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MAC inhibitors antagonize the pro-apoptotic effects of tBid and disassemble Bax / Bak oligomers

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Abstract Mitochondrial Apoptotic Channel inhibitors or iMACs are di-bromocarbazole derivatives with anti-apoptotic function which have been tested and validated in several mouse models of brain injury and neurodegeneration. Owing to the increased therapeutic potential of these compounds, we sought to expand our knowledge of their mechanism of action. We investigated the kinetics of MAC inhibition in mitochondria from wild type, Bak, and Bax knockout cell lines using patch clamp electrophysiology, fluorescence microscopy, ELISA, and semiquantitative western blot analyses. Our results show that iMACs work through at least two mechanisms: 1) by blocking relocation of the cytoplasmic Bax protein to mitochondria and 2) by disassembling Bax and Bak oligomers in the mitochondrial outer membrane. iMACs exert comparable effects on channel conductance of Bax or Bak and similarly affect cytochrome c release from Bax or Bak-containing mitochondria. Interestingly, wild type mitochondria were more susceptible to inhibition than the Bak or Bax knockouts. Western blot analysis showed that wild type mitochondria had lower steady state levels of Bak in the absence of apoptotic stimulation.

Keywords MAC inhibitors · Bax · Bak · tBid · Apoptosis · MOMP · Patch clamp

Introduction

Release of death signaling molecules from mitochondria is an early event and the point of no return during intrinsic apoptosis. The mechanisms that underlie permeabilization of the mitochondrial membranes to such molecules are still being investigated, but involve formation of a protein pore in the outer membrane – MAC (Mitochondrial Apoptosis-induced Channel) (Dejean et al. 2005; Martinez-Caballero et al. 2009; Pavlov et al. 2001; Terrones et al. 2004). The process is orchestrated by the synergistic action of three different groups of Bcl-2 family proteins, which are categorized based on their activity (i.e. pro- or anti-apoptotic) and on how many Bcl-2 homology (BH) domains are present in the protein sequence. Pro-apoptotic Bax and Bak which are core components of MAC, contain 3 BH domains (BH1-BH3), whereas the anti-apoptotic (Bcl-2, Bcl-x_L, BCL-W, MCL1 and A1/BFL-1), display all four BH domains (BH1–BH4). A third group comprises smaller pro-apoptotic proteins that contain only the BH3 domain (Bid, Bim/BOD, BAD, BMF, BIK/NBK, BLK, PUMA/BBC3, NOXA and HRK/DP5). Depending upon whether they are able to activate Bax and Bak *directly* or *indirectly* by inhibiting the anti-apoptotic members, BH3-only proteins are further categorized as *direct activators* or *sensitizers*, respectively (Kim et al. 2006; Moldoveanu et al. 2014; Ding et al. 2014; Shamas-Din et al. 2011; Leber et al. 2010).

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The BH3-only protein Bid is a pro-apoptotic direct activator, which is cleaved in the cytosol in response to apoptotic stimuli (Terrones et al. 2004; Eskes et al. 2000; Kuwana et al. 2002; Letai et al. 2002; Li et al. 1998; Lovell et al. 2008; Shamas-Din et al. 2013; Tait and Green 2010). The larger of two cleaved pieces, tBid, inserts itself into the outer membrane, where it undergoes a major conformational change facilitated by the mitochondrial carrier homolog protein. This change converts tBid in a molecular switch that upon interaction with Bax's canonical surface groove induces the insertion and oligomerization of Bax homodimers (Lovell et al. 2008; Shamas-Din et al. 2013; Tait and Green 2010; Czabotar et al. 2013). In a similar fashion, tBid activates Bak, which in a healthy cell exists as a monomer in the mitochondrial outer membrane and is held inactive by interaction with voltage-dependent anion-selective channel (VDAC2) (Dai et al. 2011; Ma et al. 2013). The end result of tBid activation of Bax and/or Bak is the formation of homo- and hetero-oligomeric forms of MAC and the ensuing mitochondrial outer membrane permeabilization (MOMP) (Pavlov et al. 2001).

Formation of MAC, and therefore onset of MOMP, can be regulated by small molecule inhibitors like the iMACs, anti-apoptotic di-bromocarbazole derivatives shown to have therapeutic potential in neurodegenerative diseases, traumatic brain injury and impaired neurogenesis (De Jesus-Cortes et al. 2012; MacMillan et al. 2011; Peixoto et al. 2009; Pieper et al. 2014). A structural modification of the iMACs, by replacement of a fluoride moiety with a hydroxyl group in iMAC1, resulted in a new, more potent compound, iMAC2. This second inhibitor was characterized as an agent with lipophilicity considerably higher than that of iMAC1, and IC_{50} values in the nanomolar range. Additionally, iMAC2 had no effect on another mitochondrial outer membrane channel, TOM, which further confirmed its specificity (Peixoto et al. 2009). Nevertheless, the mode of action of iMACs remains unknown. It is unclear whether iMAC2 directly inhibits the MAC components Bax and Bak or if it rather interacts with tBid or other pro-apoptotic activators. It is also uncertain whether iMACs can prevent MAC formation and how these compounds inhibit pore conductance and cytochrome c release. The present study aims to expand our understanding of the mechanisms underlying MAC inhibition by these agents.

