Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;26:809-820

Accepted: October 28, 2010

Pinpointing Differences in Cisplatin-induced Apoptosis in Adherent and Non-adherent Cancer Cells

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Key Words

Cell volume regulation • Glutathione • TauT activity • Programmed cell death • DNA damage • Cisplatin accumulation

Abstract

Platinum compounds are used in the treatment of cancer. We demonstrate that cisplatin-induced (10 μM) apoptosis (caspase-3 activity) is pronounced within 18 hours in non-adherent Ehrlich ascites tumour cells (EATC), whereas there is no increase in caspase-3 activity in the adherent Ehrlich Lettré ascites tumour cells (ELA). Loss of KCl and cell shrinkage are hallmarks in apoptosis and has been shown in EATC. However, we find no reduction in cell volume and only a minor loss of K^+ which is accompanied by net uptake of Na^+ following 18 hours cisplatin exposure in ELA. Glutathione and taurine have previously been demonstrated to protect cells from apoptosis. We find, however, that increase or decrease in the cellular content of glutathione and taurine has no effect on cisplatin-induced cell death in EATC and ELA. Nevertheless, knock-down of the taurine transporter TauT leads to a significant increase in apoptosis in ELA following cisplatin exposure. We find that cytosolic accumulation of cisplatin is similar in EATC and ELA. However, the nuclear accumulation and DNA-binding of cisplatin is significant lower in ELA compared to EATC. We suggest three putative reasons for the observed cisplatin insensitivity in the adherent tumor cells (ELA) compared to the non-adherent tumor cells (EATC): less nuclear cisplatin accumulation, increased TauT activity, and decreased anion and water loss.

Introduction

Cisplatin – cisplatin resistance

Platinum drugs including cisplatin (cis-diamminedichloroplatinum) are used in the treatment of more than 50% of all cancer patients with solid tumours [1]. Cisplatin is given intravenously, taken up from the blood stream by cells, translocated to the nucleus, where the major cisplatin cytotoxicity is exerted via binding to DNA (guanine, adenine). It is in general assumed that

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free cisplatin and small platinum drugs enter the cell via the copper transporter protein (CTR1) and the organic cation transporter (OCT1-3) [2-4]. A role of CTR1 in cisplatin accumulation is supported by the observation that knockdown of the transporter decreases accumulation of cisplatin [5]. Cisplatin binds to proteins in the bloodstream, in particular to those containing thiol groups, e.g. albumin, transferrin, and γ-globulin [6]. Cisplatin also binds to cytosolic sulphur-containing biomolecules, e.g. glutathione and methionine, and an elevated cellular gluthatione content has been seen in cisplatin resistant human ovarian cancer cells [7]. Cisplatin binding/trapping and hence drug inactivation in cell resistance is debated [8]. Although multi drug resistance (MDR) is normally associated with elimination of cytotoxic drugs via the P-glycoprotein in resistant cancer cells [9], resistance to cisplatin seems not to be mediated by P-glycoprotein [10]. On the other hand, P-type ATPases (ATP7A and ATP7B) have been suggested to eliminate cisplatin from cells [11]. Cisplatin resistance may involve decreased drug uptake, detoxification, drug compartmentalization, increased DNA repair, and modulation/impairment of cell death signaling [5, 12, 13].

