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Inhibitors of mitochondrial Kv1.3 channels induce Bax/Bakindependent death of cancer cells

Luigi Leanza, Brian Henry, Nicola Sassi, Mario Zoratti, K. George Chandy, Erich Gulbins and Ildikò Szabò

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 November 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

You will see that they all find the topic of your manuscript interesting but feel that the data need to be strengthened and make constructive helpful suggestions. While their reviews are explicit, I would only like to point out the most critical points: referee #1 and #3 recommend adding more controls to existing experiments (Ref.1 points 2 and 3 and Ref.3 points 1 and 2) and Ref.3 would also like to see a better characterization of the in vivo model.

Should you be able to address these criticisms in full, we would be willing to consider a revised manuscript. Please note that acceptance of the manuscript would entail a second round of review and that it is our journal's policy to allow only a single round of revision; meaning that acceptance or rejection of the manuscript will therefore depend on the completeness of your response and the satisfaction of the referees.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

This paper describes the potential of Kv1.3 channel inhibitors to induce apoptosis in tumor cells expressing this channel in the mitochondria. Evidence was provided that these inhibitors mainly act via this mitoKv1.3, while cell lines lacking Kv1.3 were resistant to these inhibitors. One of the drugs (clofazimine) also reduced tumor size in an orthotopic melanoma model in vivo.

While, the authors reported in previous work that Bax binds to Kv1.3 and mediated cytochrome C release at least in part via its interaction with Kv1.3, the new finding in this work is, that 3 distinct membrane-permeant Kv1.3 inhibitors can also activate the intrinsic apoptotic pqathway in the absence of Bax/Bak. The effect on reducing the tumor size in a B16F10 melanoma mouse model suggests a novel therapeutic option for induction of cancer cell death independent of Bax and Bak.

Although the precise role of mitoKv1.3 and the molecular mechanism was not elucidated in this study, the findings are sufficiently important and may lead to interesting new anti-cancer strategies. The data are well presented and experimentally solid. I can advise that the paper should be accepted. I have formulated some suggestions for additional experiments or controls that could further clarify the underlying mechanism.

Referee #1 (Other Remarks):

General:

This paper describes the potential of Kv1.3 channel inhibitors to induce apoptosis in tumor cells expressing this channel in the mitochondria. Evidence was provided that these inhibitors mainly act via this mitoKv1.3, while cell lines lacking Kv1.3 were resistant to these inhibitors. One of the drugs (clofazimine) also reduced tumor size in an orthotopic melanoma model in vivo.

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1) What could be the reason that in Jurkat cells, after downregulation of Kv1.3 with siRNA, there is still 50% cell death with 1 µM clofazimine (Fig 2c), whereas there was no effect of even 50 µM clofazimine on cell death in CTLL-2 cells lacking Kv1.3 (Fig 1A) ?

2) Fig 5 is important to shed more light on the underlying events responsible for Kv1.3-dependent cell death. As such it seems clear from TMRM fluorescence that the mitochondraial membrane potential may be affected by inhibiting this channel. However, the production of ROS species was only tested in Jurkat cells. It would be very advisable to measure if CTLL-2 versus Kv1.3 cells would also show a different level of ROS production. This would also be a good control that the membrane-permeable substances did not contain oxidative species that could produce a more aspecific cell death by oxidative stress (although in the Methods it was mentioned that precautions were taken to avoid this).

3) The experiment with Bax/Bak-deficient Jurkat and MEF cells convincingly shows that cell death is induced by the Kv1.3 inhibitors in the absence of Bax and Bak. However, particularly for the MEF-DKO cells from the Scorrano lab, it was previously demonstrated that these cells may undergo an autophagic cell death in some conditions of UV-induced oxidative stress (Buytaert et al., Autophagy, 2006, 2, 238-240; Buytaert et al.,FASEB J. 20: 756-758). It is therefore possible that cell death is at least to some extent due to autophagic cell death. It is advisable to measure the level of autophagy (e.g. by assaying LC3-I to LC3-II conversion) in these Bax/Bak-deficient cells.

4) In their discussion the authors speculate that the higher sensitivity of tumor cells for clofazimine may be related to increased formation of ROS species that may sensitize the tumor cells. Could it be that ROS species alone are already sufficient to inhibit the Kv1.3 channel and that the effect of clofazimine would be to catalyze such oxidation, rather than to provide a direct inhibition of the channel?
Or stated otherwise: are cells containing mitochondrial Kv1.3 channels more sensitive to oxidative stress inducers (such as menadione) as compared to agents that act on mitochondrial Ca2+ overload (such as C2-ceramide)?