Methods

Cells, growth conditions, and transfections

FL5.12 cells were cultured as previously described (Gross et al. 1998) in Iscove's modified Eagle's media, 10 % fetal bovine serum, 2 mM L-glutamine, 25 μM beta-mercapto ethanol, 10 % WEHI-3B supplement (filtered supernatant of WEHI-3B cells secreting IL-3) at 37 °C and 5 % CO_2. The cell cultures were kept at a density below 2×10^6 cells / mL. Induction of apoptosis occurred by keeping the cells in WEHI-3B-free medium for 12 h prior to the extraction of mitochondria (Pavlov et al. 2001; Guo et al. 2004).

Mouse embryonic fibroblasts (MEF) of wild-type (parental), $Bak^{-/-}$, $Bax^{-/-}$, and $Bak^{-/-}Bax^{-/-}$ (from Stanley Korsmeyer, Harvard Medical School) backgrounds were cultured below 80 % confluence as previously described (Peixoto et al. 2011) in DMEM supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, and 1 X non-essential amino acids. The same growth conditions were employed for the GFP-Bax Hela cell line (from François Ichas, European Institute for Chemistry and Biology). Induction of apoptosis occurred by addition of 1 μM staurosporine to the growth medium of MEFs or injection of 5 nM tBid (in HBSS buffer) to GFP-Bax Hela cells (Cartron et al. 2003).

Ectopic expression of inner membrane-targeted GFP (mtGFP, Richard Youle, National Institutes of Health) and the intermembrane space protein Smac fused to mCherry (Smac-cherry, David Andrews, McMaster University) was accomplished by transient transfection using polyethyleneimine as described previously (Peixoto et al. 2011). The nuclei were stained using the Hoechst 33342 dye (Life Technologies).

MAC function and inhibition in living cells

Onset of MAC formation and function was determined by monitoring relocation of GFP-Bax to mitochondria (in GFP-Bax Hela cells) and diffusion of Smac-Cherry into the cytosol (in wild type MEF), respectively, after induction of apoptosis. Time-lapse fluorescence images of cultured cells were captured using a CoolSNAP HQ2 monochrome camera (Roper Scientific-Photometrics, Tucson, AZ) on a Nikon Eclipse TE2000-E microscope equipped with a 60 X Fluor objective (numeric aperture 0.85), automated shutters, a motorized stage for autofocusing, and a heating chamber.

Direct monitoring of MAC activity using patch clamping

Mitochondria were extracted from 10 to 15 g of apoptotic FL5.12 cells (Pavlov et al. 2001; Guo et al. 2004) and treated in a French press to separate the outer membrane (Decker and Greenawalt 1977). After purification (Pavlov et al. 2001; Guo et al. 2004; Mannella 1982) the outer membranes were reconstituted in proteoliposomes by a modification of the method of Criado and Keller (Pavlov et al. 2001). Briefly, 5 μg membranes and 1 mg small phosphatidylcholine (Sigma Aldrich) liposomes were mixed in 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4) and dotted on a glass slide. The dots were dehydrated ~3 h and then rehydrated overnight with 150 mM KCl and 5 mM HEPES, pH 7.4, at 4 °C. Once reconstituted, the

membranes were harvested with 0.5 mL of the same media and stored in aliquots at −80 °C. Alternatively, 5 nM tBid and 20–50 nM recombinant full length Bax or BakΔC where included in the patch pipette to induce MAC activity directly in the liposomes.

Patch-clamp procedures and analysis used were described elsewhere (Pavlov et al. 2001; Guo et al. 2004). Unless stated otherwise, the patch solution was 150 mM KCl and 5 mM HEPES, pH 7.4 both in the pipette and in the bath (symmetrical conditions). The iMAC compounds were diluted in patch solution and introduced by perfusion of the bath. Voltage clamp was performed with the excised configuration of the patch-clamp technique using Axopatch 200 or Dagan 3900 amplifier. Currents were low-pass filtered at 2 kHz and digitized with a sampling rate of 5 kHz using a Digidata 1322A digitizer and Clampex 8.2 software (Axon Instruments). Voltage-independent currents (±50 mV) with a conductance >1.5 nS were assigned to MAC. Clampfit 8.2 (Axon Instruments) and WinEDR 2.3.3 (Strathclyde Electrophysiological Software; courtesy of J. Dempster, University of Strathclyde, UK) were used for analysis of channel activity.

Cytochrome c release

For determination of cytochrome c release, mitochondria of wild type MEF were extracted from 3 to 5×10^6 cells (Martinez-Caballero et al. 2009). The purified mitochondria (0.2 mg / mL) were then incubated with t-Bid (0–100 nM) or an equivalent amount of vehicle (150 mMKCl, 20 mM HEPES, pH 7, 30 % glycerol, 2 % octyl glucoside) for 30 min at room temperature in 70 mM sucrose, 230 mM mannitol, 1 mM EDTA, pH 7.4. Released cytochrome c was separated by centrifugation at 15,800 × *g* for 5 min and quantified by ELISA following the manufacturer's instructions (R&D Systems). Where stated, 0–5 μM iMAC1 was added to mitochondria 5 min prior to addition of tBid. Dose dependence curves were obtained to calculate the EC_{50} values for tBid induction and IC_{50} for iMAC1 inhibition of cytochrome c release. The curves fit the Hill equation $y = x^n / (k^n + x^n)$, where x is the tBid / iMAC concentration, n is the Hill coefficient, and k is the EC_{50} / IC_{50} value.

