Acquired cisplatin resistance in human ovarian A2780 cancer cells correlates with shift in taurine homeostasis and ability to volume regulate

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Sørensen BH, Thorsteinsdottir UA, Lambert IH. Acquired cisplatin resistance in human ovarian A2780 cancer cells correlates with shift in taurine homeostasis and ability to volume regulate. Am J Physiol Cell Physiol 307: C1071–C1080, 2014. First published September 24, 2014; doi:10.1152/ajpcell.00274.2014.—Cisplatin resistance is a major challenge in the treatment of cancer and develops through reduced drug accumulation and an increased ability to avoid drug-induced cell damage, cell shrinkage, and hence initiation of apoptosis. Uptake and release of the semiessential amino acid taurine contribute to cell volume homeostasis, and taurine has been reported to have antiapoptotic effects. Here we find that volume-sensitive taurine release in cisplatin-sensitive [wild-type (WT)] human ovarian cancer A2780 cells is reduced in the presence of the phospholipase A2 inhibitor bromelain lactone, the 5-lipoxygenase (5-LO) inhibitor ETH 615–139, and the cysteine leukotriene receptor 1 (CysLT1) antagonist zafirlukast and impaired by the anion channel blocker DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonate). Comparing WT and cisplatin-resistant (RES) A2780 cells we also find that evasion of cisplatin-induced cell death in RES A2780 cells correlates with an increased accumulation of taurine, due to an increased taurine uptake and a concomitant impairment of the volume-sensitive taurine release pathway, as well an inability to reduce cell volume after osmotic cell swelling. Downregulation of volume-sensitive taurine release in RES A2780 cells correlates with reduced expression of the leucine-rich repeat-containing protein 8A (LRRC8A). Furthermore, acute (18 h) exposure to cisplatin (5–10 μM) increases taurine release and LRRC8A expression in WT A2780 cells whereas cisplatin has no effect on LRRC8A expression in RES A2780 cells. It is suggested that shift in LRRC8A activity can be used as biomarker for apoptotic progress and acquisition of drug resistance.

key word: platinum drugs; drug resistance; cell volume regulation; LRRC8A; TauT

Ovarian cancer is a leading cause of death among gynecological cancer patients, and the 5-yr survival rate has been estimated to 30% (64). The main reasons for the failure in treatment outcome are that 70% of the patients have already reached advanced stages of cancer progression at the time of diagnosis, i.e., the cancer has already spread within the pelvis and abdomen (68) and that most ovarian cancer cells rapidly develop resistance or multidrug resistance (MDR) against the chemotherapy.

Cisplatin and chemotherapeutic resistance. Cisplatin was the first member of a class of platinum-based anticancer drugs, which also includes carboplatin and oxaliplatin (7, 25), and today cisplatin is frequently used in chemotherapeutic treatment of ovarian cancer, lung cancer, and lymphomas (60). Cisplatin is administered to cancer patients intravenously as a sterile saline solution. Once circulating in the bloodstream, cisplatin is taken up by the cells and accumulated in the cell nucleus, where it causes DNA lesions by formation of DNA adducts and cross links. This eventually leads to G1 cell cycle arrest and apoptosis. Chemotherapeutic resistance towards cisplatin involves 1) reduced intracellular drug accumulation following activity shift in membrane bound cisplatin carriers [MATE (multidrug and toxin extrusion), CRT1 (copper transporter 1), and OCT (organic cation transporter)1–3] and ATP-binding cassette drug pumps, 2) enhanced drug detoxification, 3) improved DNA repair, 4) enhanced DNA damage tolerance, and finally 5) diminishing in the initiation/execution of the apoptotic cell death process (5, 7, 8, 16, 43).

