Subunit composition of VRAC channels determines substrate specificity and cellular resistance to Pt-based anti-cancer drugs


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1st Editorial Decision 13 August 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the slightly extended duration of the review process here. We had sent your manuscript to three referees and we have now received their reports (included below).

As you will see from the reports, all referees express interest in the findings reported in your manuscript, although they differ somewhat in their recommendations and evaluations of the data. While ref#2 is the more positive of the three and supports publication following minor revision both refs#1 and #3 find that a number of points would have to be addressed further to support the conclusions made. In addition, they both point out the need to strongly rewrite and reorganize the study.

In light of the slight discrepancy between the recommendations from the referees, I conducted a round of cross-referee commenting and received the following additional input from ref #1:

'Although my review may seem harsh, I am much closer to reviewer 2 than to reviewer 3. This is an important study with novel findings. Reviewer 3 should be more specific about what does not "hold together." Chloride channels in general are not highly selective and even the ones that do not transport cisplatin seem to have large pore diameters. If I understand reviewer 3's somewhat vague comment, I disagree that this is a major problem.'
Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. In addition, I would ask you to follow the recommendation by refs #1 and #3 to extensively rewrite the manuscript to increase clarity for the reader.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE COMMENTS

Referee #1:

This paper describes experiments aimed to show that cisplatin uptake into cells is partially mediated by VRAC channels composed of LRRC8A/LRRC8D channels that have a higher permeability to organic osmolytes than VRAC channels composed of other LRCC8 subunits. The demonstration that LRCC8D- cells have no taurine flux is an exciting discovery and the data supporting the notion that cisplatin uptake partly depends on VRACs composed of LRRC8D subunits are very intriguing.

Although the paper describes new, interesting, and potentially important observations, these observations are presented in a confusing way that does not make a coherent story. Certainly, the fields of VRAC and apoptosis are very complex and convoluted, but by raising the question of the role of VRAC and AVD in apoptosis, the authors diffuse the impact of the paper. I suggest extensive re-writing and reorganization to focus the paper on the basic observation that cisplatin uptake into cells is partially mediated by VRAC channels composed of LRRC8A/D subunits that have a higher permeability to organic osmolytes than VRAC channels composed of other LRCC8 subunits. Any mention of AVD and apoptosis could be reserved for the discussion.

Specific comments:

The involvement of VRAC and AVD in apoptosis was muddled. On Page 4 the authors say: "In conclusion, the increased drug resistance of LRRC8D- cells cannot be explained by the postulated role of ICl.vol and AVD in apoptosis". This sentence is strange by itself but it also is incongruous with the sentence on Page 7 that "the results obtained with staurosporine are compatible with the hypothesis that VRAC-dependent AVD facilitates the progression of apoptosis". The convoluted wording and organization of the paper makes it hard to understand exactly what point the authors are trying to make. Another example: on page 7, the statement that the time-dependent increase in drug uptake suggests that LRRC8 channels are slowly activated by cisplatin is used to introduce experiments to test if cisplatin induced caspase activation depends on LRRC8 subunits. The logic behind this organization is obscure. These issues can be solved by re-writing.

There are several cases where the conclusions are not clearly substantiated by the data.

(1) On page 5, the conclusion that isotonic cisplatin uptake is associated with a slow activation of VRAC does not follow from the data in this paragraph and its associated figure. Is it not possible that VRAC is activated quickly but cisplatin accumulation is slow? Later on Page 7, the authors test this notion by measuring the effect of cisplatin on iodide uptake into cells. But, why did the authors use an iodide uptake assay rather than taurine efflux which they showed was a unique property of the LRCC8/A/D channel. Also, the experiments lack a positive control of hypotonicity and controls showing that the slowly developing iodide uptake is not a result of cell morbidity.

(2) In Fig. 2C RVD is reduced in LRCC8D-. The authors conclude that the increased drug resistance of LRCC8D- cells cannot be explained by the role of AVD in apoptosis. If RVD and cisplatin uptake are both reduced by LRCC8D-, the conclusion that RVD and cisplatin uptake does not logically follow.

There are several instances where new questions raised by the data are left unresolved.

(1) The staurosporine experiments are baffling. If I understand correctly, cisplatin is transported by LRRC8, but staurosporin is not. Yet, the effect of both drugs on caspase activation are dependent on
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LRRC8A. This implies a dual role for these channels (cisplatin uptake and AVD?), but the authors do not clarify this issue.