Gel filtration and western blot

Purified outer membranes from apoptotic FL5.12 cells were treated with vehicle (Control) or 5 μM iMACs on ice. Treated membranes were then solubilized in 2 % CHAPS and loaded onto a Superdex 200 column (16/60, GE-Amersham) pre-equilibrated with 25 mM Hepes-NaOH, 300 mM NaCl, 0.2 mM DTT, 1 % (*w*/*v*) CHAPS, pH 7.5, and eluted at a flow rate of 1 ml/min. The column was pre-calibrated with both monomeric and oligomeric recombinant Bax to determine which elution fractions contained either quaternary structure of Bax. Eluted proteins where collected in 0.5 mL fractions and examined for the presence of Bax by western blotting (N-20 antibody, Santa Cruz Biotechnology). Non-saturated western-blots bands were scanned and quantified by densitometry with the Image J 1.37v software.

Determination of MAC components in MEF lines was carried out in western blots using anti Bax (N-20, Santa Cruz Biotechnology) and anti Bak antibodies (anti-NT, Upstate Biotechnology) (Dejean et al. 2005). A standard curve of western blot band densitometries versus amount in ρg of recombinant Bax or Bak was used to interpolate the steady state levels of each MAC component in mitochondria of wild type, $Bak^{-/-}$, $Bax^{-/-}$, and $Bak^{-/-}Bax^{-/-}$ MEF. *p* values for all statistical analyses were obtained using Student's t test.

Results

In a previous study we identified inhibitors of the Mitochondrial Apoptosis-induced Channel, MAC. One such inhibitor, iMAC2 had promising therapeutic features that included a low-nanomolar IC_{50}, high LD_{50}, and moderate lipophilicity. Furthermore, treatment with iMAC2 inhibited release of mitochondrial cytochrome c in IL3-starved or staurosporine-poisoned pro-B lymphoid cell lines (Peixoto et al. 2009). In the present work we aimed to determine the mechanism of action of iMACs.

As mentioned above, formation and opening of MAC renders the mitochondrial outer membrane permeable to death factors located in the intermembrane space such as cytochrome c, apoptosis inducing factor (AIF), and second mitochondria-derived activator of caspases (Smac). We evaluated the effects of iMAC2 on staurosporine-induced permeabilization of the mitochondrial outer membranes in mouse embryonic fibroblasts (Fig. 1). Onset of permeabilization was monitored by time-lapse fluorescence microscopy in cells ectopically expressing red (Cherry) fluorescent protein with Smac presenquence. Mitochondrial localization was confirmed in cells that simultaneously expressed an outer membrane-targeted green fluorescent protein (mtGFP). For reference, the nuclei were stained blue with Hoechst 33342 dye. In the first 2 h of staurosporine treatment, the mitochondrial network appeared in yellow due to the overlapping fluorescence of Smac-Cherry and mtGFP. Subsequently, Smac-Cherry fluorescence diffused into the cytosol and mitochondria progressively became green. The cell in the image eventually detached from the culture cover glass and floated away. Note that in the top panels onset of Smac-Cherry release preceded cell collapse as is typical of intrinsic apoptosis. However, during co-treatment with 0.5 μM iMAC2 (bottom panels) Smac-Cherry release trailed the cell collapse, which is not typical of mitochondrial apoptosis. Onset of permeabilization (MOMP in

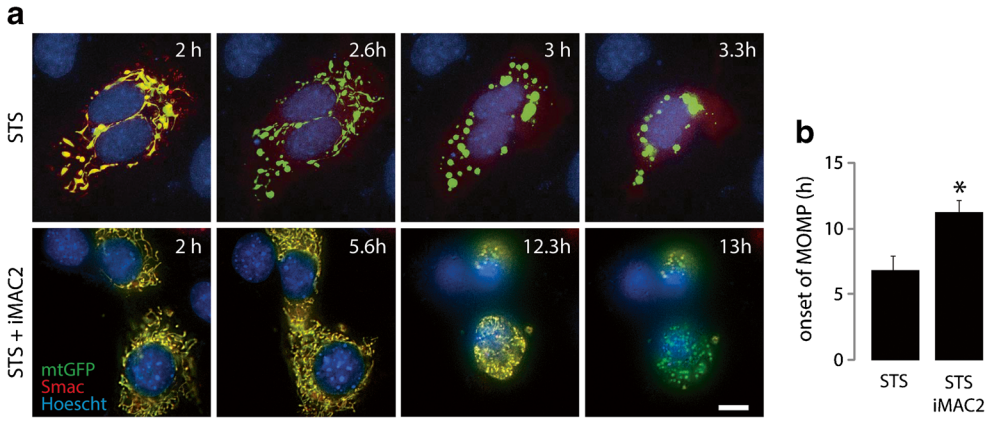


Fig. 1 Temporal dissection of outer membrane permeabilization and cell death. The effects of iMAC2 treatment on mitochondrial outer membrane permeabilization (MOMP) in living cells are shown. **a** Overlaid time-lapse fluorescence micrographs of MEF ectopically expressing inner membrane-targeted GFP (*green, mtGFP*) and intermembrane space-targeted Smac-cherry (*red, Smac*). Diffusion of red fluorescent Smac into the cytoplasm rendered the mitochondrial network green in the overlaid images, indicating outer membrane permeabilization after treatment with 1 μM staurosporine (*top*) or co-treatment with 0.5 μM iMAC2 (*bottom*). Scale bar is 5 μm. **b** Bar histograms of onset in hours ± SEM of MOMP as in (a). Staurosporine treatment led to MOMP in 6.83 ± 1.1 h ($n = 12$), while co-treatment with iMAC2 delayed onset to 12.52 ± 0.9 h ($n = 9$, $p < 0.001$)

