MAC inhibitors suppress mitochondrial apoptosis

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MAC (mitochondrial apoptosis-induced channel) forms in the mitochondrial outer membrane and unleashes cytochrome *c* to orchestrate the execution of the cell. MAC opening is the commitment step of intrinsic apoptosis. Hence closure of MAC may prevent apoptosis. Compounds that blocked the release of fluorescein from liposomes by recombinant Bax were tested for their ability to directly close MAC and suppress apoptosis in FL5.12 cells. Low doses of these compounds (IC_{50} values ranged from 19 to 966 nM) irreversibly closed MAC. These compounds also blocked cytochrome *c* release and halted the onset of apoptotic markers normally induced by IL-3 (interleukin-3) deprivation or staurosporine. Our results reveal the tight link among MAC activity, cytochrome *c* release and apoptotic death, and indicate this mitochondrial channel is a promising therapeutic target.

Key words: apoptosis, cytochrome *c* release, interleukin-3, mitochondrial apoptosis-induced channel (MAC), patch-clamp, staurosporine.

INTRODUCTION

Apoptosis evolved in higher eukaryotes as a selective and efficient mechanism for controlled cell death. It is critical for development and tissue homoeostasis as well as for the pathogenesis of a variety of diseases [1,2]. There are two main pathways of apoptosis: (i) extracellular inducers activate TNF (tumour necrosis factor) family receptors in the plasma membrane in the extrinsic pathway [3], and (ii) intracellular sentinels activate cytochrome *c* release channels in the mitochondrial outer membrane in the intrinsic pathway [4–6]. Both converge at the activation of executioner caspases [7–10]. The cell death cascade encompasses the following morphological changes: cell shrinkage, blebbing, cytoplasm reorganization, chromatin condensation and DNA fragmentation [11]. In contrast with necrosis, apoptosis converts a cell into small apoptotic bodies that will be absorbed without activation of the inflammatory machinery (for a comprehensive review on programmed cell death markers see [12]).

Release of cytochrome *c* from mitochondria occurs in many cell types in response to a variety of stresses such as heat shock [13], radiation [14], viral infection [15], interruption of kinase signalling [16] or nutrient deprivation [17]. For example, cytochrome *c* is released from mitochondria of a murine pro-B-cell line, FL5.12, upon deprivation of IL-3 (interleukin-3). Cytochrome *c* release occurs before mitochondrial depolarization or the onset of other early apoptotic markers, e.g. changes in the plasma membrane lipid asymmetry. MAC (mitochondrial apoptosis-induced channel) is formed in the outer membrane of mitochondria of FL5.12 cells after IL-3 depletion [18], but MAC involvement in the release of cytochrome *c* and the commitment of apoptosis in these cells needs further investigation.

Several lines of evidence, including directly patch-clamping apoptotic mitochondria, support the idea that MAC is the mediator of cytochrome *c* release in a variety of cell types with different inducers [4,19–21]. Our previous findings have shown that Bax and/or Bak are required for MAC formation as double knockouts for these pro-apoptotic proteins failed to display MAC activity in the mitochondrial outer membrane or release of cytochrome *c* [22]. Patch-clamp experiments show that MAC formation can be catalysed by BH3-only proteins such as t-Bid (trunctated Bid), again, only in cells expressing Bax and/or Bak [23]. On the other hand, overexpression of anti-apoptotic proteins such as Bcl2 prevents MAC formation [18].

MAC is a potential target for novel therapies as the use of agonists or antagonists of this channel could induce or prevent cell death respectively. For example, agonists of MAC could restore cytochrome *c* release and cell death in lymphomas [24]. Alternatively, antagonists of MAC could potentially protect transplanted neuronal precursor cells from apoptosis [25], as well as prevent HIV-1-induced lymphocyte depletion [26,27], severe congenital neutropenia [28] and other pathologies associated with Bax-induced cytochrome *c* release. Furthermore, iMACs (inhibitors of MAC) may provide rapid insights into underlying apoptotic mechanisms much like CSA (cyclosporine A) has done for the mitochondrial PTP (permeability transition pore) for many years [29].

In preliminary studies, compounds were identified that suppress the release of 5,6-carboxylfluorescein from liposomes triggered by recombinant Bax, a known component of MAC [30]. In the present study, the mechanisms underlying this blockade were investigated further. The effect of compounds on the progression of apoptosis induced by STS (staurosporine) was determined using flow cytometry and their toxicity was evaluated in cell viability assays. Patch-clamp techniques revealed that some of these compounds specifically, and usually irreversibly, blocked MAC. Although MAC activity was inhibited by nanomolar levels, the lethal dose of these compounds in FL5.12 cells was often $>5\ \mu$M. Our results show for the first time that blockade of MAC activity, cytochrome *c* release and apoptotic death in FL5.12 cells are tightly linked.