Referee #2 (Comments on Novelty/Model System):

This manuscript presents a wide variety of experimental approaches, which demonstrates that targeting of mitochondrial Kv1.3 with membrane permeable inhibitors can be a key to overcome resistance of cancer cells to apoptosis upon Bax/Bak deficiency. Besides in vitro studies the authors also show that clofazimine was also effective in treatment a mouse model of melanoma in vivo.

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To the reviewer's opinion the manuscript can meet the requirements of this international journal if these corrections/suggestions below are made.

1, Axis of histograms in figure 3C should be labeled as in previous figures.

2, Figure 5A: concentration of drugs is not indicated. Panel B: pJK and Kv1.3 transfected CTLL should be added accordingly.

3, Figure 8E is not mentioned in the text.

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Referee #3:

In this manuscript Authors identify a novel class of anti-cancer agents that target selectively the mitochondrial Kv1.3 channel to kill cells in a Bax, Bak independent manner. Given that downregulation of Bax, Bak is a common feature of many cancers and a key factor in their chemoresistance, finding novel drugs with validated targets is of pivotal importance for cancer therapy. In this respect, the identification of selective and potent inhibitors of this inner mitochondrial membrane channel is opening new potential avenues and must be brought to the attention of the broad interdisciplinary readership of EMM.
The authors show that killing of cells by low doses of the 3 inhibitors Psora, PAP and clofazimine (which is already approved for other conditions, making it a very attractive candidate for further development) is influenced by levels of the mitochondrial Kv1.3 channel. Interestingly, i.p.

administration of clofamizine causes death of an orthotopic model of melanoma that is known (and shown) to express high levels of Kv1.3, confirming that the approach is feasible also in vivo.

In summary, this is a very interesting and well performed study that reports a novel therapeutic target to drive Bax, Bak independent killing of cancer cell. The following points must be addressed by the authors in order to strengthen certain aspects of their work

1. authors elected to show that increased expression of the Kv1.3 channel increased death, cytochrome c release and activation of the post-mitochondrial cascade of apoptosis (figs. 3-6), confining the more meaningful experiments with siRNA to few controls. However, in order to fully validate that the inhibitors act via this channel, an experiment with the CTLL cells deficient in the Kv1.3 channel used in their previous PNAS paper is important to corroborate the selectivity of these drugs.

2. the experiments in Fig. 7 must be repeated following ablation of Kv1.3 to show that the restoration of the killing of Bax, BAk deficient cells depends on the channel.

3. the in vivo orthotopic model must be better characterized. A non cell permeant Kv1.3 inhibitor must be compared to clofazimine.

1st Revision - authors' response 07 February 2012

***** Reviewer's comments *****

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First of all we would like to thank the Reviewer for his/her very useful comments.
As to the first point, clofazimine (and also staurosporine) had an effect in cells shown on Fig 2c, because the downregulation of the channel expression by siRNA was not complete. In order to give further support to the results with siRNA-mediated downexpression, we have performed these experiments also on Jurkat double knock-out Bax/Bak-less cells. In these cells the transfection of siRNA targeting Kv1.3 has worked out more efficiently and indeed we observed an almost complete abolishment of the apoptotic effects of the three drugs upon downregulation of Kv1.3. This new data is shown in Fig. 7C.

2) Fig 5 is important to shed more light on the underlying events responsible for Kv1.3-dependent cell death. As such it seems clear from TMRM fluorescence that the mitochondraial membrane

potential may be affected by inhibiting this channel. However, the production of ROS species was only tested in Jurkat cells. It would be very advisable to measure if CTLL-2 versus Kv1.3 cells would also show a different level of ROS production. This would also be a good control that the membrane-permeable substances did not contain oxidative species that could produce a more aspecific cell death by oxidative stress (although in the Methods it was mentioned that precautions were taken to avoid this).

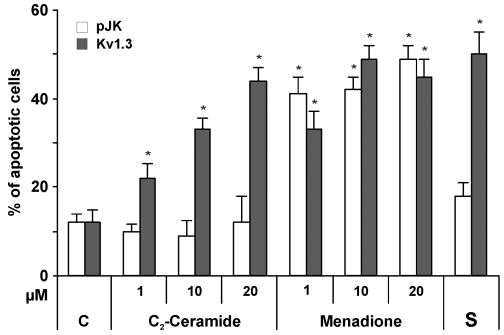
Thank you for calling our attention to the lack of this important data. We have now tested the effect of the drugs on mitochondrial ROS production in the CTLL-2 cells and included the new data in Fig. 5A. Further, we mentioned in the text that this experiment was used also as control, following your suggestion. These data confirm our previous results and show that ROS was only produced in CTLL-2/Kv1.3 cells upon various apoptotic stimuli (Gulbins et al, 2010).