the bar histograms) occurred at 6.83 ± 1.1 h ($n = 12$) after addition of staurosporine and was delayed to 12.52 ± 0.9 h ($n = 9$, $p < 0.001$) when cells were co-treated with iMAC2. Hence, iMAC2 significantly delayed onset of MOMP, the commitment step of apoptosis.

It is now established that the cascades of biochemical events that lead to MAC formation include a sudden and acute accumulation of the Bax protein in the outer membrane of mitochondria (Dejean et al. 2005; Gross et al. 1998; Wolter et al. 1997). We evaluated the effect of iMAC2 in the mitochondrial accumulation of Bax in time-lapse fluorescence microscopy videos of Hela cells stably expressing GFP-Bax (Fig. 2). Cell injection of the Bax activator tBid (white arrows) specifically changed the pattern of green fluorescence from diffuse to punctate. Co-staining with mitotracker red confirmed that punctate green fluorescence was restricted to mitochondria (not shown). In contrast, the fluorescence pattern remained diffuse when 0.5 μM iMAC2 was added to the growth medium prior to injection of tBid (Fig. 2a bottom panels). We monitored cell fate with time lapse videomicroscopy for up to 24 h post tBid injections and observed that treatment with iMAC2 significantly dropped death rates from 81 to 22 % (Fig. 2b; $n = 22$ and 24, respectively $p < 0.001$).

Our previous work showed that iMAC2 inhibited preformed MAC conductance in a stepwise manner, which was suggestive of a disassembly mechanism. We tested this hypothesis using gel-filtration chromatography analysis of MAC-enriched mitochondrial outer membranes. Apoptosis was induced in FL5.12 cells by IL-3 withdrawal as previously described and the presence of MAC in the outer membrane, as well as the mentioned stepwise inhibition by iMAC2, were confirmed by patch clamping (Fig. 3a). Western blot analysis showed that the Bax protein was present in both low (samples 21 and 22) and high (samples 15 and 16) molecular weight fractions. Calibration of the gel-filtration columns indicated that those fractions corresponded to the monomeric and oligomeric forms of Bax, respectively (Hetz et al. 2005). We compared the density of the western blot bands corresponding to monomeric and oligomeric Bax (Fig. 3b bar histograms). Relative to vehicle-treated control membranes, the ratio of oligomeric/monomeric Bax decreased significantly when the membranes where treated with 0.5 μM iMAC1 or iMAC2 (from 1 to 0.44 ± 0.05 or 0.23 ± 0.01, $p < 0.01$, respectively). An explanation to this apparent shift could be that treatment with inhibitors caused disassembly of pre-formed MACs, as supported by the previous patch clamp data.

We next investigated the possible MAC disassembly effect of iMACs in the context of Bax versus Bak oligomers in patch clamp experiments. We reconstituted MAC activity in giant liposomes by adding 5 nM tBid and 20–50 nM of either recombinant Bax or Bak protein in the patch pipette. As noted in the voltage vs. current plot of Fig. 4a, recombinant Bax formed a MAC channel that, like in mitochondrial preparations, was completely inhibited by addition of iMAC1 into the patch bath (gray current traces). Similar results were observed with recombinant Bak in accordance with the previously described structural and functional redundancy in MAC formation (Martinez-Caballero et al. 2009). Importantly, the current traces elicited by tBid and Bak were inhibited in the same stepwise manner that was previously observed for MAC, suggestive of a similar disassembly mechanism (Fig. 4b). Note that after the peak overall amplitude is achieved, the conductance is slightly reduced. However, after addition of iMAC1,