Cisplatin-induced apoptosis–apoptotic volume decrease. Programmed cell death or apoptosis is a fundamental biological process needed for the elimination of unwanted or damaged cells (17, 29, 44). Apoptosis is initiated by drug-induced DNA damage/mitochondrial dysfunction (intrinsic pathway), ligand binding to death receptors (extrinsic pathway), and/or cell shrinkage (volume sensory pathway) (16, 17). Figure 1 illustrates intracellular elements involved in initiation of apoptosis. Cisplatin-mediated DNA damage is detected by the ATM (ataxia-telangiectasia mutated kinase) and ATR (ataxia-telangiectasia and Rad3-related kinase) kinases, which subsequently activate the tumor suppressor/transcription factor p53 through phosphorylation (52). Once phosphorylated p53 escapes inactivation by ubiquitination by the ligase MDM2 (mouse double minute 2 homolog) and promotes activation of proapoptotic members of the Bcl-2 family, including Bax (Bcl-2-like protein 4) and Bak (Bcl-2 homologous antagonist), either through increased transcription of their respective genes and/or through direct activation of the proteins. Bax and Bak localize to the outer mitochondrial membrane and dimerize, facilitating release of cytochrome c from the mitochondria into the cytosol and hence stalling the energy production. Cytosolic cytochrome c binds to the Apaf-1 (apoptotic protease activating factor 1) and ATP, which then recruits procaspase-9, hence creating the apoptosome. The latter cleaves procaspase to active caspases, which subsequently activates the executor caspase-3 causing cell elimination by apoptosis. As illustrated by cisplatin-induced cell death in, e.g., Ehrlich ascites tumor cells (EATC), induction of apoptosis via the intrinsic pathway involves an initial redistribution of monovalent ions followed by a terminal net loss of negative charge.
of KCl as well as organic osmolytes and hence cell shrinkage (46). It appears that it is the decrease in the cellular K⁺ concentration, following activation of K⁺ channels in combination with an inadequate efficiency of the Na⁺–K⁺–ATPase activity, that causes activation of caspases and nucleases (32). The extrinsic pathway involves formation of the death-inducing signaling complex DISC (death-inducing signaling complex) and subsequently activation of the initiator caspase-8. Activated caspase-8 propagates the apoptotic signal by directly cleaving and activating caspase-3 and/or cleaving the Bcl-2-interacting protein, Bid. This leads to release of cytochrome c from mitochondria into the cytosol and apoptosis. Induction of cell death via the volume sensory pathway following cell shrinkage is in EATC shown to involve the monomeric GTP binding protein Rac and p38 MAPK-mediated phosphorylation/protection of p53 against ubiquitination (6). It is noted that trafficking of CD95 to the plasma membrane and caspase-3/8 activation follow osmotic cell shrinkage, and it has been suggested that an increased number of receptors in the plasma membrane contributes to sensitization of cells towards CD95 (51).

**Taurine transporters—cell volume restoration.** Taurine (β-amino-ethane sulfonic acid) is a metabolic inert amino acid that plays an important role in cell volume control, cellular metabolism, antioxidant defense, and initiation/progression of the apoptotic process (17, 23, 27–29, 31, 33, 54, 56, 57). Cellular taurine content is generally a balance between active uptake via the Na⁺-

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**Fig. 1. Extrinsic, volume sensory, and intrinsic pathways leading to apoptosis.** The extrinsic pathway is activated by a proapoptotic ligand, e.g., TNF-α. Activation of caspase-3 involves death receptors, proteins with death domains, and caspase-8. The volume sensory pathway is activated by osmotic cell shrinkage. Activation of caspase-3 involves a yet unidentified volume sensor, the monomeric GTP binding proteins (Rac), the kinase p38 MAPK, the transcription factor p53, and proapoptotic proteins as well as formation of the apoptosome (cytochrome c, released from the mitochondria, dATP/ATP, Apaf-1, procaspase-9). The intrinsic pathway is activated by radiation, toxins, viral infections, and free radicals. Activation of caspase-3 involves sequential activation of the kinases ATM/ATR and the transcription factor p53. DNA damage also activates membrane bound, volume-sensitive transporters for organic osmolytes [volume-sensitive organic anion channel (VSOAC), volume-regulated anion channel (VRAC), and leucine-rich repeat-containing protein 8A (LRRC8A)], which through a reduction in cell volume feedback to the volume sensory pathway. A putative connection between DNA damage and channels activation as well as a role of p53 in channel regulation is to be revealed.

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**Extrinsic pathway**

- **ligand e.g. TNFα**
  - Death receptor
  - Initiator caspases
  - Caspase 8
  - Bid
  - Bcl-2 family members
  - Apoptosome
  - Executor caspases
  - Caspase 3
  - Loss of taurine
  - Apoptosis

**Intrinsic pathway**

- **stress factor e.g. Cisplatin**
  - DNA damage
  - ATM / ATR
  - AVD
  - Loss of taurine

**Volume sensory pathway**

- **Cell shrinkage**
  - Volume sensor
  - Rac p38
  - p53
  - Pro-apoptotic
  - Bcl-2 family members
  - Apoptosome
  - Executor caspases
  - Caspase 3
  - AVD

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**Key compounds of VSOAC have recently been identified as**