EXPERIMENTAL

Compounds

Stock solutions of STS and CSA (Sigma) were prepared in ethanol and kept at $-20\,^{\circ}$C. Stock solutions (10 mM) of Bci1 and Bci2 (Bax channel inhibitors 1 and 2 respectively) [31], and iMACs

Abbreviations used: Bci, Bax channel inhibitor; CSA, cyclosporine A; IL-3, interleukin-3; MAC, mitochondrial apoptosis-induced channel; iMAC, inhibitor of MAC; PI, propidium iodide; PTP, permeability transition pore; STS, staurosporine; t-Bid, trunctated Bid; TOM, translocase of the outer membrane.

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(Merck Serono) [30] were freshly prepared in DMSO and were kept at −20 °C for no longer than 6 months. Solutions were held at room temperature (21 °C) for 30 min prior to use. The purity of all of the compounds was above 98 % by HPLC. The lipophilicity index ALOGP for all the compounds was calculated by Tsar Software version 3 (Accelrys), following the ALOGP computational method for determination of the partition coefficients [32].

Cells and growth conditions

FL5.12 cells were cultured as described previously [33] in Iscove's modified Eagle's medium, 10 % (v/v) fetal bovine serum and 10 % WEHI-3B supplement (filtered supernatant of WEHI-3B cells secreting IL-3) at 37 °C and 5 % CO_2. Cultures were kept at a density below 2×10^6 cells/ml. When IL-3 was used to induce apoptosis, cells were washed twice in medium without IL-3 (WEHI-3B supplement) 12 h prior to the isolation of mitochondrial outer membranes [18,34].

Flow cytometry and fluorescence microscopy

Compounds were added 1 h prior to the treatment of FL5.12 cells (0.5×10^6) with 0.1 % DMSO or 0.5 μM STS for 12 h. Cells were then harvested and washed twice with binding buffer [140 mM NaCl, 2.5 mM $CaCl_2$ and 10 mM Hepes (pH 7.4)] for FITC–Annexin V and PI (propidium iodide) labelling. Flow cytometry analysis (CellQuest Pro; BD Biosciences) was carried out immediately after PI staining. Cells negative for both Annexin V and PI were scored as healthy. Annexin-V-positive cells were scored as apoptotic, and PI-positive cells were scored as dead. Following flow cytometry analysis, DIC (differential interference contrast) and fluorescence images were acquired with a Nikon Eclipse TE2000-E fluorescence microscope using appropriate filters. Images were analysed offline using NIS-element AR 3.0 and ImageJ version 1.39.

Cytochrome *c* release and cell viability

Apoptosis was induced by either treatment with 0.5 μM STS or withdrawal of IL-3 for 12 h. Compounds were added 1 h prior to STS treatment or IL-3 withdrawal. Cytochrome *c* release was determined relative to alamethicin (1.5 μg/10^6 cells) by ELISA (Quantikine; R&D Systems), as described previously [23,35].

The effect of compounds on viability was assayed by treating cells with 0–50 μM propranolol, dibucaine, trifluoperazine, Bci1, Bci2, iMAC1, iMAC2, iMAC3, iMAC4 and iMAC5 in a 96-well plate (0.5×10^3 cells/well). Alamar Blue (Invitrogen) was added to a final concentration of 10 % and reduction/oxidation rates (560 nm/590 nm) were measured after 0, 12, 24 and 48 h, according to the manufacturer's instructions, to determine the LD_{50}.

Isolation of mitochondria and outer membrane preparations

Mitochondria were isolated from 10–15 g of FL5.12 cells, as described previously [18,34]. Outer membranes were stripped from the inner membranes by French-pressing isolated mitochondria using modifications of the method of Decker and Greenawalt [36] and were purified as reported by Mannella [37], as described previously [18,34]. Outer membranes were reconstituted as proteoliposomes by a modification of the method of Criado and Keller [18]. Briefly, outer membranes (5 μg of protein) and small liposomes (1 mg) were mixed with 5 mM Hepes (pH 7.4) and dotted on to a glass slide. Samples were dehydrated for approx. 3 h and 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 buffer and stored at −80 °C.