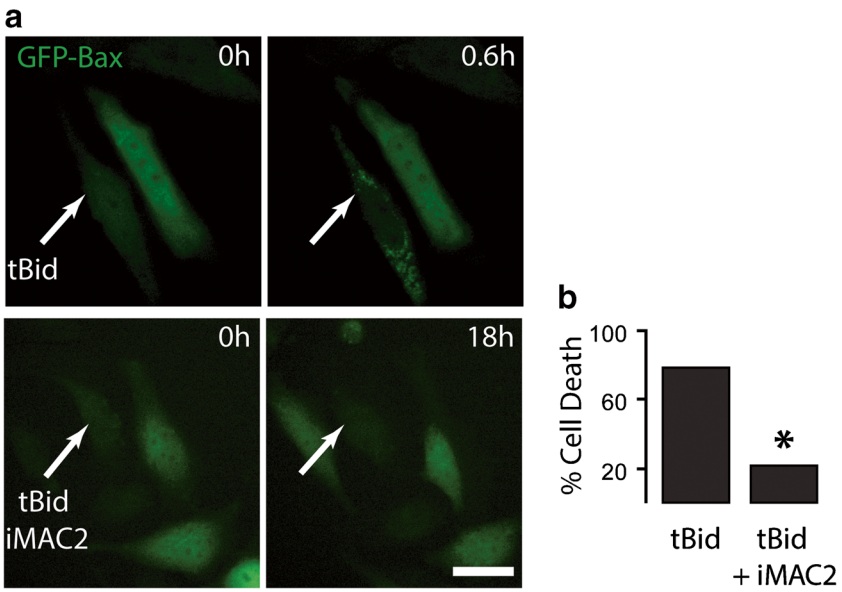


Fig. 2 Treatment with iMAC2 inhibits relocation of Bax to mitochondria. Celular distribution of Bax was monitored after injection of 2–5 nM tBid into Hela cell lines stably expressing GFP-Bax. **a** Time-lapse fluorescence micrographs of tBid injected cells (*arrows*) in the absence (*top*) or presence (*bottom*) of 0.5 mM iMAC2. Single cell injection of tBid lead to a change in Bax fluorescence pattern from diffuse (cytoplasmic) to punctate (mitochondrial) typically within 1 h. The punctation pattern was rarely observed when injected cells were pre-treated with iMAC2. Scale bar is 5 μm. **b** Bar histograms show % cell death over a period of 12 h post tBid injection with or without iMAC2 pre-treament ($n = 22$ and 24, respectively, $p < 0.001$)

conductance is lost almost completely. Inclusion of inhibitors in the pipette prevented observation of MAC activities in accordance with the results in Fig. 2a (not shown).

One limitation of the above experiments was that they did not allow for a comparison of the kinetics of Bax and Bak disassembly. We circumvented this issue by comparing tBid-induced release of cytochrome c from mitochondria of wild type, Bak, and Bax knockout cells (mouse embryonic fibroblasts). We measured release of cytochrome c by ELISA after incubation of wild type, $Bak^{-/-}$, $Bax^{-/-}$, and $Bak^{-/-}Bax^{-/-}$ mitochondria with increasing concentrations of tBid (Fig. 5) (Peixoto et al. 2009). The dose-dependent release of cytochrome c fit the Hill equation $y = x^n/(k^n + x^n)$, where x is the tBid concentration, n is the Hill coefficient, and k is the EC_{50} value. Double knockout mitochondria ($Bak^{-/-}Bax^{-/-}$) were virtually insensitive to tBid in accordance with their structural requirement for MAC function. However, no statistically sound differences were observed for wild type, $Bak^{-/-}$, and $Bax^{-/-}$ mitochondria ($EC_{50} = 8.4$, 13.6, and 9.74, respectively; $n = 5$ experiments, $p > 0.05$). Maximal release was reached at >30 nM tBid. A dose dependent decline in cytochrome c release (with 25 nM tBid) was observed when the mitochondria where pre-incubated with iMAC2 (Fig. 5b) in accordance with our previous results. Interestingly, this time there was a separation between the IC_{50} values (also determined from the Hill equation) for wild type (27.2 nM) and the single knockouts (295 nM and 207 nM for $Bak^{-/-}$ and $Bax^{-/-}$, respectively). The IC_{50} values for double knockouts were not determined due to their insensitivity to tBid (Fig. 5a).

It is possible that the inhibitory effects were linked to the steady-state levels of Bak and Bax in the mitochondrial preparations. We evaluated such possibility in western blot experiments (Fig. 6) in which we estimated the amounts of Bax or Bak content in mitochondria, interpolating the band intensities of Bak and Bax against a standard curve for both proteins. For example, the panels in Fig. 6a represent the western blot bands used to quantify Bax (top) and Bak (bottom) levels in wild type mitochondria. The average band intensities were converted into picograms (pg) of Bax (or Bak) per microgram (μg) of mitochondrial protein initially loaded in the SDS-PAGE lanes. The bar histograms in Fig. 6b show that 1 μg wild type mitochondria contained 0.04 ± 0.001 pg Bax and 0.01 ± 0.001 pg Bak (a four-fold difference, $p < 0.001$, $n = 4$). On the other hand, 1 μg of $Bax^{-/-}$ contained 0.22 ± 0.06 pg Bak while 1 μg of $Bak^{-/-}$ mitochondria contained 0.06 ± 0.01 pg of Bax. By comparison, there was a twenty-fold increase in Bak levels in $Bax^{-/-}$ relative to wild type ($p < 0.01$, $n = 4$), while the apparent increase in Bax levels of $Bak^{-/-}$ mitochondria did not reach statistical significance ($p = 0.09$).

Discussion

Since our initial characterization of the iMACs, different dibromocarbazole derivatives with anti-apoptotic function have been developed and tested in several mouse models of brain injury and neurodegeneration (De Jesus-Cortes et al. 2012; MacMillan et al. 2011; Pieper et al. 2010, 2014; Walker