- TRPC6 (a calcium-activated cation channel), which acts as a volume sensor in response to hypotonic conditions (12, 26, 28, 31).
- 11β-HSD2 (11β-hydroxysteroid dehydrogenase type 2), which catalyzes the conversion of glucocorticoids to inactive metabolites (32).
- Taurine uptake system, involving the taurine transport protein (TCTP), which plays an important role in maintaining cell volume homeostasis (33).
- VSOAC activity is modulated by phosphatases, i.e., inhibition of vanadate-sensitive protein tyrosine phosphatases by ROS, which are generated in a variety of cells in response to osmotic perturbation, or inhibition of the phosphatidylinositol (3,4,5)-trisphosphate phosphatase PTEN (phosphatase and tensin homologue), leads to significant potentiation of taurine loss following exposure to hypotonic conditions (12, 26, 28, 31).
- Key compounds of VSOAC have recently been identified as being members of the leucine-rich repeat containing 8 (LRRC8) family (49, 62). Especially LRRC8A was found to be important since the acquisition of resistance to CD95 ligation (ELA) cells, which is an adherent subline of the ascites tumor (ELA) cells, which is an adherent subline of the Ehrlich ascites tumor (ELA) cells, which is an adherent subline of the Ehrlich ascites tumor (ELA).
nonadherent EATC, chemoresistance correlates with a reduced nuclear cisplatin accumulation and an increased TauT activity (59). In line with this, Han and colleagues (9, 10) demonstrated that TauT overexpression protects kidney cells against cisplatin-induced cell death through p53 activation. More recently, Yasunaga and Matsumura (66) recently demonstrated that TauT promotes survival and multidrug resistance in colorectal cancer cells.

In this study we investigate whether acquired cisplatin resistance in human ovarian A2780 cells correlates with an increased expression/activity of TauT and/or reduced VSOAC (LRRCSA). We also test whether enzymes involved in the activation of VSOAC are affected by acquisition of resistance.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** WT and cisplatin-resistant human ovarian A2780 were grown in 75-cm² culture flasks (CellStar, Grenier Bio, Germany) in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. Both cell lines were kept at 37°C, 5% CO₂, and 100% humidity. Cells were passed on every 3–4 days using 0.25% trypsin in phosphate-buffered saline (PBS). To maintain resistance, cisplatin-resistant A2780 cells were treated with 1 μM cisplatin between every third passage. Penicillin/streptomycin, RPMI 1640 medium, fetal bovine serum, L-glutamine. Both cell lines were kept at 37°C, 5% CO₂, and 100% humidity. Cells were washed gently two times in ice cold PBS and subsequently lysed in lysis buffer containing 1% SDS, 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 0.5% Triton X-100, 1 mM Na₂VO₄, and 1% protease inhibitor mix before sonication. Lysates were centrifuged 5 min at 5°C and 20,000 rpm to separate the proteins extracts from insoluble cell debris. Protein content was estimated by a DC assay (Bio-Rad), and lysates with equivalent amount of protein (25 μg per lane) were mixed with NuPAGE sample buffer plus dithiothreitol and proceeded for SDS-PAGE gel electrophoresis (NuPAGE precast 10% Bis-Tris gels) in NOVEX chambers under reducing and denaturing conditions (NuPAGE MOPS SDS running buffer; Invitrogen). Benchmark protein ladder was used for indication of molecular weight. NuPAGE transfer buffer (Invitrogen) was used for protein transfer to nitrocellulose membranes, and protein transfer was verified by Ponceau staining. Membranes, blocked at 37°C in TBST (0.01 M Tris·HCl, 0.15 M NaCl, and 0.1% Tween 20 pH 7.4) containing 5% nonfat dry milk, were washed in TBST and subsequently incubated with secondary antibodies for 1 h at room temperature. The monoclonal anti-human-LRRCSA and polyclonal anti-human-β-actin antibodies, both produced in mouse and from Sigma-Aldrich, were used in a dilution of 1:250 and 1:1,000, respectively. TauT antibody from Yorkshire Biocience was used in a dilution of 1:10,000. The secondary AP-conjugated anti-mouse and antirabbit antibody (Sigma) was used in a dilution of 1:5,000. Following wash in TBST, membranes were developed using BCIP/NBT (KPL, Gaithersburg, MD). Membranes were scanned and bands were quantified using UN-SCAN-IT (Silk Scientific).

**Cell volume measurements.** Changes in cell volume upon hypotonic exposure were measured by electric cell sizing. WT and resistant A2780 cells, grown to 80% confluence in T75 flasks, were gently washed twice in PBS and once in 2 mM EDTA in PBS. The EDTA solution was quickly removed, and the cells were left to detach in 1 ml PBS. Following detachment, the cells were resuspended in growth media) used for cell volume measurements were microfiltered (Millipore filters; 0.45 μm) before the experiments.

**Estimation of taurine uptake and efflux.** [3H]taurine (PerkinElmer, Waltham, MA) was used to estimate the uptake of taurine via TauT as well as the efflux under isotonic and hypotonic conditions as previously described (19, 61). All the experiments were performed at room temperature.