(2) It seems odd that DMSO-cisplatin blocks VRAC currents dependent on LRRC8D, but cisplatin activates the same current. This result deserves more experimental attention.

It is not clear why the authors measure VRAC at -80mV when the outward current is so much larger. Because the inward current is relatively small it is subject to relatively more contamination by "background" currents. The authors should establish clearly that the inward currents are VRAC by ionic selectivity and pharmacology in addition to LRRC8A knockdown.

Is it possible to measure taurine currents? Taurine has a pK of 9, so at basic pH, there might be enough ionized taurine to measure a current.

The abstract reflects the lack of focus of the article: the following sentence stands out as an example: Not linked to an effect on drug uptake, and possibly related to a role of VRAC in apoptotic volume decrease, LRRC8A disruption suppressed staurosporine-induced apoptosis.

Referee #2:

In a previous study, the authors identified LRCC8 heteromers as essential components of the volume-regulated anion channels VRACs. In this study, the authors provide new data suggesting that subunit composition of VRACs determines substrate specificity, regulation and pharmacology. In particular, they propose that LRCC8D-containing channels form a pore for organic osmolytes (including taurine), an important property related to cell volume regulation and for tumor sensitivity to platinum-based anti-cancer drugs. This is a beautiful and convincing study that brings a lot of new information about VRACs. I have only one concern related to a technical issue. This study was mainly carried out using HEK cell lines lacking LRCC8 subunits. Disruption of LRCC8 expression was obtained using the CISPR-Cas9 method, which is known to produce off-target effects that may have functional consequences in other genes. This has not been tested nor even discussed by the authors. I would suggest to perform a few rescue experiments to show that heterologous re-expression of LRCC8 in the LRCC8-/-cell lines is sufficient to restore osmolyte transport (cisplatin, taurine).

Referee #3:

This manuscript describes a possible connection between the function of the newly discovered volume regulated anion channel (VRAC) and resistance to the cis-platin series of anti-tumor drugs. The authors describe the interesting finding that KMB7 cells that become resistant to cis-platin have defects in the LRRC8A and D subunits of VRAC. The authors then describe a series of experiments from which they conclude that such channels serve as transport conduits for cis-platin, which is not involved in making the chloride current that they then later show in Fig. 6 seems to be important for whatever it is that cis-platin does. All of the assays are indirect, and while the authors may indeed be correct that LRRC8A and 8D are important for the development of cisplatin resistance, the data presented do not support the very strong conclusion that VRAC makes the pathway for this drug to enter cells.

1st Revision - authors' response 05 October 2015

Detailed responses to the reviewers (Planells-Cases et al.)
In general, all three reviewers found our results interesting, with reviewer 1 and 2 being particularly positive. While reviewer 1 found our results exciting and intriguing, s/he stated that our presentation was somewhat convoluted and confusing and made several constructive suggestions for further experiments and re-wording. Reviewer 2 was enthusiastic and his/her only point concerned exclusion of off-target effects. Reviewer 3 still doubted the relevance of VRAC for the uptake of Pt drugs. In response to the specific comments of reviewers 1 and 2 we have performed several additional experiments and have extensively re-arranged and re-written our manuscript to increase the clarity of presentation. We also addressed the concern of reviewer 3 who was not convinced that VRAC transports cisplatin. We believe that the changes have greatly strengthened the conclusions of our manuscript and improved the clarity of presentation. We thank the reviewers for their time and valuable input.

Referee #1:

This paper describes experiments aimed to show that cisplatin uptake into cells is partially mediated by VRAC channels composed of LRRC8A/LRRC8D channels that have a higher permeability to organic osmolytes than VRAC channels composed of other LRCC8 subunits. The demonstration that LRCC8D- cells have no taurine flux is an exciting discovery and the data supporting the notion that cisplatin uptake partly depends on VRACs composed of LRCC8D subunits are very intriguing. Although the paper describes new, interesting, and potentially important observations, these observations are presented in a confusing way that does not make a coherent story. Certainly, the fields of VRAC and apoptosis are very complex and convoluted, but by raising the question of the role of VRAC and AVD in apoptosis, the authors diffuse the impact of the paper. I suggest extensive re-writing and reorganization to focus the paper on the basic observation that cisplatin uptake into cells is partially mediated by VRAC channels composed of LRRC8A/LRRC8D channels that have a higher permeability to organic osmolytes than VRAC channels composed of other LRCC8 subunits. Any mention of AVD and apoptosis could be reserved for the discussion.