Patch-clamp experiments and analysis

Patch-clamp procedures and analysis were as described previously [18,34]. Unless otherwise stated, the solution used was symmetrical 150 mM KCl and 5 mM Hepes (pH 7.4). Compounds were introduced to patches by perfusion of the approx. 0.5 ml bath with 3–5 ml of buffer containing the indicated compounds. Voltage clamp was performed with the excised configuration of the patch-clamp technique using an 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). Voltages were reported as pipette potentials. The conductance was typically determined from total amplitude histograms of 30 s of current traces at +20 mV. 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, Glasgow, U.K.) were used for analysis of channel activity. Curve fitting was done through OriginPro 8.0 SR0 (OriginLab).

RESULTS

Prevention of apoptosis

We have tested previously the ability of iMAC compounds to prevent the release of macromolecules in artificial systems [30]. We assessed the ability of one of these compounds to suppress apoptosis induced by STS before exploring further their mechanism of action. The onset of apoptosis markers in the absence (STS) and presence of iMAC2 (STS + iMAC) was evaluated after the addition of 0.5 μM STS (Figure 1a). The loss of lipid asymmetry in the plasma membrane, a classical marker of apoptosis, was monitored by Annexin V staining. Loss of plasma membrane integrity, a later marker of apoptosis, was detected by PI labelling; PI-positive cells were scored as dead in both fluorescence microscopy and flow cytometry experiments. At the same time, we evaluated the contribution of the mitochondrial PTP to the progression of apoptosis by determining the onset of markers in the presence of the potent inhibitor CSA. STS-treated cells that were also treated with vehicle (STS) or CSA (STS + CSA) shrank, eventually stained for both FITC–Annexin V and PI, and generated visible cell debris. In contrast with CSA pre-treatment, and yet similar to vehicle-treated controls (Control, no STS), cells pre-treated with iMAC2 had a normal volume and residual Annexin V/PI staining (Figure 1a). Figure 1(b) shows that approx. 60 % of the cells were Annexin-V-positive after 12 h of STS treatment. Although CSA did not diminish Annexin V labelling, iMAC2 reduced apoptosis by more than 50 %. Analysis of cell volume (scatter) by flow cytometry showed two main populations (Figure 1c). One represents healthy cells with normal size and granularity, and the other one represents dying cells and debris, which are smaller. STS induced the dying population to increase from 16 (in control levels) to 78 %. Pre-treatment with iMAC2 induced a significant protection at low micromolar levels ($IC_{50} = 2.5$ μM) (Figure 1d).

Blockade of cytochrome *c* release

If the anti-apoptotic effect of iMAC2 reflects a specific effect on mitochondrial permeabilization, iMACs should prevent release of cytochrome *c* during apoptotic stimulation. Compounds from two different chemical series (Figure 2) were examined further. The first chemical series corresponds to the non-aromatic compounds Bci1 and Bci2, shown previously to block cytochrome *c* release in isolated mitochondria treated with t-Bid [31]. The second series corresponds to iMAC compounds, which were shown to block the