For the influx experiments, WT and resistant A2780 cells were grown to 80% confluence in six-well polyethylene culture plates (9.6 cm² per well) in complete growth medium. Five of the six wells were used to determine the taurine uptake and the residual well was used to determine the representative protein content (Lowry method). Before
the initiation of the experiment, each well was washed three times in 1 ml isotonic NaCl medium to remove the growth media. Following the final wash to each well was added 600 μl isotonic NaCl medium. The experiment was initiated by adding 50 μl [3H]taurine stock solution containing 37,000 Bq/ml (0.005 μM taurine) to wells 1–5 at time 0, 2, 4, 6, and 8 min, respectively. Taurine uptake was terminated at 10 min by removal of the extracellular medium and rapid rinsing cells by addition and aspiration of 1 ml ice-cold MgCl2, followed by cell lysis with 200 μl 96% ethanol. After evaporation of EtOH, 600 μl ddH2O was added wells 1–5 and the plate was incubated 20 min on a shaking table to dissolve/extract the isotope. The dissolved isotope was transferred to scintillation vials, and each well was washed twice in 600 μl ddH2O. These washings were likewise transferred to scintillation vials. To all vials 3.5 ml Ultima Gold were added, and activity was determined in a PerkinElmer scintillation counter. The total [3H]taurine (counts/min) taken up by the cells/wells at a given time point was estimated as the sum of 3H activity in the cell extract and two water washouts. Cellular taurine activity (counts-min^-1, well^-1) was converted to nmol/g protein, using the extracellular specific activity (counts-min^-1-nmol^-1) and the protein content (mg protein/well), and finally plotted vs. time. Taurine uptake (nmol-g protein^-1-min^-1) was determined by linear regression.

Swelling-induced taurine efflux was estimated on cells grown to 80% confluence in six-well polyethylene culture plates. Cells were loaded in complete growth medium supplemented with [3H]taurine (18,500 Bq/well) for 2 h (37°C, 5% CO2, 100% humidity). Efflux experiment was measured at room temperature with each well representing one experiment. Before the experiment the growth media were removed and each well washed three times with 1 ml isotonic NaCl medium (300 mosM) per well to remove remaining extracellular isotope and growth media. The efflux experiment was performed by transferring the NaCl medium from the well to vials and replacing it with new medium at 2-min intervals for a total time period of 30 min, in the absence or presence of inhibitors, i.e., the 5-LO inhibitor ETH 615–139 (donated by Dr. I. Ahnfelt-Rønne, Løvens Kemiske Fabrik, Denmark; dissolved in EtOH), the CysLT1 antagonist zafirlukast {[(2S)-2-[3-endosulfan-2-yl][carbonyl]phenyl]-1-methyl-1H-indol-5-yl][carbamic acid cyclopentyl ester, dissolved in DMSO], the PLA2 inhibitor bromo-DEAmine lacton (BEL; dissolved in DMSO), and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS; dissolved in ddH2O). At time 12 min isotonic medium was replaced by hypotonic medium (200 mosM). Isotope remaining inside the cells in each well at the end of the experiments was determined by addition of 1 ml 1 M NaOH, gently shaking for 1 h, and subsequently transfer of NaOH and to timed washouts (ddH2O) to vials. Scintillation liquid was added to all vials, which were proceeded for 3H-activity determination. The sum of 3H activity released during the efflux experiment and in the NaOH/water washouts represent the total 3H activity in the cell system. The fractional rate constant (k, min^-1) for taurine release was calculated from the following equation: k = (ln(X1) – ln(X2))/((t1 – t2)), where X1 and X2 are the fraction remaining in the cell at time t1 and t2, divided by the time interval.

**Determination of cell viability**–3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The colorimetric MTT assay, i.e., conversion of the yellow soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue insoluble formazan, was, as previous described (12), used to determine cell viability after treatment of WT and resistant cells with 5–20 μM cisplatin (48 h). A2780 cells were seeded to a density of 20,000 cells per 200 μl media per well in 96-well plates and incubated for 72 h (37°C, 5% CO2, 100% humidity). Addition of TNF-α (Sigma-Aldrich) or hypertonic exposure by addition of 81.5 mM NaCl was supplemented the treatment during the last 18 h. At the end of the incubation period, 100 μl were removed, 25 μl of a MTT solution (5 mg/ml MTT in sterilized PBS) were added each well, and the plate was incubated in the cell culture incubator for 3 h. Following incubation 100 μl fresh-made 10 mM HCl containing 1% SDS was added to each well and incubated in fume hood overnight to solubilize the colored formazan crystals. Samples were measured at 540 nm using Wallac Envision Multilabel plate reader (PerkinElmer). The absorbencies measured were assumed to be directly proportional to the number of viable cells. Data were represented relative to the absorbance from untreated control cells. Each experiment was performed in triplicate.