We thank the reviewer for appreciating the novelty and importance of our paper and for her/his constructive comments. We agree that our manuscript, which addresses several different aspects of cellular cisplatin resistance, may have been confusing. We have now extensively re-written and re-organized the manuscript and hope that this has substantially increased its clarity. Concerning his/her point as to the inclusion of apoptosis/AVD, however, we politely disagree. We very much like to retain the experiments exploring the role of LRRC8 channels in the progression of apoptosis. Indeed, we feel that a paper describing cellular resistance to cisplatin/carboplatin without exploring the role of this channel in apoptosis would be truly incomplete. There is a vast literature on the role of chloride channels, in particular VRAC, in apoptosis. The resistance of cells to cisplatin and other drugs was previously attributed (by Yasunobu Okada, Else Hoffmann and others) to a role of VRAC in apoptotic volume decrease which is believed to be a prerequisite, or at least a facilitating factor, for the progression of apoptosis and cell death. After the identification of LRRC8 heteromers as VRAC components, it is now possible to rigorously test this notion and to clarify whether the same channel is responsible for regulatory volume decrease (RVD) and apoptotic volume decrease (AVD)-dependent progression of apoptosis. Indeed, a recent review (Kunzelmann, TIBS 40: 535-543 (2015)) states that it is imperative to answer the question whether the same channel (LRRC8 heteromers) is responsible for RVD and AVD-dependent progression of apoptosis.

We have now answered this question by showing that indeed LRRC8 channels are activated by pro-apoptotic stimuli (as requested by this reviewer now additionally shown with taurine efflux) and are important for the induction of apoptosis. This conclusion is particularly supported by our experiments with staurosporine. Hence, our data provide strong evidence that both RVD and AVD depend on LRRC8 channels. This is an important point which we now stress more in Discussion, third paragraph, on page 10, last paragraph.

For cisplatin/carboplatin, there was the important, unforeseen twist that VRAC/LRRC8 heteromers (mostly A/D) provide a major pathway for the uptake of these drugs. Thus, for cisplatin, VRAC plays a dual role in mediating drug uptake and resistance to apoptosis, probably by facilitating AVD (see also below).

Following the suggestion of the reviewers and the Editor, we have now changed the order of presentation. We begin with the genomic screen for drug resistance and clinical data; then try to interpret these data in the light of the VRAC-AVD-apoptosis hypothesis prevalent in the VRAC field; this hypothesis is partially confirmed experimentally, but the role of LRRC8D in cisplatin resistance
does not fit. This leads us to check for a role of VRAC in drug uptake, which we investigate in depth; finally, we extend these findings to the role of LRRC8 subunits in taurine transport.

In the discussion, we now devote a new paragraph to the VRAC-AVD-apoptosis hypothesis, then proceed with cisplatin uptake, clinical implications, and inferences for the VRAC pore. We believe that this re-ordering and re-writing makes our manuscript, which encompasses data relating to many different fields, transparent and easy to understand. We hope the reviewers agree.

Specific comments: The involvement of VRAC and AVD in apoptosis was muddled. On Page 4 the authors say: "In conclusion, the increased drug resistance of LRRC8D- cells cannot be explained by the postulated role of ICl, vol and AVD in apoptosis". This sentence is strange by itself but it also is incongruous with the sentence on Page 7 that "the results obtained with staurosporine are compatible with the hypothesis that VRAC-dependent AVD facilitates the progression of apoptosis". The convoluted wording and organization of the paper makes it hard to understand exactly what point the authors are trying to make.

We now realize that our presentation was confusing, and thank the reviewer for his/her comments. The increased resistance of haploid cells to cisplatin upon LRRC8A disruption seemed consistent with the hypothesis that VRAC-dependent AVD facilitates apoptotic cell death, a notion pioneered by Okada and others. However, the cisplatin resistance observed with LRRC8D disruption did not fit into this scheme, as we knew from our previous studies that D is not needed for VRAC currents, which we confirmed in this work for haploid cells. Our RVD measurements then showed that RVD of LRRC8D-/ cells was decreased, but not abolished.