3) The experiment with Bax/Bak-deficient Jurkat and MEF cells convincingly shows that cell death is induced by the Kv1.3 inhibitors in the absence of Bax and Bak. However, particularly for the MEF-DKO cells from the Scorrano lab, it was previously demonstrated that these cells may undergo an autophagic cell death in some conditions of UV-induced oxidative stress (Buytaert et al., Autophagy, 2006, 2, 238-240; Buytaert et al.,FASEB J. 20: 756-758). It is therefore possible that cell death is at least to some extent due to autophagic cell death. It is advisable to measure the level of autophagy (e.g. by assaying LC3-I to LC3-II conversion) in these Bax/Bak-deficient cells.

New experiments, by visualizing LC3 conversion in DKO MEFs suggest that indeed activation of autophagy occurred to some extent in this cell line upon treatment with the drugs (Fig S5B)(in accordance with data of Buytaert et al, in MEF DKO, LCII can be revealed already under control condition). On the other hand, DKO cells died to the same extent upon treatment with the drugs in the presence or absence of inhibitors of autophagy (Mizushima et al, Cell, 2010) (Fig. S5C), indicating that autophagy did not contribute importantly to the death process in our case. In this respect, it is interesting to mention that the paper by Shen et al of the Kroemer group (Shen et al, Autophagy, 2012,) reports that even though 1400 agents they tested increased autophagic flux, none of them induced autophagic cell death. Our data is briefly discussed and is shown in Fig. S5B and the above references are included into the revised manuscript.

4) In their discussion the authors speculate that the higher sensitivity of tumor cells for clofazimine may be related to increased formation of ROS species that may sensitize the tumor cells. Could it be that ROS species alone are already sufficient to inhibit the Kv1.3 channel and that the effect of clofazimine would be to catalyze such oxidation, rather than to provide a direct inhibition of the channel? Or stated otherwise: are cells containing mitochondrial Kv1.3 channels more sensitive to oxidative stress inducers (such as menadione) as compared to agents that act on mitochondrial Ca2+ overload (such as C2-ceramide)?

ROS are indeed able to inhibit Kv1.3 activity (Duprat et al, PNAS, 1995; Szabo et al, 1997, Eur. J. Physiol.), however clofazimine has been convincingly reported (and confirmed in our laboratories) to inhibit directly Kv1.3. CTLL-2/Kv1.3 and CTLL-2/pJK cells were equally susceptible to menadione (Fig. S2B of revised version), suggesting that the presence of the channel controls mitochondrial ROS production rather than determining the cell's susceptibility to ROS. Furthermore, our new data shown in Fig. 5A and mentioned above demonstrate that Kv1.3-deficient cells fail to produce ROS after clofazimine treatment, clearly indicating that ROS are downstream of Kv1.3.
Confirming previous data with C6-ceramide (Szabò et al, PNAS, 2008), C2-ceramide preferentially induced cell death in Kv1.3-containing cells (see below). It has already been shown that C2-ceramide inhibits Kv1.3 (Gulbins et al, PNAS 1997, Detre et al, Cell Signal, 2006) and presumably this is the reason for the selective death induction. At the moment we do not know whether C2-induced mitochondrial calcium overload takes place in both cell types. However, both menadione (Rimessi et al, PNAS, 2009) and C2-ceramide (Ferrari et al, BBRC 2010; Pinton et al, EMBO J, 2001) have been reported to use calcium as sensitizing co-factor and induce an initial small increase in mitochondrial calcium uptake followed by a complete release of calcium due to the opening of the permeability transition pore. Therefore, data with C2-ceramide have not been mentioned in the paper but are reported here below for the Reviewer.



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conditions, making it a very attractive candidate for further development) is influenced by levels of the mitochondrial Kv1.3 channel. Interestingly, i.p. administration of clofamizine causes death of an orthotopic model of melanoma that is known (and shown) to express high levels of Kv1.3, confirming that the approach is feasible also in vivo. In summary, this is a very interesting and well performed study that reports a novel therapeutic target to drive Bax, Bak independent killing of cancer cell. The following points must be addressed by the authors in order to strengthen certain aspects of their work.

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First of all we thank the Referee for the very constructive review. As to the first point, we apologize for not having written clearly enough that the CTLL-2 /pJK versus CTLL-2/Kv1.3 model was indeed used also in the present work, in order to correlate the observed effects of the membrane permeable drugs with the presence of the channel. In fact, the observation that these drugs do not act on CTLL-2 cells lacking Kv1.3 (but expressing the main apoptotic proteins like Bax, Bcl-xL, caspase-3 similarly to CTLL-2/Kv1.3), strongly indicates that these inhibitors do not kill the cells by a mechanism unrelated to Kv1.3. We've changed the text and put more emphasis on these experiments.

2. the experiments in Fig. 7 must be repeated following ablation of Kv1.3 to show that the restoration of the killing of Bax, BAk deficient cells depends on the channel.