**Statistics.** All data were statistically tested (SigmaPlot version 12) by Student’s t-test or one-way ANOVA with Bonferroni’s test as posttest. Statistical significant indicates P < 0.05. In bar and scatter plots, the error bars signify SE.

**RESULTS**

Chemotherapeutic resistance can be either an acquired property (extrinsic resistance) following chemotherapy or an innate property (intrinsic resistance) caused by cell differentiation or genetic changes occurred during the early stages of tumor formation. The phenotype of the cisplatin-resistant (RES) human ovarian cell line A2780 reflects extrinsic resistance and in Fig. 2A, it is seen that 48-h exposure to 5 and 10 μM cisplatin...
has no significant effect on cell viability in cisplatin RES A2780 cells whereas WT A2780 cells show a 40–50% reduction in viability under the same conditions. Increasing the cisplatin concentration to 20 μM elicits a significant increase in cell death in RES A2780 cells, i.e., induction of cell death in RES A2780 is observed although at a high cisplatin dose. To test whether cisplatin-induced cell death via the intrinsic pathway in WT A2780 cells involves other cell death pathways (see Fig. 1), we exposed WT and RES A2780 cells to cisplatin plus TNF-α (intrinsic plus extrinsic pathway) or cisplatin plus hyperosmolarity obtained by increasing the extracellular NaCl concentration (intrinsic plus volume sensory pathway). To ensure minimal independent effects of TNF-α and hyperosmolarity, we limited the exposure time to 18 h compared with the 48-h cisplatin exposure. From Fig. 2B, no significant effect of TNF-α or hyperosmolarity on cell viability is observed in WT and RES cells in the absence of cisplatin. However, TNF-α increases the toxicity of cisplatin in both WT A2780 and RES A2780 cells, whereas incubation in hypertonic medium increases the toxicity of cisplatin radically in WT A2780 and to a lesser extent RES A2780 cells (Fig. 2B). These data are taken to indicate that RES A2780 cells are less sensitive to induction of cell death via the volume sensory pathway compared with WT A2780 cells.

A hallmark in apoptosis is shift in TauT activity and/or a decrease in the volume-sensitive leak permeability towards taurine. The HPLC technique revealed that the cellular content of taurine in WT A2780 cells is 0.019 ± 0.003 μmol amino acid/mg protein (Fig. 3A), which is lower than taurine content in, e.g., ELA (0.06 - 0.07 μmol/mg protein; Refs. 59, 61) and NIH3T3 fibroblasts (0.025–0.055 μmol/mg protein; Refs. 42, 63). With the use of average values for cellular water and protein content, i.e., 3.24 ml cell water/g cell dry wt (18) and 0.78 g protein/g cell dry wt (15), the taurine content in A2780 cells corresponds to a cellular taurine concentration at 4.5 ± 0.7 mM. Plasma taurine concentration is 10–100 μM (20), indicating that the intracellular taurine concentration, due to TauT activity, is presumably 400-fold higher than the extra-

![Graph](http://ajpcell.physiology.org/)
cellular concentration in A2780 cells grown in 10% serum. From Fig. 3A, it is seen that the cellular content of the organic osmolite taurine and alanine is significantly increased in RES A2780 cells compared with WT A2780 cells, whereas cellular glycine content is concomitantly reduced. It is noted that loss in glycine almost balances gain in taurine plus alanine. As increased taurine accumulation could reflect an increased uptake and/or a reduced release, we determined the unidirectional taurine uptake and release in WT and RES A2780 cells. From Fig. 3, B and C, it is seen that taurine uptake is linear within the initial 10 min and that acquired cisplatin resistance in RES A2780 cells is accompanied by 1.7-fold increase in taurine influx. RT-qPCR and Western blotting revealed that TauT mRNA accumulation and expression of the 70-kDa TauT protein band were unaltered, i.e., TauT mRNA relative to ARP2/3 was determined in three sets of experiments at 0.9817 ± 0.0004 (WT) and 0.9823 ± 0.00002 (RES), whereas TauT protein expression relative to β-actin was determined in five sets of experiment at 0.09 ± 0.02 (WT) and 0.08 ± 0.03 (RES). This could indicate that upregulation of TauT activity in connection with cisplatin resistance reflects shift in the regulation of TauT and/or the number of TauT transporters in the plasma membrane. This was not investigated further.

