Some amphiphilic cations block the mitochondrial apoptosis-induced channel, MAC

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Abstract The mitochondrial apoptosis-induced channel (MAC) forms in the outer membrane of mitochondria early in apoptosis and this activity is altered by physiological levels of cytochrome *c*. While cyclosporine A and lidocaine have no effect, dibucaine induces a fast blockade of MAC with an IC_{50} of 39 μM. In contrast, the IC_{50} for propranolol and trifluoperazine are 52 and 0.9 μM, respectively, and these drugs likely destabilize the open state of MAC. These agents, and others not yet identified, should be valuable tools in the study of apoptosis. Profiling MAC's pharmacology may generate novel therapeutic regimes for disease.

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1. Introduction

Apoptosis is a phenomenon fundamental to higher eukaryotes and essential to the mechanisms underlying tissue homeostasis. The release of cytochrome *c* from mitochondria is considered the commitment step of apoptosis in many cell types and is tightly regulated by Bcl-2 family proteins [1–4]. While the permeability transition pore (PTP) is implicated in cytochrome *c* release in some systems [5,6], recent investigations show that cytochrome *c* can exit directly through a pore in the mitochondrial outer membrane without loss of outer membrane integrity [6–13].

A high conductance channel forms in the mitochondrial outer membrane early in apoptosis before the onset of other apoptotic markers, e.g., Annexin-V labeling [7]. The appearance of this mitochondrial apoptosis-induced channel (MAC) is prevented by overexpression of Bcl-2 [13]. Furthermore, the single channel activity of MAC is modified by physiological levels of cytochrome *c* [7]. These findings support a role for MAC in the release of cytochrome *c* and possibly other factors early in apoptosis.

MAC is a novel channel with no previous pharmacological profile. Patch-clamp techniques were used in this study to examine several potential effectors. These data show that cyclosporine A and lidocaine have no effect, and the amphiphilic cations propranolol, dibucaine and trifluoperazine are inhibitors of MAC.

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Abbreviations: MAC, mitochondrial apoptosis-induced channel; PTP, permeability transition pore; Tim, translocase of the inner membrane

2. Materials and methods

2.1. Cells and growth conditions

Parental FL5.12 cells were cultured as previously described [14] in Iscove's modified Eagle's media (IMEM), 10% fetal bovine serum, 10% WEHI-3B supplement (filtered supernatant of WEHI-3B cells secreting IL-3). Cultures were kept below 1.5 million cells/ml. Cells were washed three times in media without IL-3 (WEHI supplement) to induce apoptosis 12 h prior to the isolation of mitochondria [7,13].

2.2. Isolation of mitochondria and preparation of proteoliposomes

Mitochondria were isolated from 2 to 15 g of FL5.12 cells as previously described for outer membrane preparations [7,13]. Outer membranes were stripped from the inner membranes by French pressing isolated mitochondria using modifications of the method of Decker and Greenawalt [15]. French pressing was done at 2000 PSI in 460 mM mannitol, 140 mM sucrose, 2 mM EDTA, and 10 mM HEPES, pH 7.4. The pressed suspension was diluted 1:1 with 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 5 mM HEPES, pH 7.4, and centrifuged at 10 000 rpm (12 000 × *g*) for 10 min. Outer membranes were separated from inner membranes as described by Mannella [16].

Proteoliposomes were formed by a modification of the method of Criado and Keller [7,13,17]. Briefly, small liposomes were formed by sonication of lipid (Sigma Type IV-S soybean L-α-phosphatidylcholine) in water. Mitochondrial outer membranes (5–10 μg protein) and small liposomes (~1 mg lipid) were mixed with 5 mM HEPES, pH 7.4, and dotted on a glass slide. Samples were dehydrated ~3 h and rehydrated overnight with 150 mM KCl and 5 mM HEPES, pH 7.4, at 4°C. Proteoliposomes were harvested with ~0.5 ml of the same media and stored at −80 °C.