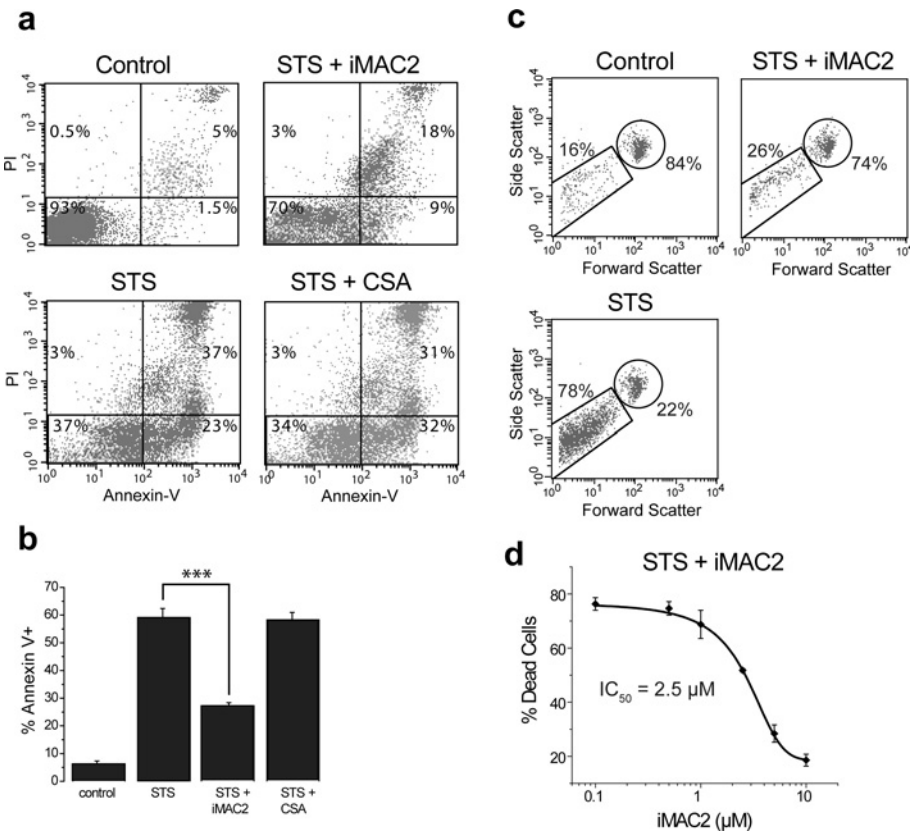

Figure 1 Effect of iMAC2 on the onset of apoptosis markers

After 12 h of treatment with DMSO (Control) or 0.5 μM STS, FL5.12 cells were stained for PI and FITC–Annexin V. When used, 5 μM iMAC2 or CSA were added 1 h prior to STS. (**a**) Distribution of PI- and FITC–Annexin-V-stained cells detected by flow cytometry. Annexin-V-positive cells were scored as positive for apoptosis. (**b**) Effect of 5 μM iMAC2 and CSA on the percentage of Annexin-V-positive cells (%Annexin V+) detected (***$P < 0.0001$). Values are means $\pm$ S.E.M. ($n = 4$ independent experiments). (**c**) Cell scatter was analysed in FL5.12 cells treated as indicated above. Trapezoid gates represent the percentage of dead cells; circular gates represent the percentage of live cells. (**d**) Curve shows the percentage of dead cells induced by treatment with 0.5 μM STS for 12 h in the presence of 0–10 μM iMAC2. Values are means $\pm$ S.E.M. ($n = 16$ determinations).

release of 5,6-carboxylfluorescein from liposomes triggered by recombinant Bax [30]. Using a more cellular approach, we tested these compounds directly for their ability to block cytochrome *c* release in cultures of apoptotic FL5.12 cells.

Apoptosis was induced either by treatment with 0.5 μM STS (Figure 3a) or by withdrawal of the obligatory supplement IL-3 (Figure 3b). Pre-treatment with 5 μM Bci1, Bci2 or iMAC1 provided a partial blockade of cytochrome *c* release after 12 h. In contrast, release of cytochrome *c* was essentially eliminated by pre-incubation with iMAC2 and iMAC3 ($P < 0.0001$), but not by iMAC4 and iMAC5 (results not shown). Interestingly, pre-treatment with the PTP inhibitor CSA failed to prevent cytochrome *c* release under these conditions.

Specific inhibition of MAC

Our results suggest iMACs exert their anti-apoptotic activity through prevention of mitochondrial outer membrane permeabilization to apoptotic factors such as cytochrome *c*. Given that CSA failed to prevent apoptosis and cytochrome *c* release in our conditions, we tested the effect of iMACs on MAC by patch-clamping reconstituted mitochondrial outer membranes of apoptotic FL5.12 cells. MAC typically occupied a long-lasting high-conductance ($\geqslant 1.5$ nS) open state that was voltage-independent (within ± 50 mV), as shown in Figure 4(a). Vehicle control (DMSO) and the TOM (translocase of the outer membrane) channel effector $CoxIV_{(1-13)}$ did not modify MAC conductance (results not shown). In contrast, nanomolar levels of iMAC2 completely blocked the current flow through MAC. Furthermore, MAC closure was often rapid, i.e. MAC activity was often inhibited before completion of the perfusion of the bath to introduce the compounds (approx. 15 s). iMACs were routinely tested on TOM channels. Figure 4(b) shows that iMAC2 had no effect on TOM activity even at 20-fold higher concentrations.

We tested further a total of seven compounds, including iMAC1–iMAC5, Bci1 and Bci2. The IC_{50} values for blocking current flow through MAC ranged from 19 to 966 nM for the various compounds (Figure 5). The dose–response curves displayed Hill coefficients of 0.85–1.28, which suggest blockade by these compounds was non-co-operative. Figure 5(d) shows that potency correlated with lipophilicity (ALOGP) of the iMACs, i.e. the most lipophilic compounds had the smallest IC_{50} values. ALOGP is a computational method for determining the partition coefficients of compounds in aqueous solutions. A saturation point appeared to exist at ALOGP values $\geqslant 4$.