We have repeated this experiment by downregulating the channel expression in Jurkat Bax/Bak-less cells and reported the results in Fig. 7 showing that indeed ablation of Kv1.3 efficiently prevented Psora-4, PAP-1 and clofazimine-induced death.

3. the in vivo orthotopic model must be better characterized. A non cell permeant Kv1.3 inhibitor must be compared to clofazimine.

We have performed a set of experiments using intravenously injected Margatoxin, a scorpion toxin which inhibits Kv1.3 with an IC_{50} of 110 pM (at higher concentrations it inhibits also other Kv shaker channels). MgTx in our in vitro experiments, used at an excess concentration of 1 μM, did not induce apoptosis as measured by Annexin binding and did not induce a significant reduction of cell viability within 12 hours in the MTT assays. The lack of apoptosis induction by MgTx was reported also in another study (Jang et al, 2011, Eur. J. Pharmacol. already cited in the ms.). However, MgTx has been shown to potently block proliferation of several cell types, including some tumoral ones. In accordance, intratumoral injection of 1 nM MgTx has recently been shown to slow down tumor growth in a lung adenocarcinoma model due to block of proliferation, but not to induction of apoptosis (Jang et al, 2011, Eur. J. Pharmacol.). In order not to overlook the possible effects of MgTx on tumor growth, we have intravenously injected even more MgTx, in particular 50-times more Kv1.3-inhibitory dose of MgTx with respect to clofazimine, taking into account the IC_{50} values (300 nM for clofazimine). At this very high concentration used, MgTx, when administered repeatedly (with the same treatment regimen as clofazimine), reduced tumor volume to $1.4 \pm 0.3\ cm^3$ (n=6). For comparison, clofazimine reduced tumor volume to $0.3 \pm 0.16\ cm^3$ (n=8). Thus, clofazimine reduces tumor volume to a larger extent than MgTx. However, please note that it is not easy to directly compare the effects of these drugs, because their pharmacokinetics probably differ significantly. For example, clofazimine is a lipophilic drug and as such, it is expected to reach the tumor at a relatively low effective concentration. In any case, clofazimine, by acting on proliferation (Ren et al, 2008, PLOS One) and in addition by inducing apoptosis very efficiently, is a more promising therapeutic target molecule with respect to non-permeant Kv1.3 inhibitors and, most importantly, is able to kill the cells also in the absence of Bax and Bak.

2nd Editorial Decision 13 February 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

The quality of the current figure images is a bit low (in the figures 4B, and 5C the bands appear blurry), could you please provide a higher resolution?
Figure 2B: when printed, it is quite difficult to differentiate the band from the background (precisely for Kv1.3), could you please decrease the background a little and maybe improve also the resolution?
Figures 4A and 6C, please provide size bars on your immunofluorescence images and correct the legends accordingly.

When providing higher resolution versions, please check to make sure that text/line-art remains clear even when zooming in. Please make sure that all labels will remain readable even if the overall figure is reduced. You may find that saving the images as EPS or PDF will better preserve the text and line-art resolution. If this does not help, you may need to remake the figures in a quality vector graphics program like Illustrator or the free opensource, alternative Inkscape.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

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Referee #1 (Other Remarks):

The authors have responded in an adequate way to the additional questions that were raised. In view of its novelty and convincing experimental evidence, I can recommend this manuscript for publication.

1) Downregulation of Kv1.3 with siRNA was now more succesfully obtained in Jurkat double-knockout (Bax/Bak-less) cells, and it was convincingly demonstrated that the apoptotic effect of 3 tested drugs entirely depended on the expression of Kv1.3 (new experimental data were added in Fig 7).

2) Important new data were included to shed more light on the underlying mechanism of cell death induced by Kv1.3 inhibitors (new data in Fig 5 concerning ROS production and mitochondrial membrane potential). It was demonstrated that ROS production was downstream of Kv1.3 inhibition

and the mechanism of these inhibitors is not due to a mere induction of oxidative stress. Their mechanism was thereby different from the effect of menadione.

3) A potential involvement of autophagy in these Bax/Bak-knockout cells was verified (new data in supplemental Fig 5). It was evident that while activation of autophagy occured in these cells, in agreement with other literature data, but autophagy was not an important factor in the cell death process as cells died to the same extent in the presence of autophagy inhibitors.

4) As to the question whether the mitochondrial Kv1.3 channel may have a role in sensitizing cells to oxidative stress, the authors have convincingly documented their point that ROS production per se was not the primary event in the action of the Kv1.3 inhibitors. Previous data with C2-ceramide also confirm this point in a convincing way.

Referee #3 (Other Remarks):

Authors fully addressed my relatively minor previous concerns. This is a very important paper that deserves to be brought to the attention of the readers of EMBO Molecular Medicine