2.3. Immunoblotting

Proteins were separated by SDS–PAGE and electro-transferred onto PVDF membranes. Indirect immuno-detection employed chemiluminescence (Amersham) using HRP-coupled secondary antibodies. Mitochondrial outer and inner membranes (0.5–2 μg per lane) were probed with primary antibodies against mammalian VDAC1 (Calbiochem 31-HL, 1:2500), cytochrome oxidase subunit IV (Molecular Probes A-6431, 1:1000) and a secondary anti-rabbit or anti-mouse antibody (Jackson Immunoresearch, 1:5000).

2.4. Patch-clamp analysis

Patch-clamp procedures and analysis used were previously described [7,13,18]. Briefly, membrane patches were excised from proteoliposomes containing purified mitochondrial outer membranes after formation of a giga-seal using micropipettes with ~0.4 μm diameter tips and resistances of 10–20 MΩ at room temperature. Unless otherwise stated, the solution was symmetrical 150 mM KCl and 5 mM HEPES,

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pH 7.4. Voltage clamp was performed with the excised configuration of the patch-clamp technique [19] using an Axopatch 200 amplifier. Voltages are reported as pipette potentials. The conductance was typically determined from total amplitude histograms of 30 s of current traces at +20 mV. MAC activity is distinct from VDAC and TOM channels [13]. Currents were assigned as MAC if the conductance was >1.5 nS and voltage independent. pClamp version 8 (Axon Instru.) and WinEDR v2.3.3 (Strathclyde Electrophysiological Software; courtesy of J. Dempster, University of Strathclyde, UK) were used for current analysis. Hill coefficients were determined as described by Moczydlowski [20].

3. Results and discussion

MAC is a potential therapeutic target because of its putative role in the commitment step of apoptosis, i.e., the release of cytochrome *c*. However, there is no pharmacological profile for MAC at this time. Apoptotic hematopoietic cells were used in this study to investigate the effect of a variety of agents on MAC activity. Apoptosis was induced by withdrawal of interleukin-3 (IL-3) from the leukemia cell line FL5.12. MAC activity is detected and cytochrome *c* is released from the mitochondria of these cells 12 h after IL-3 withdrawal [7,13,14]. Outer membranes were purified from mitochondria isolated at this time. The Western blot of Fig. 1A shows the presence of the outer membrane protein VDAC and essentially none of the inner membrane protein cytochrome oxidase subunit IV in the preparations; there is little contamination of the outer membranes by inner membranes. MAC activity was examined using patch-clamp techniques on proteoliposomes formed by the fusion of these outer membranes with liposomes (see Section 2). MAC is a heterogeneous channel with a variable high conductance and several substates. In this study, the effects of a variety of pharmacological agents were determined on MAC activity with a conductance of 1.5–5 nS and a long-lived open state [7,13].

MAC is exquisitely regulated by Bcl-2 family proteins but the molecular identity of this channel is not yet known. MAC is never detected in apoptotic cells overexpressing Bcl-2. Interestingly, MAC-like activity is detected in yeast expressing Bax and in membrane patches containing recombinant Bax [13]. These findings suggest that Bax might be involved with MAC activity. Dibucaine and propranolol (100–200 μM) prevent the release of cytochrome *c* induced by recombinant Bax plus t-Bid or BH3 peptide [21], but they do not prevent Bax insertion into membranes.

The effect of dibucaine on MAC was examined because Bax may be a component of the MAC. As shown in Fig. 1B, the current flow through MAC is rapidly reduced upon perfusion of the bath with 50 μM dibucaine. The blockade of MAC by dibucaine is reversible as removal of dibucaine from the bath usually restores the high conductance. The total amplitude histograms and current traces of Fig. 1C illustrate this reversible inhibition in another patch containing MAC. The blockade is not voltage dependent as the current voltage curves are linear between +40 and −40 mV (not shown). The blockade of MAC by dibucaine is similar in partially purified fractions of MAC from apoptotic HeLa cells treated with staurosporine (unpublished results of Dejean, Martinez-Caballero, Antonsson & Kinnally).

Dibucaine causes a fast blockade of MAC, similar to the effects of diethylamine on sodium channels [22]. These blockade events are rapid as there is no discernable increase in noise at 2 kHz accompanying the decrease in conductance in the presence of dibucaine (not shown).