Toxicity and potency

Although iMACs were potent inhibitors of MAC, they had little effect on cell viability at these nanomolar concentrations, as indicated by Alamar Blue staining. FL5.12 cells were incubated for up to 48 h in the absence (control) or presence of different

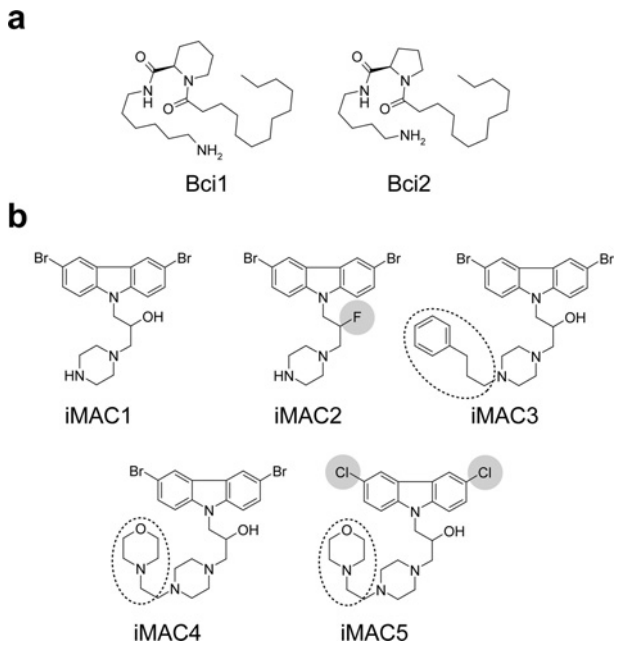

Figure 2 Structures of Bci and iMAC compounds

(**a**) Bax channel inhibitors Bci1 and Bci2. (**b**) The design of iMAC2–iMAC5 was based on the parent compound iMAC1. The structural differences are indicated. iMAC2 has a fluoride instead of a hydroxy group in the propanol linker; iMAC3 has a propylbenzene on the piperazine group; and iMAC4 has a morpholino-ethylene on the piperazine group. iMAC5 is as iMAC4, but the bromides were exchanged for chlorides on the carbazole group.

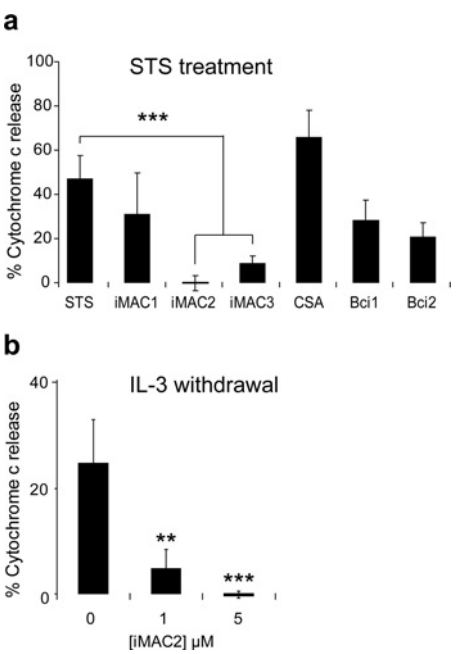

Figure 3 Effect of iMACs on the release of cytochrome *c* from mitochondria of apoptotic cells

The percentage of cytochrome *c* release in FL5.12 cells (10^6 cells) 12 h after treatment with 0.5 μM STS (**a**) or IL-3 withdrawal (**b**) is shown. Values are reported relative to the pool of cytochrome *c* released by alamethicin treatment after background (DMSO) was subtracted. (**a**) Cells were treated for 1 h prior to the addition of STS with DMSO, or 5 μM iMAC1, iMAC2, iMAC3, Bci1, Bci2 or CSA as indicated. $^{***}P < 0.0001$ compared with cells treated with STS alone. (**b**) Cells were treated for 1 h prior to IL-3 withdrawal with 0.1 % DMSO or iMAC2. Values are means $\pm$ S.E.M. for eight independent experiments. $^{**}P < 0.001$ and $^{***}P < 0.0001$ compared with cells treated 0 μM iMAC2.