Our statement ‘In conclusion, the increased drug resistance of LRRC8D- cells cannot be explained by the postulated role of ICl, vol and AVD in apoptosis’ is indeed oversimplified and unfortunate. It would have been correct only if RVD (and by extension probably AVD) would have been unchanged (like in WT). We have therefore replaced this statement by: ‘Hence it seems unlikely that LRRC8D disruption protects cells against cisplatin toxicity by impairing VRAC- and AVD-dependent apoptosis’ (page 5, last sentence).

We then discovered a role of VRAC in cisplatin uptake, which, of course, does not exclude an additional (downstream) role of the VRAC-AVD-apoptosis mechanism in cell death. For staurosporine, which is most likely not taken up through VRAC, LRRC8A (but not LRRC8D) disruption significantly reduced the activation of caspase 3 – hence VRAC currents, which depend on LRRC8A but not on LRRC8D, indeed seem to facilitate apoptosis as suggested by Okada and others. We now state clearly both in the Abstract, Result and Discussion sections that LRRC8 channels may play dual, independent roles in mediating drug uptake and in facilitating apoptosis (possibly through AVD). Again, we hope that the restructuring of our manuscript helps.

Another example: on page 7, the statement that the time-dependent increase in drug uptake suggests that LRRC8 channels are slowly activated by cisplatin is used to introduce experiments to test if cisplatin induced caspase activation depends on LRRC8 subunits. The logic behind this organization is obscure. These issues can be solved by re-writing.

As stated above, we have now re-arranged the manuscript which, we hope, results in a more logical flow of data and arguments. We begin by testing the role of LRRC8 channels in apoptosis, and can then refer in the cisplatin uptake section to the already described activation of the channels by cisplatin.

There are several cases where the conclusions are not clearly substantiated by the data. (1) On page 5, the conclusion that isotonic cisplatin uptake is associated with a slow activation of VRAC does not follow from the data in this paragraph and its associated figure. Is it not possible that VRAC is activated quickly but cisplatin accumulation is slow?

When comparing cisplatin influx from isotonic or hypotonic saline, (old Fig. 3D,F (now Fig 6D,F), old Suppl. Fig. S4B (now Fig EV4B)), we found no significant differences in isotonic 15 min or 1 hour cisplatin uptake between the genotypes. When we followed cisplatin uptake over longer times we saw a variable increase in LRRC8-dependent uptake over time. (In old Fig. 3C, now Fig. 6C (uptake with 40 µM cisplatin), there was no difference between the genotypes at 4h, first differences
have been observed at 8 hours; in old Fig. 3E, now Fig. 6E (uptake of 200 µM cisplatin), tendency of difference at 4 h, significant difference at 8h; and also in the Fig. EV4A and the new Fig. EV5; as stated in the paper, the time course varied between experiments for reasons we do not understand. VRAC regulation in general is complex and poorly understood, as summarized in many reviews.)

As cited in our discussion, previous work by others has described a time lag for the activation of VRAC currents or AVD by pro-apoptotic stimuli like staurosporine and cisplatin that ranged between 20 min to 4 hours. This agrees with the increase in the LRRC8-dependent cisplatin uptake component in our experiments. Our experiments exploring cisplatin-induced iodide influx (old Fig. 7A-C, new Fig. 5A-C,E) and the new experiments concerning cisplatin-induced taurine efflux (new Fig. 8F) show a similar time course of VRAC activation and demonstrate for the first time that the cisplatin- or staurosporine-activated channel is a LRRC8 channel, just like the channel involved in RVD. This important conclusion is now clearly stated in Discussion, page 10, last paragraph.

Cisplatin accumulation is indeed slow, as indicated by the fact that cisplatin accumulation did not reach steady-state even after 24 hours. However, if VRAC would be active from the beginning, we would detect a LRRC8-dependent uptake component also in the isotonic uptake experiments of Fig. 6D,F (previously 3D,F). In these experiments, we do see LRRC8-independent uptake above background (which probably occurs by passive diffusion), with cisplatin accumulation at 1 hour being roughly 4 times larger than at 15 min.