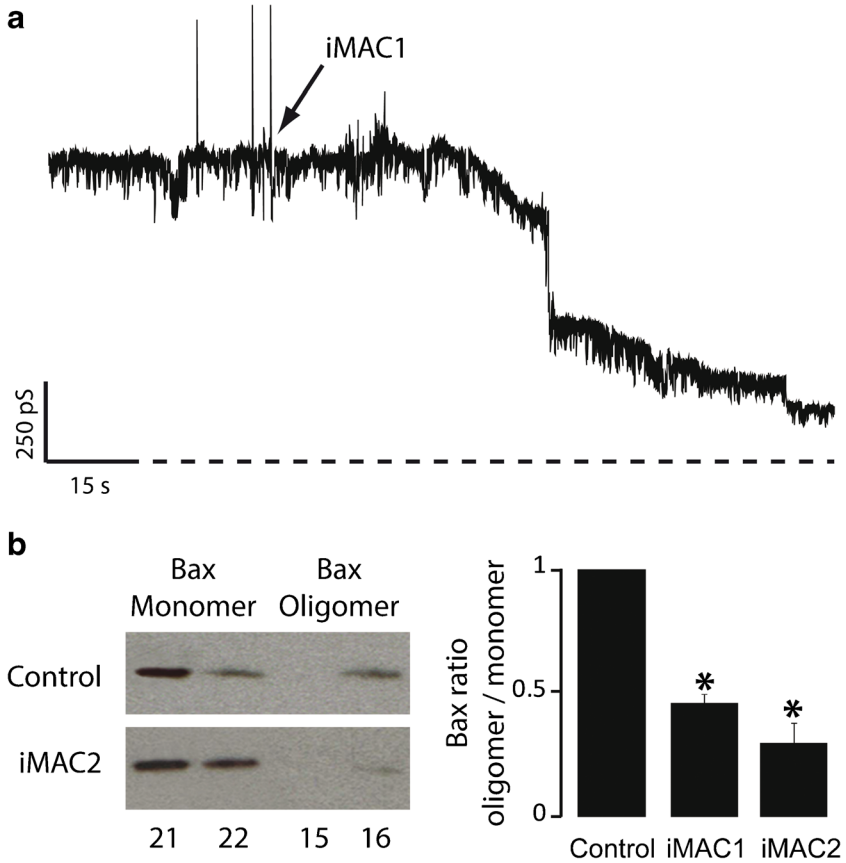


Fig. 3 Mature MAC can be disassembled after treatment with the iMACs. The effects of iMACs on channel activity and structure were determined in purified mitochondrial outer membrane preparations of apoptotic FL5.12 cells (grown in absence of IL-3). **a** Representative current traces of MAC activity initially in the fully open state and after progressive (step-wise) inhibition in the presence of 300 nM iMAC1 (*arrow*). The dotted lines indicate 0 pS conductance. **b** Representative western blots of apoptotic outer membrane fractions that were solubilized in 2 % CHAPS and separated by gel-fitration to distinguish monomeric and oligomeric Bax. Pre-treatment of membranes with 5 μM iMACs (*bottom*) decreased the amounts of oligomeric Bax in comparison to vehicle-treated controls (*top*). The bar histograms show the average (± SEM) band densitometry ratios of oligomeric and monomeric Bax in membrane fractions treated with iMAC1 (blot not shown) and iMAC2 relative to membrane fractions treated with DMSO ($n = 4$, $p < 0.001$)

et al. 2014; Naidoo et al. 2014; Blaya et al. 2014; Asai-Coakwell et al. 2013; Tesla et al. 2012). One recent advance in the modification of such derivatives included replacement of a hydroxyl for a fluoride moiety in the propyl linker, similar to the modification that produced iMAC2 from iMAC1 (Peixoto et al. 2009). Owing to the increased therapeutic potential of anti-apoptotic di-bromocarbazole derivatives, we sought to expand our knowledge of the mechanism of action of these small molecule inhibitors.

The high specificity of iMACs had been inferred from the low nanomolar concentration needed to inhibit MAC, the lack of effect on another mitochondrial channel, TOM, and the inhibition of cytochrome c release in presence of staurosporine (Peixoto et al. 2009). The present observation that iMAC2 halted onset of mitochondrial permeabilization even after cell shrinkage is another indicator of MAC-specificity (Fig. 1). Further evidence of iMACs binding to MAC components was obtained by patch clamping of recombinant Bax and Bak proteins after reconstitution in liposomes (Fig. 4) and by measurement of cytochrome c release inhibition from Bax and Bak knockout mitochondria (Fig. 5). It is interesting to note that delaying mitochondrial apoptosis was not sufficient to overcome the toxic effects of kinase inhibitor staurosporine, a result that should be considered especially in models where the nature of the insult is unknown, for example, during neurodegeneration.

A more robust inhibition of death was observed when the cells were challenged with tBid, a specific activator of MAC (Martinez-Caballero et al. 2009) (Fig. 2). Occasional relocation of Bax to mitochondria and onset of cell death was observed in negative controls (not shown) and could at least partially account for the background death in presence of iMAC2. Another possibility was that tBid had secondary effects (Liu et al. 2004), but in the vast majority of the experiments, prevention of Bax accumulation in the outer membrane (and formation of MAC) abolished the apoptotic effect of tBid. In our conditions, iMAC2 was introduced in the growth