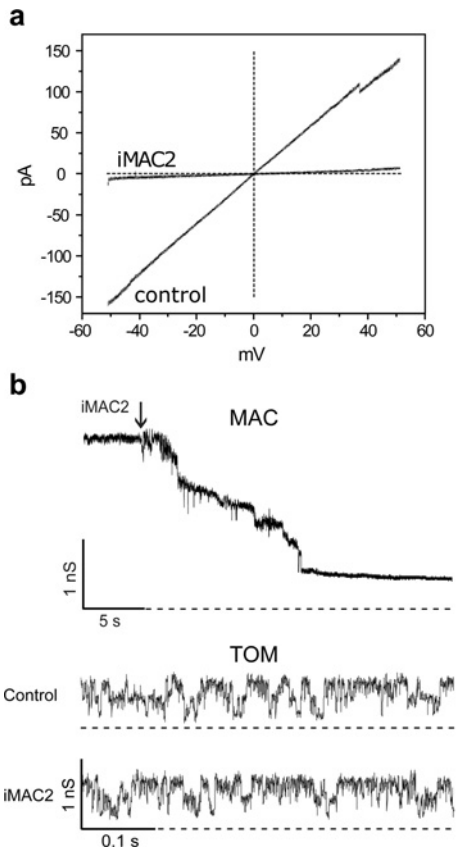

Figure 4 Effect of iMAC2 on MAC and TOM channels

MAC and TOM activities were recorded in patches excised from reconstituted mitochondrial outer membranes of apoptotic FL5.12 cells. (**a**) Current–voltage plots of MAC are shown after perfusion of the bath with buffer containing DMSO (control) and then 100 nM iMAC2. (**b**) Representative current traces at +20 mV of MAC and TOM upon perfusion with buffer containing 25 nM and 500 nM iMAC2 respectively. The current trace of TOM in the presence of iMAC2 was recorded approx. 1 min after perfusion. Broken lines indicate 0 current levels.

concentrations of iMACs, which typically had LD_{50} values $\geqslant 5$ μM, as shown in Figure 5(b). Figure 5(c) shows the LD_{50}/IC_{50} ratios for the various compounds tested. In earlier studies, propranolol, dibucaine and trifluoperazine were identified as inhibitors of MAC [38], and these were included for comparison. Although those original compounds were toxic at concentrations close to their effective blocking doses, iMACs had LD_{50} values at least 15 times higher than their respective IC_{50} doses. It may be of some importance that the LD_{50} values for iMAC2 and iMAC3 were more than 500 times higher than their IC_{50} doses.

DISCUSSION

Specific inhibitors of MAC have the potential to identify modulators of intrinsic apoptosis and enable a further functional dissection of this process. They could also be useful therapeutic tools, providing they can cross the plasma membrane to reach the mitochondria and close MAC. In the present study, several compounds, some of which are derivatives and structurally related, were assessed for their ability to suppress apoptosis by closing MAC and, hence, the pathway for cytochrome *c* release. The therapeutic window of each of those compounds was also determined. The piperazine derivatives of 2-propanol, iMAC2 and iMAC3, were found to have the lowest toxicity and highest efficacy of MAC blockade of the agents tested.