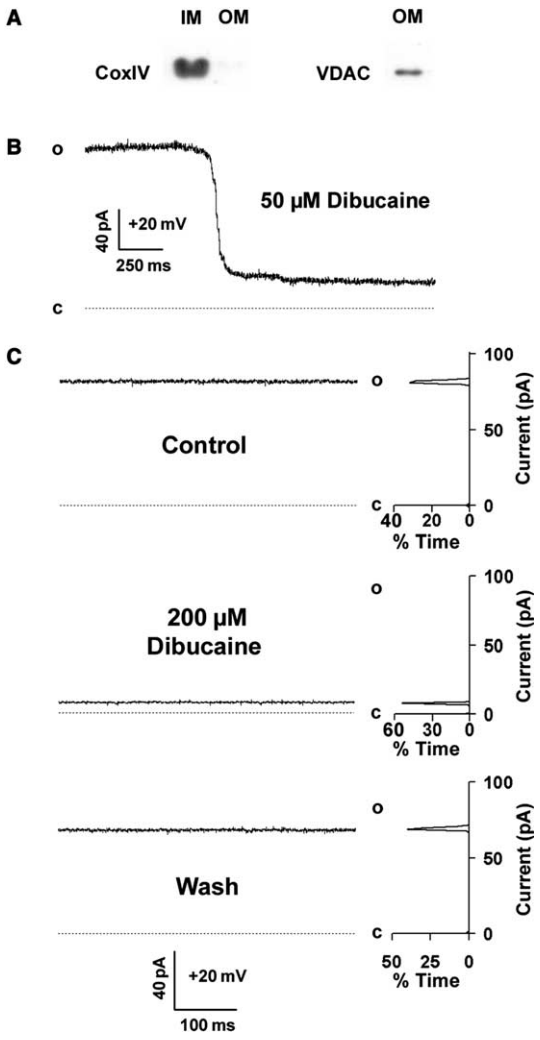

Fig. 1. Fast blockade of MAC by dibucaine. (A) Immunoblots show the presence of the outer membrane protein VDAC but not the inner membrane protein cytochrome oxidase subunit IV (CoxIV) in the outer membranes (OM, 2 μg) purified from mitochondria of apoptotic FL5.12 cells. Inner membranes (IM, 2 μg) are the positive control for CoxIV. (B) Representative current trace of a MAC recorded at +20 mV with 2 kHz filtration is shown 10 s after perfusion of the bath with 50 μM Dibucaine. o and c indicate open and closed conductance states. (C) The dibucaine induced closure of MAC is reversible. Current traces (right) and total amplitude histograms (left) of MAC are shown before and after perfusion with media containing 200 μM dibucaine and after perfusion with 150 mM KCl and 5 mM HEPES to wash out dibucaine. o and c correspond to open and closed conductance states.

Like dibucaine, propranolol blocks the release of cytochrome *c* induced by Bax plus either a synthetic BH3 peptide or t-Bid [21]. Propranolol also blocks the current flow through MAC as shown in the current traces and amplitude histograms of Fig. 2A. The inhibition of MAC conductance by propranolol is not voltage dependent as the current voltage curves are linear between +40 and −40 mV (not shown). In contrast to dibucaine, the effects of propranolol on MAC activity are not reversible as removal by perfusion of the bath does not restore MAC's conductance. Trifluoperazine also blocks MAC as shown in the current traces and amplitude histograms of

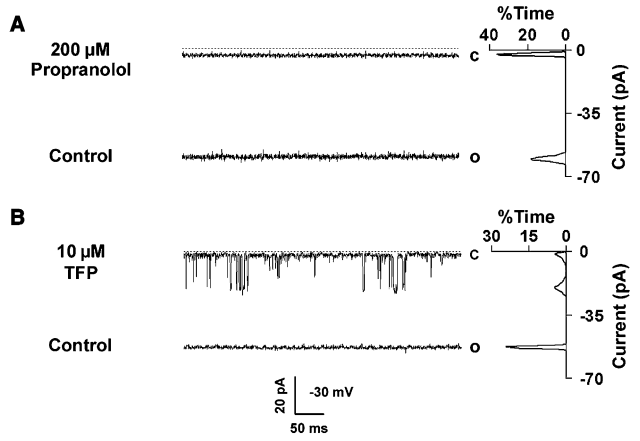

Fig. 2. Propranolol and trifluoperazine block the conductance of MAC. Current traces (right) and total amplitude histograms (left) of two patches in which MAC was recorded at −30 mV before and after application of (A) 200 μM propranolol and (B) 10 μM trifluoperazine (TFP). c and o indicate closed and open conductance states.