From Fig. 3D, it is seen that taurine is released from WT A2780 cells under isotonic conditions and that release increases dramatically and transiently upon hypertonic exposure, reaching a maximal rate constant for taurine release within 8 min. However, in RES A2780 cells the volume-sensitive taurine release was absent (Fig. 3D). It is noted that rate constant for taurine release under isotonic conditions in A2780 cells was not affected by acquisition of resistance, i.e., the fractional rate constant was determined at 0.0033 ± 0.0002 min⁻¹ (n = 10) for WT A2780 cells and 0.0039 ± 0.0004 min⁻¹ (n = 9) for RES A2780 cells. However, with the use of values for the cellular taurine content (Fig. 3A), and the fractional rate constant for taurine release, it is seen that the actual taurine release under isotonic conditions is almost tripled in RES A2780 (1.7·10⁻⁴ mol·mg prot⁻¹·min⁻¹) compared with WT A2780 cells (0.6·10⁻⁴ mol·mg prot⁻¹·min⁻¹) due to the steeper cellular to extracellular taurine gradient. It is noticed that values for taurine efflux under isotonic conditions are larger than values for taurine influx (Fig. 3B), primarily due to the experimental setups, i.e., influx is determined at a low extracellular taurine concentration (0.005 μM), which is significantly lower than the concentration required for half saturation of TauT (20, 27, 31) and the concentration in the growth medium, and secondly because the specific activity, used for the calculations of taurine influx, might be smaller than the specific activity determined from the added isotope due to taurine release during the experiment. It is assumed that the taurine leak pathway, evoked during apoptosis, represents taurine loss via the volume-sensitive leak pathway designated VSOAC (17). Hence, the increased taurine content (Fig. 3A) in resistant cells reflects an increased taurine uptake via TauT and impaired taurine release via the volume-sensitive leak pathway VSOAC.

Osmotic cell swelling normally elicits a volume regulatory response [regulatory volume decrease (RVD)], which reflects net loss of K⁺, Cl⁻, and the organic osmolites (17). From Fig. 3, E and F, it is seen that WT A2780 swell within 2 min following exposure to hypotonic environment where after they regulate their volume towards its original value. RES A2780 cells likewise swell as WT A2780 cells but show no RVD (Fig. 3, E and F). Previous findings with EATC indicate that the volume-sensitive Cl⁻ and K⁺ conductances are also impaired during acquisition of drug resistance (46). In the case of A2780, the cellular taurine concentration (4.5 mM, see above) is low compared with the cellular K⁺ and Cl⁻ concentration and taurine loss as well as loss of glycine and alanine will only account for a minor fraction of the total loss of osmolites during RVD. Hence, acquired resistance in A2780 cells just like resistance in EATC most probably implies reduction in the volume-sensitive transporters for organic osmolites as well as for ions. Downregulation of the volume-sensitive ion transporters was not investigated further.

Reduced VSOAC activity could reflect either reduced activation and/or expression of the transporter. From Fig. 4A, it is seen that activation of the volume-sensitive taurine release from WT A2780 cells in congruence with findings from EATC (27) and human lung cells (19) is significantly reduced following inhibition of PLA₂ activity (iPLA₂ inhibited by BEL), 5-LO activity (inhibited by ETH), and blockage of the CysLT1 receptor with the antagonist zafirlukast. VSOAC activity is also inhibited following acute exposure to the anion channel blocker DIDS (Fig. 4A). As inhibition of the channel in WT A2780 cells with DIDS reduces the VSOAC activity to an even lower value than the activity in RES A2780 (Fig. 4A), we tested for the expression of LRRC8A, which has recently been identified as a VSOAC component (49, 62). From Fig. 4, B and C, it is seen that the expression of LRRC8A is significantly reduced although not eliminated in RES A2780 cells compared with WT A2780 cells. In congruence we find that acute DIDS exposure reduces VSOAC activity in RES A2780 cells by 18 ± 3% compared with the 82 ± 1% seen in WT A2780 cells. Using QPCR we find in three sets of experiments that LRRC8A mRNA relative to ARP2/3 mRNA is reduced from 0.00045 ± 0.00004 (WT) to 0.00033 ± 0.00005 (RES). Hence, reduced expression of LRRC8A (Fig. 4C) correlates the reduced volume-sensitive taurine release (Fig. 3D) and ability to volume regulate after hypertonic exposure (Fig. 3F). It is noted that 5-LO and CysLT1 mRNA/protein expression could also be reduced as part of acquisition of drug resistance but that the effect on taurine release would be masked by the concomitant reduction in LRRC8A expression/activity.