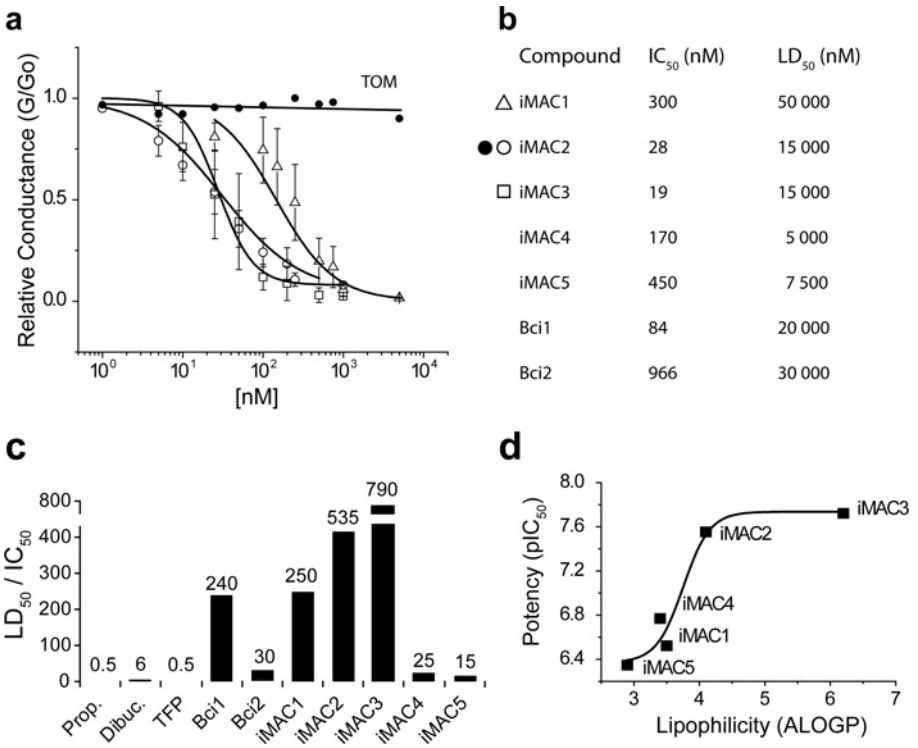

Figure 5 Effects of iMACs on MAC activity and cell viability

(**a**) The relative conductance (G/G_0) at +20 mV as a function of the concentration of iMAC compounds for MAC (open symbols) and TOM (closed circles) channels is shown. G_0 and G are the conductances before and after perfusion with the compounds indicated by the symbols in (**b**) respectively. Results shown are relative conductance means ± S.D. for a minimum of ten independent experiments for each compound tested. (**b**) IC_{50} values were calculated from the fitted curves of the Hill equation $y = x^n/(k^n + x^n)$, where x is the drug concentration, n is the Hill coefficient, and k is the IC_{50} value from the patch-clamp data as in (**a**). LD_{50} values were determined using Alamar Blue (six replicates/point) as described in the Experimental section. (**c**) LD_{50}/IC_{50} ratios for the indicated compounds showing the separation of the toxic and blocking doses. Prop. propranolol; Dibuc., dibucaine; TFP, trifluoperazine. (**d**) IC_{50} and lipophilicity indices ALOGP of the iMACs fit in a dose–response curve ($r^2 = 0.9$) with formula $y = A1 + (A2 - A1)/1 + 10^{(Logx0-x)p}$, where A1 and A2 are the bottom and top asymptotes respectively, Logx0 is the centre, and p is the Hill slope. Other conditions are as in Figure 4.

Mitochondrial apoptosis seemingly begins by initiating the formation of MAC, a channel that permeabilizes the mitochondrial outer membrane to death factors. If the rationale that MAC is pivotal to mitochondrial apoptosis is correct, then iMACs are also expected to suppress the downstream events of apoptosis. Indeed, ELISA and flow cytometry experiments showed that iMAC2 was effective in curbing or delaying the early and late markers of apoptosis, including the release of cytochrome *c*, cell shrinkage, and the loss of plasma membrane lipid asymmetry and integrity after 12 h of STS treatment (Figure 1). In fact, iMAC2 also suppressed cytochrome *c* release after IL-3 withdrawal (Figure 3b). Hence this inhibition suggests that MAC function is essential to mitochondrial apoptosis induced by at least two different means.

If MAC provides the pathway for cytochrome *c* release, then blockade of this process should correlate with the direct blockade of the ion flow, or current, through the channel. In order to test this possibility, the effects of compounds on MAC activity were assessed using patch-clamp techniques on preparations containing outer membranes from apoptotic mitochondria (Figures 4 and 5). Similar to the cytochrome *c* release experiments, iMAC2 and iMAC3 were the most potent, with the lowest IC_{50} values. The action of these iMACs resembled MAC depletion in Bax/Bak-double knockouts in which the channel activity of MAC, cytochrome *c* release and apoptosis were each eliminated [23,39].

MAC blockade by the iMACs showed interesting features in electrophysiological experiments. Multiple stepwise decreases in current flow were typically observed (Figure 3b), consistent with a destabilization of the open state. It is not likely that these small compounds (approx. 0.5 kDa) physically block the pore of the channel as does cytochrome *c* [4,34]. Hence the observed effects could be due either to closure or disassembly of MAC. Although some high-affinity compounds can irreversibly close channels, classical drug-induced closure is often reversible. In our hands, extensive washes usually failed to recover MAC conductance even 30 min after treatment with each of the iMACs (results not shown). The results shown in the present study could be interpreted as an iMAC-induced disassembly mechanism. Such a mechanism was proposed for ceramide channels in the presence of Bcl-x_L, where an irreversible multiple stepwise closure was also observed [40]. Strikingly, MAC often displays multiple stepwise conductance increments during its assembly [23]. Thus the apparent assembly and probable disassembly of MAC present similar behaviour. Alternatively, there may be an inactivation or conformational change in Bax and/or Bak, which might lead to a large but inactive complex. Finally, one could also speculate that the effects of these compounds on membrane fluidity may modify the stability of the open state of MAC, particularly if lipids play a significant role in this channel.

The PTP is an inner membrane channel of mitochondria that gained prominence for its possible role in apoptosis [41]. In lieu of MAC, PTP provides an alternative way of releasing cytochrome *c* from mitochondria and is critical to the processes of ischaemia/reperfusion injury and necrosis [41,42]. Because the molecular identity of PTP is not well-defined, involvement of this channel in apoptosis is typically indicated by a delay or blockade

of the onset of apoptotic markers by the potent PTP blocker CSA [41]. In contrast with iMAC2, CSA did not modify the onset of the apoptotic indicators Annexin V and PI staining under the same conditions (Figure 1). Furthermore, pre-treatment with 5 μM CSA failed to prevent cytochrome *c* release (Figure 3a). These results indicate that cytochrome *c* release and apoptosis progression in this system is not dependent on the opening of PTP. Instead, these findings support the idea that MAC, but not PTP, mediates apoptosis in STS-treated or IL-3-withdrawn FL5.12 cells.