Coming back to the suggestion of the reviewer that VRAC may be activated quickly, but that cisplatin accumulation is slow: Is this meant to suggest that maybe cisplatin enters, at constant rates from the beginning (channel open from beginning, intracellular concentration far from equilibrium), but that some other transport process extrudes cisplatin initially, but not at later times? Such a mechanism could, in principle, explain a time lag in accumulation, but we have no indication for such a mechanism (and it seems difficult to explain why such an extruder should be active initially, and inactive at longer time points (a couple of hours)). By contrast, our experiments and the scientific literature suggests that VRAC can be activated with an appropriate time course by pro-apoptotic stimuli.

We believe that the re-arrangement of the manuscript, with the section on apoptosis and VRAC-activation by pro-apoptotic stimuli now moved before the uptake experiments, results in a much more logical sequence of arguments.

Later on Page 7, the authors test this notion by measuring the effect of cisplatin on Iodide uptake into cells. But, why did the authors use an Iodide uptake assay rather than taurine efflux which they showed was a unique property of the LRRC8A/D channel?

We performed this experiment to test the published notion that VRAC anion currents are activated in the course of apoptosis induction. Therefore, iodide flux (as substitute for chloride) seemed better suited than taurine (or cisplatin). By moving this experiment (now Fig. 5) directly after the caspase-induction experiments the logical connection should be clear.

Nonetheless, following the suggestion of this reviewer, we have performed new experiments on cisplatin-induced taurine efflux (new Fig. 8F). Indeed, like iodide influx, also taurine efflux is stimulated by preincubation with cisplatin and depends on LRRC8A. Also the time course of activation was similar. Importantly, cisplatin-induced taurine efflux was not due to a crude leak owed to cell morbidity for two reasons: (1) Leaky cells should have largely lost their radioactive intracellular taurine during the five washes before the start of the 30 min efflux period. (2) Taurine efflux could be blocked by the VRAC-inhibitor carbenoxolone. This is mentioned in Results on page 9.

Also, the experiments lack a positive control of hypotonicity and controls showing that the slowly developing Iodide uptake is not a result of cell morbidity.

We have added a trace showing swelling-induced YFP quenching, similar to those published previously (Voss et al., 2014; this was the basis for our screening assay that identified LRRC8A) (new Fig. 5D). The rate of hypotonicity-induced quenching is higher than the one induced by cisplatin because VRAC is opened much more (this also applies for taurine fluxes: Higher efflux rates with swelling-activation than with cisplatin-stimulation in our new experiments (new Fig. 8F). However, as mentioned on page 6, second paragraph, comparing the slopes of quenching (between cisplatin- and tonicity-stimulation) severely underestimates hypotonicity-induced VRAC opening because hypotonic medium, in contrast to cisplatin (with which cells are pre-incubated), is added.
acutely – hence the slope also reflects the slow kinetic of cell swelling and subsequent VRAC opening (see current traces in new Appendix Fig S2A,E). The small degree of LRRC8 channel activation by cisplatin fits well to the low VRAC-like currents observed by others (e.g. Shimizu...Okada, PNAS 2004) upon stimulation by pro-apoptotic drugs. We do not think that non-specific leaks caused by 'cell morbidity' significantly contaminated our YFP-quenching curves. We follow the kinetics of I-induced YFP quenching over several minutes. Large, non-specific leaks in the plasma membrane should lead to quenching that is much faster than the one caused by channel-mediated I-influx (as we have verified in our primary screen for VRAC (Voss et al.) where we added, as control, TritonX100 to permeabilize cells). With a significant proportion of leaky cells, we would expect a fast quenching component (representing leaky cells), followed by a slow quenching as observed in (new) Fig. 5A-C. Such a fast quenching component has not been observed. We have now added two sentences in the legend to Fig. 5: 'Note that increased YFP quenching with cisplatin preincubation is not due to large non-specific leaks as a consequence of cell morbidity. Such leaks should lead to a fast component of YFP quenching.' We have also added an additional original trace of iodide quenching to Fig. 5 (S5C) that shows that even at 8.5 hours there is no fast component of YFP quenching – hence, even at this longer time, no unspecific, morbidity-related leak.

(2) In Fig. 2C RVD is reduced in LRCC8D-. The authors conclude that the increased drug resistance of LRCC8D- cells cannot be explained by the role of AVD in apoptosis. If RVD and cisplatin uptake are both reduced by LRCC8D-, the conclusion that RVD and cisplatin uptake does not logically follow.

We have addressed this point already above. Indeed, our conclusion that loss of AVD cannot be invoked was not warranted in the strict sense, as RVD was only reduced, but not abolished. We have made the appropriate changes mentioned above and now clearly state in discussion that LRRC8 heteromers may have a dual role in (1) cisplatin uptake, and (2) in VRAC/AVD-dependent apoptosis. This second mechanism may be more important with a loss of LRRC8A, because LRRC8D-/ cells have unchanged ICl,swell and retain, albeit reduced, RVD.

There are several instances where new questions raised by the data are left unresolved. (1) The staurosporine experiments are baffling. If I understand correctly, cisplatin is transported by LRRC8, but staurosporin is not. Yet, the effect of both drugs on caspase activation are dependent on LRRC8A. This implies a dual role for these channels (cisplatin uptake and AVD?), but the authors do not clarify this issue.

This again relates to the points discussed above. The staurosporine experiments indeed support the role of VRAC/AVD in apoptosis as suggested by others. Therefore the inclusion of these experiments is so important: they highlight the dual role of VRAC. As stated above, this is now discussed in more detail.

(2) It seems odd that DMSO-cisplatin blocks VRAC currents dependent on LRRC8D, but cisplatin activates the same current. This result deserves more experimental attention.

Most likely, the activating action of cisplatin and the blocking action of cisplatin-DMSO adducts occur through very different mechanisms, which we cannot elucidate at this point. Activation by cisplatin, which takes up to several hours in the present case (Fig. 5E), most likely shares much of the pathways by which staurosporine and other pro-apoptotic drugs activate VRAC. This pathway is not known in detail, but several mechanisms (like ROS production, as now mentioned and cited on page 12, 3rd paragraph) have been invoked. By contrast, block of VRAC currents by cisplatin-DMSO adducts occurred within minutes. We originally thought that this larger derivate of a permeant species may block the pore, but could not obtain electrophysiological evidence for a classical open-pore block, as we had hoped. Although the mechanism of block remains unknown, we believe that these data are important as they show that different heteromers (in this case those containing D) may be targeted specifically by pharmacologic means. This may have broad implications if, e.g., blockers specifically blocking glutamate-conducting heteromers can be found (they might e.g. be useful for diminishing glutamate toxicity in stroke, without abolishing cell volume regulation altogether).

It is not clear why the authors measure VRAC at -80mV when the outward current is so much...
larger. Because the inward current is relatively small it is subject to relatively more contamination by "background" currents. The authors should establish clearly that the inward currents are VRAC by ionic selectivity and pharmacology in addition to LRRC8A knockdown.

Like many other studies investigating VRAC (e.g. Nilius.. Droogmans, J. Gen. Physiol. 103: 787-805 (1994); Meyer & Korbmacher, J. Gen. Physiol. 108: 177-193 (1996); Voets...Nilius, Br. J. Pharmacol. 118: 1867-1871 (1996)), we evaluated currents at negative potentials as they can be measured more reliably than currents at positive voltages which may be affected by voltage-dependent channel inactivation. Even in the negative voltage range, current amplitudes are well separated from background.

Nonetheless, as other readers may have the same doubts as the reviewer, we have now replaced the bar diagrams in former Fig. 2 (new Figure 3) by averaged I/V curves that show the currents over the entire voltage range. Swelling-activation (now directly shown in Appendix Fig S2) and LRRC8A-dependence already identify the KBM7 and HAP1 currents shown in Fig. 3 as being mediated by VRAC/VSOAC. We have now confirmed their I-\rightarrow Cl- selectivity and show their inhibition by the VRAC inhibitor DCPIB in Appendix Fig S2. These results are now mentioned in the legend to Fig. 3. 'Consistent with being VRAC currents they needed hypotonic swelling for activation, displayed an I- \rightarrow Cl- permeability sequence, and were blocked by DCPIB.' We refer to Appendix Fig S2 also in the main text (page 5, 2nd paragraph).

Is it possible to measure taurine currents? Taurine has a pK of 9, so at basic pH, there might be enough ionized taurine to measure a current.

In principle, this can be done, as shown e.g, by Kevin Strange in the past. However, we are afraid of possible changes in VRAC transport activities by the required strongly alkaline pH. Moreover, we consider these experiments beyond the scope of this manuscript which already contains a lot of different data and touches upon many different areas of biophysics, biology, and medicine.

The abstract reflects the lack of focus of the article: the following sentence stands out as an example: Not linked to an effect on drug uptake, and possibly related to a role of VRAC in apoptotic volume decrease, LRRC8A disruption suppressed staurosporine-induced apoptosis.

Thank you for pointing this out. We have re-written the abstract and believe that it now summarizes the main points of the paper in a succinct and clear manner.

Referee #2:

In a previous study, the authors identified LRCC8 heteromers as essential components of the volume-regulated anion channels VRACs. In this study, the authors provide new data suggesting that subunit composition of VRACs determines substrate specificity, regulation and pharmacology. In particular, they propose that LRCC8D-containing channels form a pore for organic osmolytes (including taurine), an important property related to cell volume regulation and for tumor sensitivity to platinum-based anti-cancer drugs. This is a beautiful and convincing study that brings a lot of new information about VRACs. I have only one concern related to a technical issue. This study was mainly carried out using HEK cell lines lacking LRCC8 subunits. Disruption of LRCC8 expression was obtained using the CISPR-Cas9 method, which is known to produce off-target effects that may have functional consequences in other genes. This has not been tested nor even discussed by the authors. I would suggest to perform a few rescue experiments to show that heterologous re-expression of LRCC8 in the LRCC8-/- cell lines is sufficient to restore osmolyte transport (cisplatin, taurine).

We thank the reviewer for appreciating the novelty and quality of our study. We also thank the referee for putting forward the risk of off-target effects. We are very well aware of these, but we strongly believe that off-target effects can be neglected as underlying cause of our findings. We have taken care to study several different cell lines (KBM7, HAP1, HEK and HCT116) and saw qualitative similar effects in all these lines. Furthermore, the initial finding of LRRC8A and LRRC8D came out of an insertional mutagenesis screen using haploid cells. The setup of this screen is fundamentally different and there is no involvement of CRISPR/Cas9. Moreover, the risk of off-target effects is minimized by the fact that a significant number of random integrations (P<0.001) affecting LRRC8A or LRRC8D is enriched in the resistant cells (Fig. 1B). For the subsequent
validation, the disruption of LRRC8D was done differently in HAP1 and KBM7 cells (KBM7 clones were from the random insertional mutagenesis study of Lee et al., whereas CRISPR-Cas9 was used for HAP1 cells). The sgRNA used to target LRRC8D in the HAP1 cells differed also from those used in CRISPR-Cas9 disruption in HEK and HCTT16 cells, which further reduces the risk of off-target effects. Moreover, we used different CRISPR-Cas9 constructs (which should have different off-target effects, if any) to construct two independent LRRC8A (one of them newly generated, see Figure R1 for the reviewers below) and LRRC8D HEK KO cell lines, with similar transport properties. Importantly, our results with LRRC8D-/- cells (which showed that LRRC8D is important for taurine and cisplatin transport) matches perfectly with our results for LRRC8(B,C,E)-/- cells (A/D left) that show efficient taurine and cisplatin transport, whereas LRRC8(B,D,E)-/- (A/C left) or LRRC8(B,C,D)-/- cells (A/E left; newly added Fig. EV5) showed little transport. This virtually excludes off-target effects on those results. We now mention this point shortly on page 11 (2nd paragraph) in discussing the evidence for VRAC mediating cisplatin transport.

Nonetheless, we have followed the suggestion of this reviewer and have performed a rescue experiment. Such experiments are problematic with VRAC because its transport properties strongly depend on the subunit composition of the (probably hexameric) LRRC8 heteromer. We have shown previously (Voss et al., 2014) that overexpression of LRRC8A in WT HCTT16 cells strongly reduced VRAC currents, probably because of the formation of transport-incompetent heteromers containing too many LRRC8A subunits (a similar suppression of currents by overexpression was mentioned by Qiu et al. 2014).

In new experiments we partially rescued the swelling-induced taurine efflux of LRRC8D-/- HEK cells by transient overexpression of LRRC8D (new Figure 8B). LRRC8D is notoriously difficult to express heterologously (Voss et al., 2014), but nonetheless taurine efflux was increased to about twice the levels that had remained in LRRC8D-/- cells. We believe to have excluded a distortion of our results by off-target effects beyond reasonable doubt, and hope that the reviewer agrees.

Referee #3:

This manuscript describes a possible connection between the function of the newly discovered volume regulated anion channel (VRAC) and resistance to the cis-platin series of anti-tumor drugs. The authors describe the interesting finding that KMB7 cells that become resistant to cis-platin have defects in the LRRC8A and D subunits of VRAC. The authors then describe a series of experiments from which they conclude that such channels serve as transport conduits for cis-platin and the related compound carboplatin, but not oxaliplatin. These are all extremely similar drugs and the basic contention that the authors make regarding VRAC as the main entry pathway does not hold together when one considers that the pore is highly selective for halide ions and is proposed to also pass blasticidin (which is has a nearly 3x larger radius). The authors show that one of the subunits they identify, LRRC8D is not involved in making the chloride current that they then later show in Fig. 6 seems to be important for whatever it is that cis-platin does. All of the assays are indirect, and while the authors may indeed be correct that LRRC8A and 8D are important for the development of cisplatin resistance, the data presented do not support the very strong conclusion that VRAC makes the pathway for this drug to enter cells.

We thank referee 3 for this concern. However, we think that the evidence that VRAC is involved in the uptake of cis- and carboplatin is unambiguous. We directly measured uptake into cells expressing, or lacking, VRAC subunits: uptake could be stimulated by the activation of VRAC by hypotonic swelling and inhibited by the VRAC blocker carbenoxolone. We used four different cell lines (HCTT16, HEK, HAP1, KBM7) and indeed many different KO cell lines, excluding off-target effects as stated above. In addition, we performed a new rescue experiment. We think that this provides a strong basis for our conclusion.

The reviewer seems also puzzled by the fact that VRAC is both selective for small anions over cations, but also conducts large organic substance as shown here. We agree that this is unexpected, and we do not understand the molecular basis in detail (but see below). Somewhat similar findings were reported for pannexins, hexameric plasma membrane channels that display sequence homology to LRRC8 proteins (Abascal and Zardoya, as cited in our ms.). Pannexin 1 may form anion-selective channels (Ma et al., Pfügers Archiv 463: 585-592 (2012)), but also conducts large organic molecules including ATP (for recent review, see Bond and Naus, Frontiers Physiol. 5: 58 (2014)). Moreover, it is known that other transporters can distinguish between different Pt-based
drugs: for instance, the OCT2 transporter, which is expressed in the renal proximal tubule, transports cisplatin, but not carboplatin – explaining the larger nephrotoxicity of cisplatin compared to carboplatin.

Most importantly, a crucial point of our paper is the finding that different subunit compositions of LRRC8 heteromers yield channels with different substrate selectivities. We have shown, for instance, that A/D heteromers have a much larger cisplatin/Cl transport ratio than A/C heteromers. Similar findings were made for taurine. Hence, a major conclusion of our paper is that this ‘puzzle’ may be solved by the presence of not only one VRAC, but several VRACs that display different pore properties. This is discussed in the second-last paragraph of Discussion (page 14).

Figure R1 for the reviewers
LRRC8A-dependent cisplatin transport in two independent LRRC8A clones as compared to wild type HEK cells.

Long term cisplatin (200 µM) uptake as a function of time of two LRRC8A knockout clones generated in HEK cells using different CRISPR-Cas9 sgRNAs to control for off-target effects. LRRC8A/- (A1.13), one of the clones used in the main body of the manuscript, was generated using the guide sgRNA sequence: 5´ TGATGATTGCCGTCTTCGGGGGG 3´ (Voss et al., 2014). The newly generated LRRC8A/- (Z65) used the sgRNA: 5´ TCCTGCAATGATTCGTTCCGGGG 3´. Both clones LRRC8A/- (A1.13) and LRRC8A/- (Z65) contain premature LRRC8A truncations in or just after the first transmembrane domain (TMD1) of the protein, respectively. The disruption of LRRC8A was confirmed by Western blot analysis. n=4; error bars, SEM. ** p<0.01; *** p<0.001.

2nd Editorial Decision 05 October 2015

Thank you for submitting the revised version of your manuscript to The EMBO Journal. It has now already been seen by two of the original referees and their comments are included below.

As you will see they both find that all criticisms have been sufficiently addressed and I am therefore happy to inform you that your manuscript has been accepted for publication in The EMBO Journal.

Referee #1:
I commend the authors for their thorough and detailed responses to my comments. The paper is greatly improved and represents an important contribution to the field.

Referee #2:
The authors have addressed my earlier comments in a satisfactory manner.