7. At various time periods after reperfusion as indicated, the animals were killed, and the hippocampi were excised and gently homogenized in buffer A. The homogenate was cleared from particulate cellular structures, including mitochondria, by centrifugation at $12,000 \times g$ for 10 min. The cytochrome c content in the supernatant (corresponding to the cytosolic fraction) was determined by Western blotting with anti-cytochrome c antibody (A). The intensity of the cytochrome c bands was determined by densitometry (B). The intensity of the cytochrome c band in non-ischemic animals was taken as 1 in each experiment, and the bands in the ischemic animals were normalized to the non-ischemic control (C). *d*, days; AU, arbitrary units. B and D, shown is the effect of Bcl1 on cytochrome c (cyt. c) release 5 days after global ischemia in gerbils. The three animal groups ($n = 4$) studied were as follows: non-ischemic animals (*Sham*), ischemic animals (*Ischem.*; receiving vehicle), ischemic animals intraperitoneally treated with 30 mg/kg Bcl1 at 15 min, 24 h, and 48 h after ischemia (*Ischem. + Bcl1*). The animals were killed 5 days post-ischemia, corresponding to the peak in cytochrome c release as shown in A. Cytosolic cytochrome c levels were determined as described above. $***, p < 0.001$; *ns*, not significant.



Compound treatment almost completely inhibited cytochrome c release. These results show that the mitochondrial apoptosis pathway, which is normally activated after the ischemic insult, is inhibited by the Bax channel inhibitors in the animals.

The results presented here show that the protective effect of the compounds in cells *in vitro* is dependent on Bax activity. In Bax-deficient cells, the compounds showed no protective effect, whereas in the presence of Bax, they were highly protective. The compounds prevented cytochrome c release both *in vitro* and *in vivo*, strongly indicating that Bax channel activity is required for cytochrome c release. Furthermore, the results demonstrate that inhibition of Bax channel activity protects neurons *in vivo* against apoptosis, showing that the mitochondrial apoptosis pathway is the major pathway in ischemia-related apoptosis, at least in the brain. Finally, the results indicate that inhibition of Bax channel activity by low molecular weight compounds could be an efficient target to prevent apoptotic cell death and to reduce tissue damage following ischemic injuries in the brain and presumably other tissues.

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