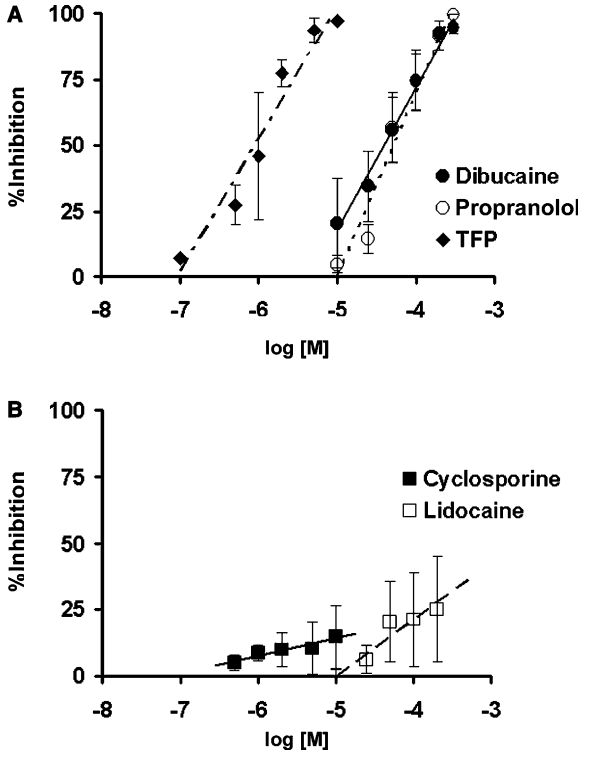

Fig. 3. Inhibitory effects of dibucaine, propanolol and trifluoperazine. % Inhibition of conductance (% mean conductance with/without drug) is plotted as a function of the log concentration [M] of dibucaine, propranolol and trifluoperazine (A), and lidocaine and cyclosporine A (B). The data are best fit with lines for dibucaine, propranolol and trifluoperazine with IC_{50} of 39, 52 and 0.9 μM and correlation coefficients (R^2) of 0.99, 0.96 and 0.95, respectively. The correlation coefficients (R^2) for the best fits for lidocaine and cyclosporine A are 0.92 and 0.90, respectively.

Fig. 2B. Like propranolol, the blockade by trifluoperazine is not voltage dependent or reversible.

The mechanism of blockade of MAC by dibucaine is not the same as that of trifluoperazine and propranolol. Propranolol and trifluoperazine decrease the conductance of MAC, but the effects are not reversible. Repeated washing out of trifluoperazine and propranolol does not result in a re-opening of the channel. Therefore, the mechanism(s) underlying the effects of trifluoperazine and propranolol are likely either a tight binding that "plugs" the pore of MAC or a destabilization of the open state. The latter is more likely as the effects are not reversed several minutes after removal of the agents. Hence, the effects of trifluoperazine and propranolol are similar to the Type 2 effects of cytochrome *c* [7].

The three pharmacological agents inhibit MAC in a dose-dependent manner as shown in % inhibition curves of Fig. 3A. The IC_{50} for trifluoperazine, propranolol, and dibucaine blockade of MAC (and Hill coefficients) are 0.9 μM (1.4 ± 0.2), 52 μM (2.1 ± 0.2), and 39 μM (1.3 ± 0.1). While the mechanism of blockade for the three agents is not identical, the Hill coefficients for all of these inhibitors are more than 1. Typically, this finding indicates that there is some degree of cooperativity involved in the blockade for each of these cationic amphiphilic drugs.