The potencies of these compounds for the blockade of MAC, some of which are derivatives and structurally related, varied from 19 to 966 nM in patch-clamp experiments. The overall efficacy of apoptosis blockade was the highest for iMAC2 and iMAC3, which also completely blocked the release of cytochrome *c* in apoptotic cells (Figure 3). Surprisingly, Bci1 and Bci2 were not as potent as expected from previous studies [31]. Differences in these potencies may be accounted for by the different experimental approaches used for evaluation. In the present study, agents were introduced to cell cultures 1 h prior to the addition of STS or withdrawal of IL-3. In previous studies, Bci compounds were tested for their ability to block cytochrome *c* release from isolated mitochondria treated with t-Bid. As with Bci1 and Bci2, iMAC1 also failed to significantly block cytochrome *c* release in our hands.

Specific structural modifications of iMAC1 (Figure 2) dramatically decreased the IC_{50} value and increased the lipophilicity of the derivatives. iMAC2 was formed from iMAC1 by substituting a fluoride for a hydroxy group at the propanol linker, which caused an increase in potency. Similarly, an increase in potency was observed in yet another derivative, iMAC3, which was formed by adding a propylbenzene to the piperazine group, again on iMAC1 (Figure 5). The lipophilicity of both iMAC2 and iMAC3 was considerably larger than that of iMAC1, with ALOGP values above 4. Two other structural modifications of iMAC1 resulted in iMAC4 and iMAC5, whose ALOGP values were below 4 (Figure 5). Although their lipophilicity was smaller than that of iMAC1, their IC_{50} values were in the same range of a few hundred nanomolar. These findings suggested that a correlation exists between lipophilicity and potency (Figure 5).

A variety of factors influence potential applications of these agents. Unlike several of the experimental approaches used in the present study, the IC_{50} value determined by direct application of compounds to MAC in our patch-clamp experiments were not influenced by factors that might reduce the local concentration. That is, the permeability barriers for the compounds that exist in cells or animals are bypassed by such an optimal system for drug delivery. These barriers can be significant and may have an impact on the useful applications as suggested by the differences in IC_{50} values for blocking MAC conductance (Figure 5) and changes in scatter (Figure 1c). As with permeability, specificity may also be a limiting factor in defining potency. Although other channels and cellular functions may be examined further for pleiotropic effects, iMACs routinely had no effect on TOM channel activity. This finding, added to the observation that iMACs strongly co-localize with mitotracker in HeLa cells [30], reinforces the notion that iMACs interact with mitochondria and specifically with MAC. Blocking MAC through agents such as iMACs may in fact delay but not prevent cell death. The effectiveness of these agents may also be a function of the pleiotropic effects of the inducer of apoptosis, e.g. STS is a broad kinase inhibitor that affects many processes. Hence there may be variability in the sensitivity of onset of some apoptotic markers to iMACs. Finally, studies have shown that cells lacking both Bax and Bak, which then cannot form MAC [22,23], often eventually die through autophagy or necrosis [43].

In summary, iMAC2 specifically suppressed the onset of apoptotic markers by blocking MAC and suppressing cytochrome *c* release. This iMAC mimicked the behaviour of Bax/Bak-double knockouts in which MAC activity, cytochrome *c* release and apoptosis were eliminated and cell death was delayed. Hence these results reveal the tight link between MAC activity, cytochrome *c* release and apoptotic death. In addition, these findings identify this mitochondrial channel as a promising therapeutic target, as the lethal dose and effective blocking doses are widely separated for multiple derivatives. Without diminishing the therapeutic potential of iMAC2, this compound may also be useful to functionally dissect the mechanisms underlying the apoptotic cascade in various cell types. Finally, iMAC2 may be valuable for the study of HIV-1-induced lymphocyte depletion [26,27], neutropenia [28], Parkinson's disease [44] and other disorders associated with Bax-induced cytochrome *c* release.

AUTHOR CONTRIBUTION

Pablo Peixoto performed the patch-clamp, cytochrome *c* release, cell viability, microscopy and flow cytometry experiments and wrote the paper; Shin-Young Ryu performed the patch-clamp experiments; Agnes Bombrun and Bruno Antonsson synthesized the Bci and iMAC compounds; and Kathleen Kinnally designed the research.

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