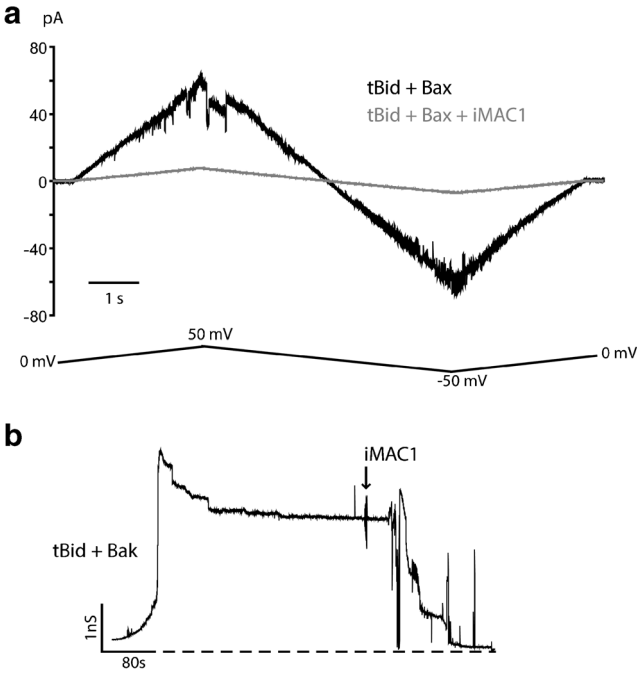
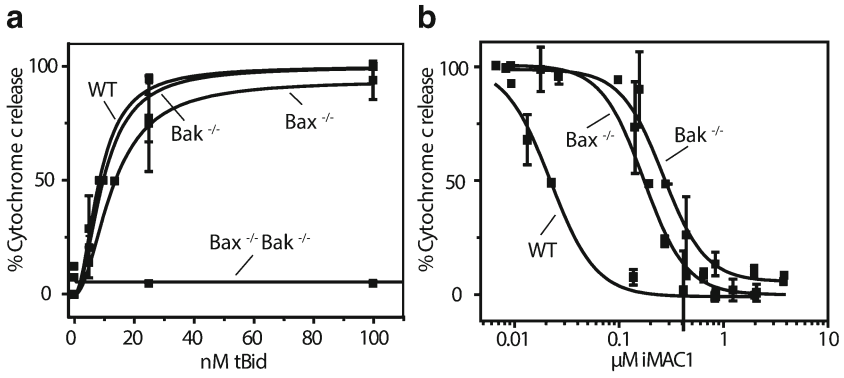


Fig. 4 MAC inhibition does not require other mitochondrial components. Recombinant Bax and Bak channel activities were reconstituted in artificial phosphatidylcholine liposomes by adding 20–50 nM of either MAC component and 5 nM tBid in the patch pipette. **a** Graph shows Bax currents (ρA) elicited by ramp voltages between −50 and +50 mV before (*black*) and after adition of 300 nM iMAC1 (*gray*). **b** Representative current traces elicited by a holding potential of +20 mV show the progressive inhibition of Bak conductance after addition of 300 nM iMAC1 (*arrow*) as in Fig. 3. The dotted lines indicate 0 pS conductance

medium while tBid was injected into single cells. Co-injection of tBid and iMAC2 may help determine whether the inhibitors bound to tBid or MAC components directly.

Previous patch clamp data on mitochondria extracted in the absence of apoptotic inducers showed that introduction of tBid in the pipette summoned MAC activity in a stepwise manner (MAC identity was confirmed with Bax and Bak knockouts) (Martinez-Caballero et al. 2009). A model was proposed of a MAC channel growing through the progressive *addition* of Bax and/or Bak trans-membrane helices. Our patch clamp recordings of MAC inhibition with iMAC2 (Peixoto et al. 2009) and iMAC1 (Fig. 3) displayed stepwise decreases in conductance compared to those increases recorded in the presence of tBid. These decreases may reflect a progressive *subtraction* of Bax and/or Bak trans-membrane helices in the shrinking channel. The western blot data showing the oligomeric-to-monomeric shift of Bax induced by iMACS supports a disassembly mechanism underlying MAC inhibition. This mechanism may not be exclusive to Bax, as indicated by the similar iMAC-induced stepwise inhibition of Bak conductance (Fig. 4b).

As mentioned above, the inhibition of recombinant Bax and Bak activities indicates that these MAC components are the most likely targets of the iMACs. Moreover, the experimental setup in Fig. 4 was such that tBid and the iMACs where physically separated by the lipid bilayer prior to MAC formation (tBid was in the patch pipette and the iMACs where introduced into the patch bath). Therefore, disassembly of MAC was likely independent of tBid interactions. Inclusion of the iMACs in



c

	WT	Bax$^{-/-}$	Bak$^{-/-}$	Bax$^{-/-}$Bak$^{-/-}$
tBid EC_{50} (nM)	8.4 ± 0.02	13.6 ± 0.72	9.74 ± 0.75	>500
iMAC1 IC_{50} (nM)	27.2±0.0012	207 ± 0.01	295 ± 0.02	n.d.