Loss of amino acids in, e.g., EATC, is in full progress within the initial 20 h following exposure to cisplatin (46). Similarly we find that exposure of WT A2780 cells for 18 h to 10 μM cisplatin reduces the cellular content of taurine, glycine, and alanine significantly to 62 ± 11, 78 ± 5, and 86 ± 3% of the initial value (data from 3 sets of experiments). From Fig. 5, A and B, it is seen that acute exposure (18 h) of WT A2780 cells to 5 or 10 μM cisplatin induces a significant increase in LRRC8A expression. In contrast, acute exposure of RES A2780 cells to cisplatin had no effect on LRRC8A expression, i.e., the LRRC8A to β-actin protein expression ratio, when corrected for ions. Downregulation of the volume-sensitive ion transporters was not investigated further.
A \[KCl\] via \(K^+\), \(Cl^-\) channels, as well as organic osmolytes and that drug resistance in cancer cells implies downregulation of \(K^+\) (16, 46) and \(Cl^-\) (39, 46) channel activities. In addition, prevention of \(KCl\) loss through inhibition of \(K^+\) channels (36, 46) and \(Cl^-\) channels (39, 46) is found to limit or postpone apoptosis in WT EATC. The \(Na^+\)-\(K^+\)-\(2Cl^-\) cotransporter NKCC1, the \(Na-K-ATPase\), cation channels, and the \(Na^+/H^+\) exchanger NHE1 have been designated antiapoptotic transporters as they compensate for loss of osmolytes and hence limits cell shrinkage and onset of apoptosis (16). On the other hand, \(K^+\) and \(Cl^-\) channels, which are responsible for AVD, as well as \(Ca^{2+}\) channels, which are involved in \(Ca^{2+}\) influx and modulation of \(Ca^{2+}\)-sensitive steps during apoptosis, have been designated proapoptotic ion channels (16). It is noted that inhibition of \(Cl^-\) loss not only limits cell shrinkage but also prevents DNA degradation in human Jurkat cells following intrinsic activation of the apoptotic process (14). Inhibition of \(K^+\) loss will likewise limit the initial cell shrinkage but presumably also restrict decay of the membrane potential, which would normally favor \(Ca^{2+}\) influx and activation of apoptotic enzymes (32, 35, 67).

In the present study we find that hyperosmotic exposure, i.e., cell shrinkage, obtained by increasing the NaCl concentration in the RPMI growth medium, increases the toxicity of cisplatin in WT A2780 cells but has only a minor effect in RES A2780

**DISCUSSION**

**Deregulation of proapoptotic, volume-sensitive taurine release correlates with drug resistance.** In recent years it has become clear that cisplatin-induced cell shrinkage [apoptotic volume decrease (AVD)] in, e.g., EATC, reflects net loss of...
cells. In pig LLC-PK1 kidney cells, it has been shown that an increase in the extracellular NaCl concentration per se has no effect on cisplatin accumulation although a dramatic effect on cisplatin toxicity (11). In the case of EATC, the osmotic cell shrinkage has previously been shown to induce apoptosis via activation of a volume sensory pathway, which like the cisplatin-induced intrinsic pathway involves phosphorylation and activation of the transcription factor p53 and subsequently caspase-3 (see Fig. 1; Ref. 6). Hence, cisplatin-induced cell death in WT A2780 cells most probably reflects activation of the intrinsic pathway and a concomitant activation of the volume sensory pathway due to activation of transporters for ions/organic osmolytes (LRRC8A) and a subsequent reduction in cell volume. In RES A2780 cells, reduced sensitivity to cisplatin could partly reflect reduced activation of the volume sensory pathway due to the reduced LRRC8A expression and the inability of RES A2780 cells to activate their volume-sensitive transporters for ions, which in the present study is seen as an inability to perform RVD. A shift in p53 activation/downstream signaling in RES A2780 cell cannot be excluded from the present study.

Loss of amino acids including taurine is part of AVD, and it has been determined that EATC reduce their cellular content from 350 to 100 μmol/g dry wt within 20 h in the presence of 5 μM cisplatin (46). Cisplatin resistance was in EATC shown to correlate with a reduced activity of the volume-sensitive taurine transporter VSOAC (46), i.e., VSOAC could be characterized as a proapoptotic transporter. In daunorubicin-resistant EATC cells, it was demonstrated that TauT mRNA as well as TauT protein expression and activity was significantly reduced compared with WT EATC (47). On the other hand, taurine uptake in MDRI-transfected NIH3T3 cells was increased compared with parental fibroblasts, i.e., downregulation of taurine transporter in EATC with acquired drug resistance occurs independently of P-glycoprotein overexpression (47). In ELA cells, which have an innate resistance to cisplatin, TauT could be designated an antiapoptotic transporter as it has been demonstrated that cisplatin resistance correlated with an increased TauT expression/activity (59). Accordingly, Yasunaga and Matsumuru (66) characterize TauT as a colorectal cancer-specific cell surface marker, as TauT knockdown attenuates survival and enhanced drug sensitivity, whereas TauT overexpression increases cell survival and drug resistance. In the present work acquired resistance in A2780, similar to innate resistance in ELA, correlates with an increased TauT activity, which does not reflect shift in TauT mRNA or protein but more likely increased activity or translocation of TauT protein to the plasma membrane.