Apoptosis – Apoptotic volume decrease

Programmed cell death or apoptosis is a fundamental biological mechanism, characterized by activation of the caspase cascade leading to elimination of unwanted or damaged cells from a tissue [14-16]. Apoptosis is mediated through extrinsic (death-receptor) or intrinsic (mitochondrial) pathways and involves cysteine-aspartic acid proteases (caspases) that are grouped into initiator caspases and executioner caspases (caspase-3, 6 and 7). The intrinsic pathway can be activated by cellular stress, for instance chemotherapeutic drugs and DNA-damage. DNA-damage, caused by the chemotherapeutic drug cisplatin, is detected by the serine/threonine-specific protein kinase (ataxia telangiectasia mutated, ATM) that subsequently activates the tumor suppressor gene p53 through phosphorylation [17]. p53 is capable of initiating the intrinsic pathway by signaling to the downstream Bcl-2 family proteins. As a response to stress, the pro-apoptotic Bcl-2 family proteins Bak and Bax in conjunction cause the mitochondrial outer membrane to become leaky which not only compromises the cells energy production but also releases cytochrome c into the cytosol. In the cytosol cytochrome c, dATP and Apaf-1 together with the procaspase-9 form the apoptosome complex which cleaves and activates caspase-9. Activated caspase-9 then activates the executioner caspase-3 and the cell subsequently becomes eliminated by apoptosis. In Ehrlich ascites tumor cells (EATC), it has been demonstrated that caspase-3 activity is significantly increased within 10 hours exposure to 5 μM cisplatin [18]. An important feature of apoptosis is the redistribution of intracellular monovalent ions plus loss of organic osmolytes, which accounts for one of the classical hallmarks of apoptosis, i.e., cell shrinkage, also known as "Apoptotic Volume Decrease" (AVD). In a recent paper, we described three distinctive AVD stages in EATC following exposure to cisplatin: An early AVD_1 (4-12 h), associated with a 30% cell water loss, a transition stage AVD_T (12 to 32 h) where cell volume is partly recovered, and a secondary AVD_2 (past 32 h) where the cell volume was further reduced [18]. AVD_1 and AVD_2 were coupled to net loss of Cl^-, K^+, and amino acids, whereas Na^+ and Cl^- were accumulated during AVD_T [18].

Cell-protective roles of taurine

Taurine is an inert sulphonic amino acid which is accumulated via the Na^+-dependent transporter TauT and released through a volume sensitive leak pathway [19]. Taurine is known through its contribution to the cellular pool of organic osmolytes and hence cell volume. However, within recent years it has turned out that taurine protects cells through stabilization of membrane/proteins, elimination of reactive oxygen species (ROS), and modulation of ion channel activity [19]. Several experiments have indicated that taurine also prevents apoptosis. Taurine inhibits ischemia-induced apoptosis in rat neonatal cardiomyocytes by prevention of apoptosome assembly, cleavage and activation of caspase-3/-9 [20, 21]. In mouse hypothalamic nuclei taurine reduced ischemia-induced caspase-8 and caspase-9 expression thus attenuating apoptosis [22]. It has been reported that in rat testes, pre-treatment with taurine, suppressed arsenic-induced activation of caspase-3 thus suppressing apoptosis [23]. *In vivo* experiments in rats have shown that taurine reduces the cisplatin-induced nephrotoxicity by effectively attenuating the accumulation of platinum (cisplatin) in the renal cells [24]. Furthermore, preloading Jurkat T-lymphocytes with 40 mM taurine is demonstrated to significantly inhibit CD95-induced DNA fragmentation and apoptotic cell shrinkage [25].

In multidrug resistant EATC it has been shown that drug resistance involves down regulation of TauT [26] and down regulation of the volume regulated Cl^- channel (VRAC), which prevents AVD_1 and hence induction of apoptosis [18]. Cisplatin exposure is known to increase the production of ROS via NADPH oxidases [27], and

we have recently demonstrated that ROS activate Cl^- loss via a KCl cotransporter in non-adherent EATC but via VRAC in adherent Ehrlich Lettré ascites cells (ELA) [28]. In a recent work, it was also shown that cisplatin-induced apoptosis was prevented in kidney cells following overexpression of TauT [29]. Hence, TauT over-expression may prevent/attenuate cell shrinkage and protect cells from apoptotic cell death. As ion movement and TauT expression are important players in the apoptotic process we have compared the sensitivity to cisplatin in the non-adherent EATC and the adherent ELA, as well as the role of TauT activity and ion movement in cisplatin-induced apoptosis in the two cell types.

Materials and Methods

^{3}H-labelled taurine (GE Healthcare, UK). Lipofectamine 2000 (Invitrogen, Denmark). Sodium dihydrogen phosphate anhydrous, suprapur, sodium chloride, HNO_3 65% pro analysis (Merck, Darmstadt, Germany). Albumin from human serum 96-99%, cis-diammineplatinum (II) Sodium hydroxid (1.0/0.1M prepared from 35% NaOH), HCl 30% and ammonia solution 28%, (Prolabo, VWR International, Pennsylvania, USA). Ammonium acetate (Riedel-de Haën, Seelze, Germany). Platinum standard, 1000 µg/mL in 10% HCl, SCP Science (Clark Graham, Canada), ClinChek® