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

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Abstract

Although platinum-based drugs are widely used chemotherapeutics for cancer treatment, the determinants of tumor cell responsiveness remain poorly understood. We show that the loss of subunits LRRC8A and LRRC8D of the heteromeric LRRC8 volume-regulated anion channels (VRACs) increased resistance to clinically relevant cisplatin/carboplatin concentrations. Under isotonic conditions, about 50% of cisplatin uptake depended on LRRC8A and LRRC8D, but neither on LRRC8C nor on LRRC8. Cell swelling strongly enhanced LRRC8-dependent cisplatin uptake, bolstering the notion that cisplatin enters cells through VRAC. LRRC8A disruption also suppressed drug-induced apoptosis independently from drug uptake, possibly by impairing VRAC-dependent apoptotic cell volume decrease. Hence, by mediating cisplatin uptake and facilitating apoptosis, VRAC plays a dual role in the cellular drug response. Incorporation of the LRRC8D subunit into VRAC substantially increased its permeability for cisplatin and the cellular osmolyte taurine, indicating that LRRC8 proteins form the channel pore. Our work suggests that LRRC8D-containing VRACs are crucial for cell volume regulation by an important organic osmolyte and may influence cisplatin/carboplatin responsiveness of tumors.

Keywords chloride channel; haploid cell screen; swelling-activated; VSOAC; VSOR

Subject Categories Autophagy & Cell Death; Cancer; Membrane & Intracellular Transport

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Introduction

The platinum-containing drugs cisplatin, carboplatin, and oxaliplatin are among the most successful drugs used for treating cancer (Kelland, 2007). Pt drugs introduce covalent adducts in DNA which eventually cause cell death. Tumor cells treated with therapeutic doses of Pt drugs probably die predominantly by mitotic catastrophe/necrosis (Borst et al, 2001; Brown & Attardi, 2005). Attempts to link Pt drug sensitivity to apoptosis remain prominent (Speirs et al, 2011), however, even though the induction of apoptosis by high drug concentrations has been shown to be due to a cytoplasmic off-target effect of Pt drugs (Mandic et al, 2003; Berndtsson et al, 2007; Fayad et al, 2009).

In cultured cells, the induction of apoptosis by cisplatin and other drugs such as staurosporine is characterized by an early cell shrinkage denominated apoptotic volume decrease (AVD) which is followed by other apoptotic hallmarks like the activation of caspases and DNA fragmentation. AVD and the induction of apoptosis could be inhibited by blockers of the ubiquitously expressed volume-regulated anion channel VRAC (also known as VSOR or VSOAC) (Maeno et al, 2000; Ise et al, 2005; Okada et al, 2006; Lang & Hoffmann, 2012). Being closed under resting conditions, VRAC opens upon cell swelling and releases chloride and organic osmolytes in the course of regulatory volume decrease (RVD). The effect of VRAC blockers on drug-induced apoptosis was attributed to a loss of VRAC-dependent AVD (Maeno et al, 2000). However, the blockers used to substantiate a role of VRAC in apoptosis are all non-specific, and the notion that AVD facilitates apoptosis is controversial (Orlov et al, 2013). VRAC has been characterized biophysically and physiologically for decades, but the failure to identify the underlying proteins (Okada, 1997; Pedersen et al, 2015)
precluded conclusive genetic and biochemical studies. Only recently, LRRC8 heteromers were identified as essential VRAC components. LRRC8A is an obligatory VRAC subunit (Qiu et al., 2014; Voss et al., 2014), but it needs at least one other LRRC8 homolog (LRRC8B, LRRC8C, LRRC8D, or LRRC8E) to mediate volume-activated anion currents (I_{Cl,vol}) or to release organic osmolytes like taurine (Voss et al., 2014). A weak homology to pannexins suggests that LRRC8 proteins may assemble to hexameric channels (Abascal & Zardoya, 2012). The formation of different LRRC8 heteromers indicates that there are several different VRACs that may vary in properties and tissue distribution. For instance, the subunit composition of LRRC8 heteromers might specify the channel's preference for chloride over taurine or vice versa. Such a scenario would support the contentious notion (Lambert & Hoffmann, 1994; Stutzin et al., 1999; Shennan, 2008) that VRAC is molecularly distinct from a postulated volume-stimulated organic osmolyte/anion channel VSOAC (Jackson & Strange, 1993). However, the impact of specific LRRC8 subunits on VRAC’s selectivity has not yet been investigated.

An unbiased genomic screen now revealed that the loss of either of two VRAC subunits, LRRC8A or LRRC8D, increased resistance against carboplatin and cisplatin. This finding can be largely explained by an unsuspected role of VRAC in drug transport. This transport required the obligatory channel subunit LRRC8A and also depended on LRRC8D, a subunit that strongly increased VRAC’s permeability to cisplatin/carboplatin. Exposure to staurosporine revealed an additional, drug uptake-independent, but possibly AVD-related effect of LRRC8A on drug-induced apoptosis. Moreover, LRRC8D increased VRAC’s permeability to taurine, and cells lacking this subunit showed reduced cell volume regulation. Our work uncovers a novel role of VRAC in cisplatin and carboplatin uptake and resistance, identifies LRRC8A/LRRC8D-containing VRACs as physiologically important organic osmolyte channels, and reveals differences in substrate selectivity and pharmacology between differently composed VRACs.

**Results**

A genomewide screen identifies LRRC8A and LRRC8D as mediators of carboplatin and cisplatin resistance

To identify genes affecting platinum drug sensitivity, we performed genomewide loss-of-function screens of haploid KBM7 cells that were subjected to insertional mutagenesis using a gene-trap virus (Carette et al., 2009) (Fig 1A). Cells resistant exposure to 7 μM carboplatin for three weeks showed a significant enrichment of insertions in the LRRC8D and LRRC8A genes (Fig 1B and Appendix Table S1). Most sense insertions were localized in introns upstream of the ORF-containing exon 3 (Fig 1C). These positions are consistent with gene-inactivating mutations. Two LRRC8D-deficient KBM7 clones, which display reduced blasticidin S uptake (Lee et al., 2014) and which lack the LRRC8D protein (Fig EV1), were indeed more resistant to both carboplatin and cisplatin, but not to the larger compound oxaliplatin (Fig 1D and Appendix Table S2). Likewise, KBM7-derived haploid HAP1 cells (Carette et al., 2011) were resistant to carboplatin and cisplatin, but not to oxaliplatin, when LRRC8A or LRRC8D was disrupted (Fig EV2). Hence, two genes, LRRC8A and LRRC8D, affected carboplatin and cisplatin sensitivity at drug concentrations that can be achieved in patients.

**Low LRRC8D expression correlates with reduced survival of Pt drug-treated ovarian cancer patients**

To determine whether LRRC8A or LRRC8D expression might affect chemotherapy in patients, we examined The Cancer Genome Atlas (TCGA) data collection of ovarian cancer patients who were treated with platinum drugs. We analyzed the survival of patients with a low tumor expression of LRRC8A or LRRC8D versus the remaining patients. We used the lower tertile of the distribution of LRRC8A and LRRC8D expressions as cutoff. Whereas low LRRC8A expression had no influence on survival (Fig 2A), patients with a low LRRC8D gene expression in their ovarian cancers displayed a significantly reduced survival (Fig 2B). Most patients had also received taxane, but disruption of LRRC8A or LRRC8D did not provide resistance against docetaxel (Appendix Fig S1). To corroborate these results, we investigated the data derived from ovarian cancer patients that were recently published by Patch et al. (2015). Although the available data are derived from fewer patients, also in this analysis a low expression of LRRC8D, but not LRRC8A, correlated with a modest, but significant decrease in survival (Fig 2C and D). Thus, LRRC8D might also affect platinum drug responses in cancer patients.

**Pt drug resistance is not strictly correlated with lack of VRAC currents or volume regulation**

LRRC8A and LRRC8D are subunits of the volume-regulated anion channel VRAC. The finding that the loss of LRRC8D conferred drug resistance was surprising because this subunit, unlike LRRC8A, is dispensable for VRAC Cl\(^-\) currents (I_{Cl,vol}) in HCT116 cells (Voss et al., 2014), a finding we now confirmed for haploid HAP1 and KBM7 cells (Fig 3A and B, Appendix Fig S2). Hence, Pt drug resistance is not correlated with a loss of VRAC Cl\(^-\) channel activity.

In view of the postulated role of VRAC activation in cisplatin-induced cell shrinkage and subsequent apoptotic cell death (Maeno et al., 2000; Okada et al., 2006; Lang & Hoffmann, 2012), we explored the role of LRRC8A and LRRC8D in regulatory volume decrease (RVD) that can be determined more reliably than AVD. Like I_{Cl,vol} (Voss et al., 2014), RVD depended on LRRC8 heteromers because it was similarly impaired in LRRC8\(^{+/−}\) and LRRC8B(C,D,E)\(^{-}\) cells that lack LRRC8 isoforms B through E (Fig 3C). In LRRC8D\(^{-}\) cells, notwithstanding unchanged I_{Cl,vol} amplitudes (Fig 3A and B; Voss et al., 2014), RVD was significantly reduced but not abolished (Fig 3C). Hence, it seems unlikely that LRRC8D disruption protects cells against cisplatin toxicity by impairing VRAC- and AVD-dependent apoptosis.

**LRRC8-dependent induction of apoptosis by cisplatin and staurosporine**

We next measured drug-induced activation of caspase-3 to test whether the induction of apoptosis by staurosporine or high concentrations of cisplatin depends on LRRC8 subunits. Cisplatin-induced caspase activation was indeed suppressed in LRRC8A\(^{+/−}\) and LRRC8D\(^{-}\) cells (Fig EV3). These findings are consistent with the idea that LRRC8D may be involved in the induction of cisplatin-induced apoptosis in human ovarian cancer cells.
LRRC8D−/− and LRRC8−/− cells that lack all LRRC8 subunits (Fig 4A). To explore whether activation of VRAC by hypotonic swelling facilitated cisplatin-induced apoptosis, we exposed cells during 1 h to 200 μM cisplatin in hypotonic medium (−25%) and measured caspase activity after 1 to 3 days. This procedure drastically increased caspase activation in WT cells (Fig 4B).
Swelling-enhanced caspase induction by cisplatin depended on VRAC as it was abolished in LRRC8A<sup>-/-</sup> and LRRC8D<sup>-/-</sup> cells. It was strongly reduced, but not abolished, in LRRC8D<sup>-/-</sup> cells (Fig 4B).

By contrast, staurosporine-induced caspase activation was not enhanced by hypotonic swelling and was suppressed in LRRC8A<sup>-/-</sup>, but not in LRRC8D<sup>-/-</sup> cells (Fig 4C). Since both I<sub>Cl,vol</sub> and caspase induction by staurosporine depend on LRRC8A but not LRRC8D, these results are compatible with the hypothesis that VRAC activation-dependent AVD facilitates the progression of apoptosis (Maeno et al., 2000; Shimizu et al., 2004). VRAC activation by pro-apoptotic stimuli was monitored by the quenching of YFP fluorescence by externally added iodide (Voss et al., 2014). Iodide permeates VRAC efficiently and hence VRAC opening increases YFP quenching rates. The slow emergence of an LRRC8A-dependent quenching component upon incubation with 200 μM cisplatin (Fig 5A–C) or 4 μM staurosporine indicated that both drugs slowly activated VRAC (Fig 5E and F). Drug-induced VRAC activation was small compared to that elicited by acute hypotonic cell swelling (Fig 5D). A direct comparison of quenching slopes, however, underestimates the difference in activation because quenching rates after acute exposure to hypotonicity (but not with drug preincubation) also reflect the time course of cell swelling and ensuing VRAC opening (Appendix Fig S2A and E).

VRAC mediates cisplatin uptake which depends on LRRC8 subunit composition

The observation that cisplatin activated caspase-3 in WT cells more efficiently when applied in hypotonic medium suggested that cisplatin might enter cells through VRACs. Indeed, VRACs not only conduct halide ions, but also organic compounds like taurine,
glutamate, and 5-bromo-2-deoxyuridine (Jackson & Strange, 1993; Hyzinski-Garcia et al., 2014; Lee et al., 2014; Qiu et al., 2014; Voss et al., 2014). Importantly, early studies on a variety of cultured cell lines have led to the conclusion that cisplatin/carboplatin uptake involves two components: About half the drug enters cells by passive diffusion through the plasma membrane and the other half via a protein component, possibly a channel (reviewed by Gately & Howell, 1993). We therefore measured the cellular accumulation of Pt-based drugs by determining Pt concentrations in extracts of cells of various LRRC8 genotypes.

Under isotonic conditions, drug uptake performed at therapeutic carboplatin concentrations (7 μM) by drug-resistant LRRC8D−/− KBM7 cell clones was indeed reduced ~twofold compared to control (Fig 6A). The effect of VRAC subunits in Pt drug uptake was explored in detail for cisplatin in HEK and HCT116 cells of different LRRC8 genotypes. The underlying uptake processes did not saturate with cisplatin concentrations up to 400 μM in both WT and LRRC8A−/− cells (Fig EV3), allowing us to use concentrations exceeding therapeutic levels to increase the sensitivity of the assay. When uptake was performed over several hours under isotonic conditions, cisplatin accumulation was reduced by roughly 70 and 50% upon disruption of LRRC8A or LRRC8D, respectively (Fig 6C) (HEK cells). This subunit dependence resembled that observed with cellular carboplatin and cisplatin resistance (Figs 1 and EV2). The LRRC8-dependent uptake component increased with variable time course over the first few hours (Figs 6C and D). When uptake was studied in isotonic saline for 1 h or less, no significant influence of either LRRC8A or LRRC8D was detected (Fig 6D) (HEK and HCT116 cells). Importantly, early studies on a variety of cultured cell lines have led to the conclusion that cisplatin uptake involves two components: About half the drug enters cells by passive diffusion through the plasma membrane and the other half via a protein component, possibly a channel (reviewed by Gately & Howell, 1993). We therefore measured the cellular accumulation of Pt-based drugs by determining Pt concentrations in extracts of cells of various LRRC8 genotypes.

Figure 3. Disruption of LRRC8A, but not of LRRC8D, abolishes IC_{vol} and blocks volume regulation.
A, B VRAC currents (IC_{vol}) of the HAP1 (A) and KBM7 (B) haploid cell lines. Left panels, example current traces of IC_{vol} fully activated by hypotonic cell swelling measured with the voltage-clamp protocol shown in (A). Dashed lines indicate zero current. Right panels, averaged current/voltage relationships of maximally activated IC_{vol}. Consistent with VRAC currents, they needed hypotonic swelling for activation, displayed an $F^-_>/Cl^-$ permeability sequence, and were blocked by DCPB (Appendix Fig S2A–H). The difference in current inactivation between HAP1 and KBM7 cells can be explained by the fact that KBM7 cells hardly express LRRC8E (Fig EV1) which accelerates VRAC inactivation (Voss et al, 2014). At potentials >100 mV, also KBM7 currents inactivated (Appendix Fig S2I). Data are presented as mean ± SEM; n = 5–10.
C Dependence of regulatory volume decrease (RVD) of HEK cells on LRRC8 genes. Cells were exposed to hypotonic medium starting at t = 0, and intracellular calcein fluorescence was followed over ~1 h as semiquantitative measure of cell volume. Data are presented as mean values ± SEM from sixteen wells.
much smaller degree than LRRC8A/D heteromers or WT VRAC (Fig 6F). Importantly, amplitudes of swelling-activated Cl\(^-\) currents of A/C-expressing cells were similar to WT I_{Cl,vol}, but were much smaller with A/D heteromers (Fig 6G)—a finding agreeing with heterologous expression studies (Voss et al., 2014). Hence, LRRC8D-containing heteromers display a considerably higher cisplatin/Cl\(^-\) transport ratio than other LRRC8 heteromers (Fig 6H).

VRAC Cl\(^-\) currents are inhibited by cisplatin–DMSO complexes in a LRRC8D-dependent manner

In a high-throughput drug screen for VRAC transport inhibitors, in which compounds were routinely dissolved in DMSO, we identified cisplatin as putative VRAC blocker (data not shown). However, suppression of I_{Cl,vol} required DMSO, with which cisplatin forms adducts like Pt(NH\(_3\))\(_2\)(Cl)(DMSO) (Fischer et al., 2008). Cisplatin–DMSO complexes inhibited native I_{Cl,vol} of HEK cells in a dose-dependent manner (Fig 7A), but had no effect on currents of LRRC8 D-dependent manner (Fig 7B). Hence, differently composed LRRC8 channels can also differ in their pharmacology, and cisplatin-conducting VRACs might be specifically targeted without abolishing I_{Cl,vol} and cell volume regulation.

Swelling-induced release of the cellular osmolyte taurine depends on LRRC8D

Whereas LRRC8D\(^{-/-}\) cells displayed unchanged swelling-activated Cl\(^-\) currents (I_{Cl,vol}) (Figs 3A and B, and 6G; Voss et al., 2014), their RVD was markedly decreased (Fig 3C). We therefore hypothesized that LRRC8D is not only important for the transport of cis- and carboplatin, but also of organic osmolytes that do not contribute to electric currents. Indeed, LRRC8D disruption drastically reduced swelling-induced efflux of the important cellular osmolyte taurine (Fig 8A), but had less of an effect than disruption of LRRC8A that apparently totally abolishes swelling-induced taurine efflux (Voss et al., 2014). Transient transfection of LRRC8D, which is difficult to express heterologously (Voss et al., 2014), partially rescued taurine efflux (Fig 8B). Taurine efflux from A/D-expressing cells resembled WT fluxes (Fig 8C), whereas LRRC8C or LRRC8E failed to support significant taurine flux as revealed by experiments with LRRC8(B,D,E)\(^{-/-}\) and LRRC8(B,C,D)\(^{-/-}\) cells in which only LRRC8A and LRRC8C or LRRC8E, respectively, remain (Fig 8D and E). The reduced RVD of LRRC8D\(^{-/-}\) cells (Fig 3C) thus exposes the importance of taurine and possibly other organic osmolytes in cell volume regulation.

Like VRAC-dependent I\(^-\) influx (Fig 5A–C), also VRAC-mediated taurine efflux was stimulated by preincubation with cisplatin (Fig 8F). In congruence with the weaker stimulation of iodide flux by cisplatin than by hypotonicity (Fig 3), cisplatin preincubation stimulated taurine efflux much less than hypotonic swelling. The cisplatin-induced efflux component increased with the time of

Data information: Data are presented as mean ± SEM, n = 3–6. *P < 0.05, **P < 0.01, and ***P < 0.001. Similar results were obtained in three independent experiments. Fold change in (A) refers to t = 0. Control experiments indicated that hypotonicity per se had no effect (Appendix Fig S4).
Figure 5. Activation of LRRC8 channels by pro-apoptotic stimuli.

A–C Cisplatin-induced iodide influx into WT (black □) but not LRRC8A⁻/⁻ (green ▼) HEK cells indicates VRAC halide current activation during apoptosis. Cells expressing an iodide-sensitive YFP variant were exposed to 200 μM cisplatin for periods of 0.5 h (A), 4.5 h (B), or 8.5 h (C) before adding extracellular I⁻ (50 mM final). The difference in slopes of YFP fluorescence quenching between control and cisplatin-treated cells semiquantitatively reflects VRAC current activation. Note that increased YFP quenching with cisplatin preincubation is not due to large non-specific leaks resulting from cell morbidity. Such leaks should lead to a fast component of YFP quenching in WT, but not LRRC8A⁻/⁻ cells after the pipetting artifact that immediately follows addition of iodide (indicated by arrows).

D Swelling-induced iodide influx into WT (black □) and LRRC8A⁻/⁻ (green ▼) HEK cells for comparison. Iodide (50 mM final) was added in isotonic or hypotonic (230 mOsm final) solution at the time indicated by arrow.

E, F Time course of VRAC activation by 200 μM cisplatin (E) or 4 μM staurosporine (F) determined as in (A–C). Averaged maximal slopes of YFP quenching from eight wells (E) or 16 wells (F) each were evaluated to estimate iodide influx rates. WT (black □) and LRRC8A⁻/⁻ (green ▼). Data are presented as mean ± SEM.
preincubation and was abolished in LRRC8A−/− cells. Taurine efflux did not occur through cell morbidity-related unspecific leaks since it could be blocked by carbenoxolone (Fig. 8F). WT VRACs seemed to transport taurine at low rates also without stimulation by cisplatin as indicated by the reduced efflux from cells lacking LRRC8A. However, at these low transport rates, we cannot exclude that slight and variable osmotic imbalances marginally opened VRAC as steady-state efflux differed between experiments (compare Fig 8A–E).

**Discussion**

Our work indicates that heteromeric LRRC8 channels (VRACs) can transport cisplatin and carboplatin, widely used anti-cancer drugs, and that the substrate selectivity and pharmacology of VRAC depend on its subunit composition. LRRC8D plays important pharmacological and physiological roles in supporting the transport of anti-cancer drugs and of the organic osmolyte taurine. LRRC8 channels also facilitated drug-induced apoptosis independent of drug transport, revealing a dual role of VRAC in cisplatin toxicity.

**LRRC8 channels in drug-induced apoptosis**

Several groups have reported that inhibition of *bona fide* VRAC (VSOR, VSOAC) by various non-specific compounds impaired the induction of apoptosis by drugs such as cisplatin and staurosporine (Maeno et al, 2000; Ise et al, 2005; Okada et al, 2006; d’Anglemont de Tassigny et al, 2008; Poulsen et al, 2010). These findings were attributed to an impairment of VRAC-dependent apoptotic volume decrease (AVD) which is thought to be crucial for the progression of apoptosis (Maeno et al, 2000) (for recent reviews, see Hofmann & Lambert, 2014; Lang & Hoffmann, 2012). Indeed, cisplatin and other drugs lead to a slow activation of currents with typical characteristics of \( I_{\text{Cl},\text{vol}} \), and the resulting AVD could be reduced by non-specific VRAC inhibitors (Shimizu et al, 2004; Poulsen et al, 2010; Min et al, 2011; Cai et al, 2015). Moreover, several cancer drug-resistant cell lines display less \( I_{\text{Cl},\text{vol}} \), AVD, RVD, and swelling-activated taurine efflux than their parent cells (Lee et al, 2007; Poulsen et al, 2010; Min et al, 2011; Sørensen et al, 2014). In KCP-4 cells, both cisplatin sensitivity and \( I_{\text{Cl},\text{vol}} \) were partially restored in parallel by non-specific changes in gene expression using a histone deacetylase inhibitor (Lee et al, 2007). Since some of those cell lines are resistant to various anti-cancer drugs that probably enter cells through different pathways, these reports suggested that VRAC downregulation entailed drug resistance through an AVD-dependent mechanism.

Because LRRC8A was required for both \( I_{\text{Cl},\text{vol}} \) and pro-apoptotic drug-induced iodide influx or taurine efflux, our data now strongly suggest that LRRC8 channels are involved in both RVD and AVD. Since disruption of the obligatory VRAC subunit LRRC8A also reduced the activation of caspase-3 by two pro-apoptotic stimuli, staurosporine and cisplatin, our results are compatible with the notion that apoptosis is facilitated by an LRRC8A-dependent volume decrease. The requirement for LRRC8D in cisplatin-induced, but not in staurosporine-induced caspase activation, can be explained by different mechanisms of cellular uptake. Staurosporine, a large hydrophobic compound that may cross membranes by passive diffusion, apparently does not enter cells through VRAC since it activated caspases regardless of whether it was applied in iso- or hypotonic medium. By contrast, cisplatin permeates through VRACs, in particular those containing LRRC8D subunits. This uptake mechanism imposes an additional LRRC8D dependence on caspase induction by cisplatin.

**Cisplatin and carboplatin uptake through VRAC**

Like \( I_{\text{Cl},\text{vol}} \) and taurine efflux (Voss et al, 2014), cellular cisplatin uptake was strongly stimulated by hypotonic swelling, as reported previously (Smith & Brock, 1989). This uptake component was abolished in LRRC8A−/− and LRRC8(B,C,D,E)−/− cells which do not express functional VRACs and was inhibited by the VRAC inhibitor carbenoxolone. Furthermore, caspase activation by cisplatin showed a similar dependence on LRRC8 isoforms and medium toxicity as cisplatin uptake as is expected for an intracellular effect of cisplatin. Our results, which were obtained with up to four different cell line and several independent LRRC8 knockout clones that excluded off-target effects, constitute overwhelming evidence that LRRC8 channels directly transport cisplatin and carboplatin.

About 50 to 70% of long-term isotonic cisplatin uptake was dependent on LRRC8D and LRRC8A, respectively. The uptake component remaining in LRRC8A−/− and LRRC8(B,C,D,E)−/− cells probably represents passive diffusion across the plasma membrane (Gately & Howell, 1993). The dependence on LRRC8D was surprising since this isoform is dispensable for \( I_{\text{Cl},\text{vol}} \) (Voss et al, 2014). Drug uptake through VRAC was also unexpected since this channel is thought to be closed under isotonic conditions (Nilius et al, 1997), which prevents a continuous loss of cellular constituents like amino acids. However, LRRC8-dependent isotonic uptake of cisplatin in HEK cells developed with a time course that was consistent with a diffusion process.

**Figure 6.** LRRC8 subunit- and osmolarity-dependent carboplatin/cisplatin transport.

- **A** Carboplatin uptake into control KBM7 cells and two LRRC8D-deficient clones (\( n = 6 \)).
- **B** Hypotonicity-stimulated cisplatin uptake in HEK WT cells was selectively blocked by 100 \( \mu \)M carbenoxolone (CBX), a non-specific blocker of VRAC (\( n = 3 \)).
- **C, D** Cisplatin uptake into HEK cells of indicated genotypes using 40 \( \mu \)M cisplatin under long-term isotonic (C) or 200 \( \mu \)M cisplatin in short-term hypotonic (D) conditions as function of time (\( n = 3 \)). Similar results were obtained in HCT116 cells (Fig EV4).
- **E, F** Cisplatin uptake (200 \( \mu \)M) into HEK cells of indicated genotypes: LRRC8(B,C,D,E)−/− and LRRC8(B,D,E)−/− cells express only LRRC8A and LRRC8D, and LRRC8A and LRRC8C, respectively (\( n = 3 \) for WT, \( n = 6 \) for LRRC8−/−, \( n = 9 \) for LRRC8A−/− in E; \( n = 3 \) in F).
- **G** Mean current densities of maximally activated \( I_{\text{Cl},\text{vol}} \) at \(-80 \) mV. The number of cells is indicated for each column.
- **H** Ratio of LRRC8-dependent swelling-activated cisplatin uptake (60 min) to mean \( I_{\text{Cl},\text{vol}} \) as (in Q) as function of genotype.

Data information: Data are presented as mean ± SEM. *\( p < 0.05 \), **\( p < 0.01 \), and ***\( p < 0.001 \) (for [C, E] compared to LRRC8A−/− cells). Dotted lines in (D) and (F) highlight that there is no significant difference in isotonic 60-min uptake between the genotypes.
Figure 6.

A (7 μM carboplatin)

B (200 μM cisplatin)

C (40 μM cisplatin)

D (200 μM cisplatin)

E (200 μM cisplatin)

F (200 μM cisplatin)

G

H

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cisplatin has been observed after time lags that ranged from bona fide the activation of consistent with a slow drug-induced activation of VRAC. Previously, cisplatin uptake, downregulation of LRRC8 subunits could have significant impact on tumor drug resistance. While requiring confirmation by prospective studies, this notion is supported by our analysis of two independent data sets from ovarian cancer patients: LRRC8D down-regulation correlated with poor survival of Pt drug-treated patients. No correlation was seen with reduced LRRC8A expression, possibly because LRRC8A-dependent volume regulation is a viability factor for cells.

In addition to its role in cisplatin/carboplatin uptake, VRAC facilitated apoptosis through an independent, possibly AVD-related mechanism. An impairment of apoptosis may further contribute to drug resistance (Kelly & Strasser, 2011; Speirs et al., 2011), although tumor cells treated with therapeutic doses of Pt drugs probably die predominantly by other mechanisms (Borst et al., 2001; Brown & Wilson, 2003; Brown & Attardi, 2005). Acute induction of apoptosis in HCT116 cells, as analyzed in Fig 4, is thought to require cisplatin concentrations that are ~10-fold higher than therapeutic ones (Berndtsson et al., 2007). Such an induction is independent of nuclear DNA damage (Mandic et al., 2003) and depends on the production of reactive oxygen species (ROS) (Berndtsson et al., 2007) which can activate VRACs (Shimizu et al., 2004; Varela et al., 2004). Therefore, the relevance of VRAC-facilitated apoptosis at therapeutic doses remains to be determined in tumors of patients.

LRRC8D increases VRAC’s permeability for cisplatin/carboplatin and taurine

Whereas the obligatory LRRC8A subunit is required for the surface expression of VRAC, no specific roles could be assigned so far to the other subunits, except for an influence on the kinetics of inactivation at non-physiological voltages (Voss et al., 2014). We have now discovered a role of LRRC8D in taurine and cisplatin/carboplatin transport that is biologically and medically important. Although LRRC8D disruption did not decrease swelling-activated Cl− currents (I_{Cl,vol}) (Fig 3A and B; Voss et al., 2014), it reduced both isotonic and swelling-activated cisplatin uptake and blunted the ensuing activation of caspases. Comparison of swelling-induced cisplatin and Cl− transport (I_{Cl,vol}) revealed that the incorporation of LRRC8D substantially increased VRAC’s cisplatin/Cl− transport ratio, as most strikingly demonstrated by the >10-fold different transport ratio between LRRC8A/D and LRRC8A/C channels. The substantial swelling-activated cisplatin transport remaining in LRRC8D−/− cells (Figs 6D and EV4B) indicates that the cisplatin permeability of VRAC is not absolutely dependent on LRRC8D. LRRC8A/C and LRRC8A/E heteromers transported cisplatin very poorly under isotonic conditions (Figs 6E and EV5), but significant, albeit small, transport through A/C channels could be elicited by cell swelling (Fig 6F). Hence, even A/C channels may, in principle, transport
LRRC8A/LRRC8D-containing channels transport the cellular osmolyte taurine.

A, B Swelling-induced efflux of $[^3]$H-taurine from WT and LRRC8D$^{−/−}$ HEK cells (A, B) and partial rescue by transient transfection of LRRC8D (B). Rescue is incomplete due to low transfection/expression efficiency of LRRC8D (Voss et al., 2014).

C–E Swelling-induced efflux of $[^3]$H-taurine from LRRC8(B,C,E)$^{−/−}$ (C), LRRC8(B,D,E)$^{−/−}$ (D), or LRRC8(B,C,D)$^{−/−}$ (E) HEK cells compared to WT cells.

F Cisplatin-induced taurine efflux (over 30 min) from WT and LRRC8A$^{−/−}$ HEK cells after preincubation with 200 μM cisplatin for 4 or 12 h, or without cisplatin (0 h). Carbenoxolone (CBX, 100 μM) blocks taurine efflux from WT cells treated for 12 h with cisplatin, excluding taurine flux through unspecific leaks.

Data information: Data are presented as mean ± SEM. Dashed lines in (A–E) represent isotonic efflux from the same individual experiments. Red arrows, change to hypotonic solution. Each panel shows the average of two independent experiments (n = 4 for each experiments, i.e., n = 8 in total).
cisplatin. Possible roles of other subunits and subunit combinations remain to be explored.

A mere loss of LRRC8D-containing, cisplatin-preferring channels should have quantitatively similar effects on isotonic and hypotonic uptake. However, whereas under long-term isotonic conditions, LRRC8D disruption reduced the LRRC8-dependent component (as defined by the difference between WT and LRRC8A/−/− cells) by ~70%, the hypotonicity-stimulated cisplatin uptake component was diminished by only ~50% or less in LRRC8D/−/− cells. This disparity suggests a differential activation of LRRC8D-containing heteromers by pro-apoptotic drugs or cell swelling.

LRRC8D also plays a prominent physiological role. It crucially determines VRAC’s taurine permeability, but mirroring its role in cisplatin transport, it is not absolutely required for taurine transport. Taurine, the cellular concentrations of which range from 5 to 50 mM, is an important cellular osmolyte and has several additional biological roles (Lambert et al., 2015). LRRC8D/−/− cells can now be used to estimate the contribution of organic osmolytes to cell volume regulation. Our RVD experiments suggest that this contribution is substantial. The simultaneous presence in the plasma membrane of VRACs incorporating or lacking LRRC8D subunits probably explains previous observations which suggested that the volume-regulated anion channel VRAC is molecularly distinct from a volume-sensitive organic osmolyte and anion channel VSOAC (Lambert & Hoffmann, 1994; Stutzin et al., 1999; Shennan, 2008).

Implications for the pore of VRACs

The dependence of the substrate selectivity on subunit composition is compelling evidence that LRRC8 heteromers form the VRAC pore. The structural basis of VRAC selectivity is currently unknown. Remarkably, VRACs are thought to display a substantial anion > cation selectivity for inorganic ions (Nilius et al., 1997), while also transporting rather large organic substrates. The radius of the VRAC pore has been estimated to be 0.6−0.7 nm (Droogmans et al., 1999; Ternovsky et al., 2004). This value is compatible with the release of organic osmolytes like taurine and amino acids and also with the uptake of cisplatin and carboplatin (maximal radius of ~0.15 and ~0.28 nm, respectively). On the other hand, this simple consideration does not explain the observation that oxaliplatin (r ~ 0.45 nm) did not permeate, whereas blasticidin S (r ~ 0.7 nm) apparently does (Lee et al., 2014).

The conundrum of a highly halide-selective pore that also conducts much larger and differently charged organic substrates rests on the assumption that there is just one VRAC, and not a whole spectrum of differently composed LRRC8 heteromers, that is, different VRACs. Given the wide tissue distribution of most LRRC8 isoforms, it is likely that various VRACs with diverse properties are co-expressed in the same cell. Our work is compatible with the possibility that some subunit combinations form channels that only conduct organic substrates, whereas others only conduct small anions. However, since, for example, LRRC8(B,C,E)/−/− cells transport both organic molecules and Cl−, this hypothesis demands that LRRC8A and LRRC8D can assemble with different stoichiometries and thereby yield channels with different substrate selectivities. This proposition, which could apply for other subunit combinations as well, is difficult to test.

Our work revealed that the specific subunit composition of VRACs determines their substrate specificity, regulation, and pharmacology. LRRC8A/LRRC8D-containing VRACs, which are amenable to specific pharmacological modulation, are important for cell volume regulation by organic osmolytes. They may also play an important dual role in tumor drug sensitivity by mediating cisplatin/carboplatin uptake and possibly by modulating the progression of apoptosis.

Materials and Methods

Loss-of-function screens

A haploid-genetic screen for carboplatin resistance was performed as described (Carette et al., 2011). 10⁸ KBM7 cells were transduced with a gene-trap retrovirus using spin infection. 10⁹ mutagenized cells were then exposed to 7 μM carboplatin and distributed over 96-well plates (10⁶ cells/well). Surviving clones were pooled after 21 days and subjected to density centrifugation using lymphocyte separation medium (Lonza, 30 min at 400 g) to remove cell debris. Next, 3 × 10⁷ cells were pelleted for genomic DNA isolation (QiAmp DNA mini kit, Qiagen). Gene-trap insertion sites were amplified by inverse PCR. First, genomic DNA was digested using the restriction enzymes NalI and Msel (New England Biolabs) overnight at 37°C. Fragments were then circularized using T4 DNA ligase (New England Biolabs) (o/n at RT). Fragments containing the gene-trap sequence and flanking genomic DNA were amplified by PCR using primers containing the illumina adapters P5 and P7 (for NalI-digested DNA: 5′-AATGATACGCACAGATCATGTGATGGTTCTCTAGCTTGCC-3′ and 5′-CAACAGCAAAGACGCAACATGACACCCAGGTAAAGATCAAGGTC-3′; for Msel-digested DNA: 5′-AATGATACGCCACACAGATCATGTGATGGTTCTCTAGCTTGCC-3′ and 5′-CAACAGCAAAGACGCAACATGACACCCAGGTAAAGATCAAGGTC-3′).

Products were purified (PCR purification kit, Qiagen) and sequenced on a HiSeq 2000 (Illumina) using a custom sequencing primer 5′-CTAGCTTTGCAACCGTACGTTGGGTGCTTTCA-3′.

Sequence reads were cropped to 36 bp for comparability with previously performed screens and aligned to the human genome (hg18) using bowtie (Langmead et al., 2009). Only reads that align uniquely without mismatches were considered. Additionally, reads aligning non-uniquely when 1 to 3 mismatches were allowed were removed, as well as reads falling within 2-bp distance of each other. As the gene-trap construct is unidirectional, only insertion sites in the sense orientation of intronic regions, and integrations in sense or antisense of exonic sequences were considered. To identify genes enriched in mutations following carboplatin treatment, the number of insertion sites per gene was compared to an unselected mutagenized population (Carette et al., 2011) using a one-sided Fisher exact test. Correction for multiple testing for P-values was carried out using Benjamini and Hochberg FDR correction in R.

Cytotoxicity and colony formation assays

LRRC8D-expressing and LRRC8D-deficient KBM7 cells were seeded in 96-well plates in triplicates (3 × 10⁵ cells/well) and treated with various concentrations of carboplatin, cisplatin, and oxaliplatin for 96 h. Relative numbers of viable cells were measured in
comparison with the untreated control and the solvent control using the fluorimetric, resazurin-based Cell Titer Blue® assay (Promega). Measurements were performed according to the manufacturer’s instructions at 560Ex/590Em nm in a Tecan counter (Infinite M200). Cell growth was expressed as percentage of the untreated control.

Regarding HAP1 cells, 10,000 LRRCA8−/− HAP1 and 8,000 HAP1 vector control cells were seeded per well into 6-well plates to account for differences in growth rate. Cells were assayed for colony formation in the presence of five different concentrations of carboplatin, cisplatin, or oxaliplatin. After 7 days, surviving colonies were formalin-fixed and stained with crystal violet. The optical absorption was determined at 590 nm after extracting the dye with 10% acetic acid.

LRRCA8 knockout cell lines

The generation of stably diploid HCT116 cells and polyploid HEK293 cells with disruptions in one or several LRRCA8 genes using the CRISPR-Cas9 method has been described previously (Voss et al., 2014). HEK cells lacking LRRCA8-E singly or in various combinations, and HAP1 cells lacking LRRCA8 or LRRCA8D, were newly generated using the same procedure. KBM7 clones lacking LRRCA8D were obtained from Hidde Ploegh (Lee et al., 2014). Disruption of LRRCA8 genes was confirmed by genomic sequencing and Western blotting (Fig EV1).

Antibodies and Western blots

Polyclonal rabbit antibodies against LRRCA8A and LRRCA8E have been described previously (Voss et al., 2014). New antibodies were raised against LRRCA8B, LRRCA8C and LRRCA8D in rabbits using the following peptides that were coupled through N-terminally added cysteines to keyhole limpet hemocyanin: 5QSLPYPQPGLESPGIESPT, LRRCA8B; EDALFETLPSDVREQMKAD, LRRCA8C; LEVKEALNQDVNEKNS, LRRCA8D. Sera were purified by affinity to the respective peptides. Western blots of native HCT116 and HEK cells, together with sera (γ-LRRCA8C, α-LRRCA8D) were made against mouse peptide sequences that differed by a few amino acids from the human counterparts. Western blots were done following standard procedures using antibodies against tubulin or actin as loading controls.

Electrophysiology

VRAC-mediated swelling-activated anion currents ICl,vol were measured in the whole-cell patch-clamp configuration as described (Voss et al., 2014). Cells were swollen by exposure to hypotonic solution (240 mM) that differed from the isotonic solution (320 mM: 150 NaCl, 6 KCl, 1 MgCl2, 1.5 CaCl2, 10 glucose, and 10 HEPES, pH 7.4) by containing 105 instead of 150 mM NaCl. In order to isolate Cl− currents, the pipette solution contained (in mM) the following: 40 CsCl, 100 Cs-methanesulfonate, 1 MgCl2, 1.9 CaCl2, 5 EGTA, 4 Na2ATP, and 10 HEPES, pH 7.2 (290 mM). Whole-cell currents were monitored by voltage ramps from −100 to +100 mV and voltage-clamp traces recorded using the protocol shown in Fig 3A.

Measurement of regulatory volume decrease (RVD)

Cell volume regulation was measured semiquantitatively using the calcein method (Capó-Aponte et al., 2005) in a fluorimetric imaging plate reader (FLIPRTM; Molecular Devices) in a 384-well format as described (Voss et al., 2014). Cell swelling was initiated by decreasing extracellular osmolarity to 94 mOsm, and calcein fluorescence was followed at λ = 515–575 nm for ~65 min.

Drug-induced iodide influx

Iodide influx into HEK cells expressing an iodide-sensitive YFP variant (Galietta et al., 2001) was carried out using the FLIPRTM as described (Voss et al., 2014), but using both LRRCA8−/− and LRRCA8−/− cells. Cells in half of the wells of 384-well plates were preincubated with 4 μM staurosporine (in 0.4% DMSO; also added to control wells) or 200 μM cisplatin (without DMSO) for the times indicated in Fig 7. Iodide uptake (always under isotonic conditions) was initiated by pipetting 25 μl of a solution containing 70 mM iodide to the well, resulting in a final iodide concentration of 50 mM. YFP quenching was followed over time using illumination at λ = 495–505 nm and fluorescence measurement at λ = 526–585 nm using the FluorLED/Filter set. Maximal fluorescence quenching rates were determined as semiquantitative measure of iodide uptake. Values were averaged from 16 wells for each condition.

Caspase-3 activity determination

Cells covering (~90% confluency) the bottom of 6-cm plates were incubated at 37°C/5% CO2 in DMEM/FCS containing 4 μM staurosporine, or 0.4% DMSO as vehicle control, or 200 μM cisplatin (without DMSO). Cells were collected at different times of drug exposure. To explore the dependence on osmolarity, cells were incubated with 4 μM staurosporine or 200 μM cisplatin in isotonic (320 mM) or hypotonic (240 mM) solution (as described for electrophysiology) for 1 h at 37°C. The drug-containing solutions were then replaced by normal medium and cells incubated for different times. Caspase activity from cytosolic extracts was monitored fluorometrically in a Safire2 plate reader (Tecan AG, CH) set at 37°C using as substrate 30 μM Ac-DEVD-AMC (Enzo). Specific enzymatic activity was calculated from the slopes of the best fit curves and normalized to total protein. For long-term isotonic protocols, the activity was further normalized to “time zero” samples.

Measurement of taurine efflux

HEK293 cells were grown to ~80% confluency (48–72 h after plating) in 35-mm diameter plates coated with poly-L-lysine. For rescue experiments, cells were transfected one day before flux measurements with an LRRCA8D expression plasmid using Fugene HD. WT and LRRCA8D−/− control cells were mock transfected with a GFP expression vector. Cells were loaded with 3H-taurine (2 μCi/ml; Hartmann Analytics) for 2 to 2.5 h in culture medium (without FCS) at 37°C. They were then washed five times at room temperature with isotonic solution (same as used in electrophysiology). After washing, external media were removed in 5-min intervals and replaced with fresh isotonic or hypotonic solution (same as in electrophysiology) and saved for counting. To measure cisplatin-induced
taurine efflux, cells were preincubated with cisplatin (200 μM) in cell culture medium for 2 or 10 h. Cells were then loaded with $^3$H]-taurine for 2 h in cell culture medium (±200 μM cisplatin, w/o FCS and w/o DMSO) at 37°C. Cells were washed 5 times and incubated for 30 min at 37°C with fresh isotonic solution. As a control for LRRRC8 contribution to cisplatin-induced taurine efflux, isotonic solution containing 100 μM carbenoxolone was used throughout the washes. At the end of the experiments, cells were lysed with 0.75 ml of 0.1 M NaOH. The radioactivity of cell supernatants and of the final cell lysate was determined in a liquid scintillation counter. Rate constants were calculated as previously described (Holm et al., 2013).

Uptake of cisplatin into cells

Intracellular platinum drug concentrations were measured as described (Brouwers et al., 2005) or by ICP-MS (Brouwers et al., 2006) (NKI and Institut für Medizinische Diagnostik (IMD), Berlin). Cells in 6-cm plates (~90% confluency) were incubated at 37°C/5% CO₂ in DMEM/FCS containing cisplatin (without DMSO) for different times as indicated. For determination of uptake on osmolarity, cells were incubated with 200 μM cisplatin in isotonic or hypotonic solution (same as used in electrophysiology) for the indicated time periods at 37°C. At the end of the uptake period, cells were quickly washed 5 times with 3 ml ice-cold PBS, collected, gently spun down (1,000 g, 5 min, 4°C), and the pellet was resuspended in 0.1 M HCl. For carboxplatin uptake in KBM7 cells grown in suspension, 2 × 10⁷ cells per group (in T25 flasks) were incubated at 37°C/5% CO₂ in IMDM Glutamax (10% FBS) containing 7 μM carboxplatin (without DMSO) and samples were collected at the indicated time points after drug treatment. Cells were quickly washed twice with 10 ml cold PBS (400 g, 5 min), and the pellet was resuspended in 1 ml cold PBS. Of this suspension, 100 μl was taken for protein concentration determination using the BCA assay. The rest (900 μl) was spun down (400 g, 5 min) and resuspended in 200 μl 0.1 M HCl for ICP-MS.

Statistics

Data are presented as mean ± SEM, with n denoting the number of observations (samples). For RDF measurements (Fig 3C), cisplatin uptake in Figs 6C–F and EV4, caspase induction (Fig 4A), activation of VRAC iodide flux (Fig 5), and Western blots (Fig EV1), a minimum of two independent experiments were performed with similar results. Student’s t-tests for paired or unpaired data were employed as appropriate and considered as significant as follows: *P < 0.05; **P < 0.01; and ***P < 0.001.

Gene expression and survival analysis based on published data concerning ovarian cancer patients

Gene expression data were downloaded from the TCGA (http://cancergenome.nih.gov/). The expression data are derived from RNAseq with expression quantification using RSEM. The density plot shows the log of the gene expression of the individual LRRRC8 family members. The survival analysis uses the clinical data provided by the TCGA. The number of days to death is scored as an event. As time interval definition, we used “days_to_last_follow-up.” This is the time interval from the date of last follow-up to the date of initial pathologic diagnosis, represented as a calculated number of days (https://tcga-data.nci.nih.gov/docs/dictionary/). The patients were stratified on the expression levels of LRRRC8A or LRRRC8D. Those that were in the lowest tertile (< 33%) of the levels were classed as having low expression. The analysis is performed with the package “survival” from the R-project.

The Australian ovarian cancer (OV AU) expression data of the study performed by Patch et al (2015) were gene-wise matched to the TCGA panel after which the selected RSEM was normalized. A survival analysis was performed contrasting the low (< 33%) expression of LRRRC8A or LRRRC8D against the rest.

Expanded View for this article is available online.

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Author contributions

RPC planned, performed, and analyzed experiments (cisplatin uptake (HEK and HCT116 cells), caspase induction, Western blots, iodide influx, cell volume regulation) and wrote the paper; DL generated HEK LRRRC8 KD cell lines and performed and analyzed taurine flux experiments; CG performed and analyzed haploid cell resistance screens and KBM7 cytotoxicity assays; NMG and AK performed and analyzed KBM7 cytotoxicity assays, HAP1 clonogenic assays, and Pt uptake into haploid cells; AK measured cellular Pt concentrations; FU and SMR performed and analyzed electrophysiological experiments; DAE performed cisplatin uptake (HEK and HCT116); CX performed and analyzed KBM7 Pt uptake experiments; FKV performed and analyzed cell volume regulation and iodide influx experiments; TS analyzed LRRRC8 antibodies; VAB analyzed haploid screens; DJV and LFW performed cancer database analysis; TRB planned and advised on haploid screens; PB advised, analyzed experiments, and wrote the paper; SR planned and analyzed experiments and wrote the paper; TJ planned and analyzed experiments and wrote the paper. All authors read and commented on the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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