Taurine accumulates in A2780 cells as they develop resistance, and taurine per se has previously been reported to play a role in progression of apoptosis, i.e., taurine supplementation reduces apoptosis induced by drug exposure, ischemia, hypoxia, and glucose supplementation through mechanisms that involve 1) prevention of mitochondrial permeability transition/dysfunction and reduction in cytochrome c release (3); 2) prevention in drug-induced upregulation of Bax and Fas (37); 3) prevention of p53 activity; ROS generation, and Ca2+ mobilization (4, 65); 4) elevation in cellular Bcl-2 (55); 5) suppression of Apaf-1/caspase-9 apotosome assembly (57); as well as 6) reduction in caspase-8 and caspase-9 expression (58).

Reduced LRRC8A expression and volume-sensitive taurine release correlate with acquirement of cisplatin resistance. Members of the LRRC8 family have been found to be important entities of the swelling activated Cl− and taurine channel(s) (49, 62). Sequence comparisons have revealed that LRRC8 shares a common ancestor with pannexines, a protein family considered to form hexameric channels like LRRC8 and known to be involved in leakage of Ca2+ from the endoplasmic reticulum and ATP-dependent cell death (1, 50). The LRRC8 proteins are like pannexines composed of four transmembrane segments located at the NH2-terminal half of the protein and a leucine-rich repeat domain (LRRD) carrying up to 17 leucine-rich repeats located at the COOH-terminal end (1). Furthermore, LRRC8 contains putative sites that could be regulated through phosphorylation and ubiquitinylation (1). To date not much is known about the LRRC8 family and its cellular functions. The LRRC8 family was first described in a girl with congenital agamma-globulinemia and minor facial anomalies due to lack of B cells in peripheral blood (53). The patient was found to carry a chromosomal translocation on chromosome 9 causing a truncated LRRC8 gene, and the truncation was found to result in pro-B-cell arrest and thereby prevents B-cell maturation (53). In the present context it is of interest that LRRC8A has been demonstrated to be an essential component of VSOAC, i.e., LRRC8A knockdown almost blunted RVD and the concomitant swelling-induced taurine release in cancer cells (49, 62). In congruence we find that the impairment of swelling-induced taurine efflux and ability to volume regulate observed in cisplatin resistance in A2780 cells correlate with a decreased protein level of LRRC8A. LRRC8A mRNA is also reduced in RES A2780 cells compared with WT A2780 cells although not to the same extent as the LRRC8A protein expression, indicating that posttranslational ubiquitination and degradation of LRRC8A could be increased during acquirement of cisplatin resistance in A2780 cells as indicated by Bradley et al. (2). It is assumed that the functional VSOAC transport pathway is formed by six proteins from the LRRC8 family and that shift in stoichiometry of LRRC8A and other LRRC8 members affects channel activity (62). According to Qiu et al. (49) and Voss et al. (62), LRRC8 hexamers constitute to a common volume leak pathway for Cl− and amino acids including taurine. If this is the case, the differences observed with respect to time frame for activation and inactivation of volume-regulated anion channel (VRAC) and VSOAC (27) and sensitivity to cholesterol depletion (24, 61) following osmotic cell swelling could represent heterogeneity in the stoichiometry of LRRC8 components and/or that the substrate selectivity is differently regulated. Qiu et al. (49) show that mutation of T44 at the external part of TM1, which lines the permeability pore, has a considerable impact on the anion permeability.

In conclusion our data indicate that induction of apoptosis, i.e., cisplatin-induced loss of osmolites in WT A2780 cells correlates with increased LRRC8A expression whereas cisplatin resistance in RES A2780 cells correlates with reduced LRRC8A expression. Hence, shift in LRRC8A activity could be used as biomarker for apoptotic progress and acquirement of drug resistance. It is noted that other LRRD-containing proteins have been associated with induction of cell death, i.e., Nod1, which is a Apaf-1 like, LRRC8-containing protein, was found to regulate apoptosis through caspase-9 activation (in-
trinic path way but not caspase-8 (extrinsic pathway) (21). Likewise leucine-rich repeat and Ig domain containing 1 (LINGO-1) potenti ates neuronal apoptosis by inhibiting with no lysine kinase 3 (WNK3) activity (69).

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AUTHOR CONTRIBUTIONS


REFERENCES