Fig. 5 Comparison of tBid-sensitivity of Bak and/or Bax channels in presence of iMAC1. Sensitivity of Bax and/or Bak channels to tBid was estimated in mitochondria of wild type, Bak$^{-/-}$, Bax$^{-/-}$, and Bax$^{-/-}$Bak$^{-/-}$ MEFs by measuring release of cytochrome c. **a** Graph shows the dose-dependent effect of 0–100 nM tBid on release of cytochrome c. **b** Graph shows the dose-dependent inhibition of cytochrome c release by treatment with 0–4 μM iMAC1 prior to incubation with 25 nM tBid. Plotted data were average ± SEM ($n = 5$). **c** The table summarizes the EC_{50} and IC_{50} values for tBid induction and iMAC1 inhibition of cytochrome c release, respective. The values were calculated from a Hill equation as described in the methods section

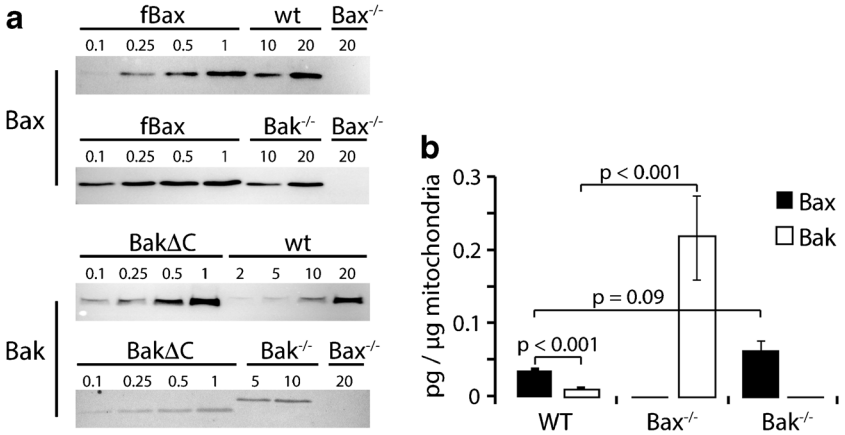


Fig. 6 Semi quantification of Bax and Bak in mitochondria of wild type, $Bak^{-/-}$, and $Bax^{-/-}$ MEFs. Mitochondria were isolated from the indicated MEF lines and evaluated for the relative amounts of Bax and Bak by western blot. **a** Representative western blot bands reactive to Bax (N20, top) and Bak (NT, bottom) antibodies. The indicated amounts in ρg of full length Bax (fBax) and truncated Bak (BakΔC) were loaded in the SDS-PAGE as a reference for estimation of the Bax and Bak contents in wild type, $Bak^{-/-}$, and $Bax^{-/-}$ mitochondria (loaded amounts indicated in µg). **b** Histogram bars show the average (± SEM, $n = 4$) amounts of Bax and Bak in ρg / µg mitochondrial proteins interpolated from the standard curves (not shown) of band densitometry vs ρg fBax or BakΔC

the patch pipette prevented MAC formation, as discussed above. Future studies shall determine if the iMACs interfere with BH3:groove and/or α6-α6 interactions shown to promote Bax and Bak oligomerization (Czabotar et al. 2013; Ma et al. 2013; Dewson et al. 2009, 2012).

Bax and Bak have been found to undergo similar conformational changes after interaction with tBid (Dewson et al. 2012). We evaluated the dose dependence of tBid-induced cytochrome c release from mitochondria isolated in the absence of apoptotic treatment (Fig. 5a). The finding that Bak and Bax single knockouts exhibited similar sensitivities to tBid may seem unsurprising given their structural and functional redundancy towards MAC function (Martinez-Caballero et al. 2009). However, analysis of MAC composition in wild type mitochondria determined that the steady-state levels of Bax were four-fold higher than those of Bak, a bona-fide mitochondrial outer membrane protein (Fig. 6). Mitochondria from $Bax^{-/-}$ cells had 20-fold increased levels of the Bak protein, seemingly as a compensatory mechanism. In contrast, no reciprocal Bax increase was observed in $Bak^{-/-}$ mitochondria.

A few interesting observations arose from the inhibition of cytochrome c release in these mitochondria. The iMAC1 IC_{50} values for single knockouts were ten-fold higher than those for wild type mitochondria (Fig. 5b and c). This could indicate that MAC in wild type mitochondria is structurally different from that in the single knockouts, i.e., it might be formed by heteroligomers of Bax and Bak. The different levels of Bax and Bak may reflect an asymmetric stoichiometry. A hetero-oligomeric composition for MAC has been suggested by studies of co-immunoprecipitation, and more recently in cysteine linkage studies (Dewson et al. 2012; Mikhailov et al. 2003; Sundararajan et al. 2001). The latter study suggested that etoposide treatment of wild type mouse embryonic fibroblasts induced formation of Bax/Bak heterodimers, but that they were less abundant than homodimers. In our conditions in which we directly treated isolated mitochondria with tBid, the clear separation in the iMAC IC_{50} values indicated that, in steady-state levels, MAC might be mainly formed by heteroligomers. It was unclear whether or not the heteroligomers were formed by homodimers. Finally, the similar IC_{50} values for homo-oligomeric MAC (in single knockouts) despite the significantly increased levels of Bak suggest that the iMACs may have a higher affinity for Bax.

In summary, our results indicate that the iMACs work through at least two mechanisms: 1) by blocking relocation of the cytoplasmic Bax protein to mitochondria and 2) by disassembling Bax oligomers in the outer membrane. A comparison of the inhibitory effects over channel conductance and cytochrome c release suggests that the iMACs interacted with both Bax and Bak with similar kinetics. Future studies will examine structural interactions between iMACs and Bax/Bak proteins, the composition of Bax and Bak hetero-oligomers in MAC, and the stepwise mechanism of MAC disassembly in greater detail. Aside from the potential protective effects on impaired neurogenesis and ischemia reperfusion injury the iMACs may become an invaluable tool to dissect the mechanisms of permeabilization of the mitochondrial outer membrane during apoptosis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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