Cyclosporine A and lidocaine have limited effects on MAC activity as shown in Fig. 3B. The IC_{50} for these agents are mM to M, which is much larger than that of dibucaine, propranolol and trifluoperazine. The structures of lidocaine and dibucaine are similar [21], but 300 μM lidocaine has little effect on MAC activity. Cyclosporine A (0.1–1 μM) blocks the PTP in mitochondria [23–25]. However, the putative PTP inhibitors trifluoperazine (10–20 μM) and dibucaine (50–100 μM) [26] also block MAC, but the IC_{50} for MAC is lower than it is for the PTP. Similarly, propranolol blocks MCC, an electrophysiological manifestation of the PTP, but the IC_{50} is ~700 μM [27] compared to ~50 μM for MAC. Interestingly, trifluoperazine and propranolol block apoptosis in some cell lines [28,29]. Furthermore, trifluoperazine (10–20 μM) and dibucaine (50–100 μM) also block mitochondrial depolarization induced by glutamate in neurons [26]. Hoyt et al. [26] interpreted this protection as an inhibition of the permeability transition by these agents. Nevertheless, 0.1–1 μM cyclosporine A blocks the PTP in mitochondria [23–25] and up to 10 μM has no effect on MAC activity. These findings suggest that MAC and the PTP are independent.

While the IC_{50} for trifluoperazine is in the high nanomolar range, none of these drugs are highly specific. In fact, trifluoperazine, propranolol and dibucaine also inhibit mitochondrial protein import [30] and inhibit the permeability transition induced by signal peptides, which likely corresponds to opening of the protein import channel (translocase of the inner membrane, Tim) [21,31,32]. While lidocaine and dibucaine increase membrane fluidity [33,34], lidocaine has no effect on MAC. In contrast, there are conflicting reports of the effects of propranolol on membrane fluidity [33,35]. As dibucaine and propranolol may have opposing effects on bilayer fluidity, MAC opening may be somehow sensitive to this parameter. Others have suggested a role for lipids in the release of cytochrome *c* [36–38] and the molecular identity of MAC is not known. These agents may modify as yet unidentified lipid components of the MAC. In fact, the results of Polster et al. [21] suggest that dibucaine and propranolol inhibit Bax-induced permeability changes through a direct interaction with the lipid membrane. Finally, dibucaine and trifluoperazine inhibit phospholipase A2 [39], which again may implicate a role for lipids in MAC activity. However, it is unlikely that

there is functional phospholipase A2 in the reconstituted system used in this study.

Trifluoperazine, dibucaine and propranolol reduce the conductance of MAC below 1.3 nS. It will be important to compare these agents with those identified as blockers of Bax channels [21,40], as Bax may be a component of MAC. Our previous studies indicate that cytochrome *c* does not affect MAC if the conductance is below 1.9 nS [7], i.e., cytochrome *c* likely does not transit a pore with a conductance below 1.9 nS. Hence, trifluoperazine, dibucaine, and propranolol effectively eliminate the MAC's permeability for cytochrome *c* and should short circuit the death cascade. Future studies will include examination of the effect of pharmacological agents that modify MAC on the progression of apoptosis in vitro and in vivo. Ultimately, agents may be identified that reduce the volume of cell death associated with, e.g., stroke and myocardial infarction.

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References

[1] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell 86, 147–157.
[2] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) Science 275, 1132–1136.
[3] Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P. and Wang, X. (1997) Science 275, 1129–1132.
[4] Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Science 292, 727–730.
[5] Kroemer, G. and Reed, J.C. (2000) Nat. Med. 6, 513–519.
[6] Brenner, C. and Kroemer, G. (2000) Science 289, 1150–1151.
[7] Guo, L., Pietkiewicz, D., Pavlov, E.V., Kasianowicz, J.J., Korsmeyer, S.J., Antonsson, B. and Kinnally, K.W. (2004) Am. J. Physiol. Cell Biol. 286, C1109–C1117.
[8] Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R. and Martinou, J.C. (1997) Science 277, 370–372.
[9] Martinou, J.C. and Green, D.R. (2001) Nat. Rev. Mol. Cell Biol. 2, 63–66.
[10] Saito, M., Korsmeyer, S.J. and Schlesinger, P.H. (2000) Nat. Cell Biol. 2, 553–555.
[11] Shimizu, S., Matsuoka, Y., Shinohara, Y., Yoneda, Y. and Tsujimoto, Y. (2001) J. Cell Biol. 152, 237–250.
[12] De Giorgi, F., Lartigue, L., Bauer, M.K., Schubert, A., Grimm, S., Hanson, G.T., Remington, S.J., Youle, R.J. and Ichas, F. (2002) FASEB J. 16, 607–609.
[13] Pavlov, E.V., Priault, M., Pietkiewicz, D., Cheng, E.H.-Y., Antonsson, B., Manon, S., Korsmeyer, S.J., Mannella, C.A. and Kinnally, K.W. (2001) J. Cell Biol. 155, 725–732.
[14] Gross, A., Jockel, J., Wei, M.C. and Korsmeyer, S.J. (1998) EMBO J. 17, 3878–3885.
[15] Decker, G.L. and Greenawalt, J.W. (1977) J. Ultrastr. Res. 59, 44–56.
[16] Mannella, C.A. (1982) J. Cell Biol. 94, 680–687.
[17] Criado, M. and Keller, B.U. (1987) FEBS Lett. 224, 172–176.
[18] Lohret, T.A., Jensen, R. and Kinnally, K.W. (1997) J. Cell Biol. 137, 377–386.
[19] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. Eur. J. Physiol. 381, 85–100.
[20] Moczydlowski, E. (1986) in: Ion Channel Reconstitution (Miller, C., Ed.), pp. 75–113, Plenum Press, New York.
[21] Polster, B.M., Basanez, G., Young, M., Suzuki, M. and Fiskum, G. (2003) J. Neurosci. 23, 2735–2743.
[22] Zamponi, G.W. and French, R.J. (1993) Biophys. J. 65, 2335–2347.
[23] Lenartowicz, E., Bernardi, P. and Azzone, G.F. (1991) J. Bioenerg. Biomembr. 23, 679–688.
[24] Broekemeier, K.M., Carpenter-Deyo, L., Reed, D.J. and Pfeiffer, D.R. (1992) FEBS Lett. 304, 192–194.
[25] Szabo, I., Bernardi, P. and Zoratti, M. (1992) J. Biol. Chem. 267, 2940–2946.
[26] Hoyt, K.R., Sharma, T.A. and Reynolds, I.J. (1997) Br. J. Pharmacol. 122, 803–808.
[27] Antonenko, Y.N., Kinnally, K.W., Perini, S. and Tedeschi, H. (1991) FEBS Lett. 285, 89–93.
[28] Freedman, A.M., Kramer, J.H., Mak, I.T., Cassidy, M.M. and Weglicki, W.B. (1991) Free Radic. Biol. Med. 11, 197–206.
[29] Nieminen, A.L., Saylor, A.K., Tesfai, S.A., Herman, B. and Lemasters, J.J. (1995) Biochem. J. 307 (Pt 1), 99–106.
[30] Pavlov, P.F. and Glaser, E. (1998) Biochem. Biophys. Res. Commun. 252, 84–91.
[31] Kushnareva, Y.E., Polster, B.M., Sokolove, P.M., Kinnally, K.W. and Fiskum, G. (2001) Arch. Biochem. Biophys. 386, 251–260.
[32] Sokolove, P.M. and Kinnally, K.W. (1996) Arch. Biochem. Biophys. 336, 69–76.
[33] Jutila, A., Rytomaa, M. and Kinnunen, P.K. (1998) Mol. Pharmacol. 54, 722–732.
[34] Kingston, C., Ladha, S., Manning, R. and Bowler, K. (1993) Anticancer Res 13, 2335–2340.
[35] Varga, E., Szollosi, J., Antal, K., Kovacs, P. and Szabo, J.Z. (1999) Pharmazie 54, 380–384.
[36] Siskind, L.J., Davoody, A., Lewin, N., Marshall, S. and Colombini, M. (2003) Biophys. J. 85, 1560–1575.
[37] Siskind, L.J., Kolesnick, R.N. and Colombini, M. (2002) J. Biol. Chem. 277, 26796–29803.
[38] Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R. and Newmeyer, D.D. (2002) Cell 111, 331–342.
[39] Broekemeier, K.M., Schmid, P.C., Schmid, H.H. and Pfeiffer, D.R. (1985) J. Biol. Chem. 260, 105–113.
[40] Bombrun, A., Gerber, P., Casi, G., Terradillos, O., Antonsson, B. and Halazy, S. (2003) J. Med. Chem. 46, 4365–4368.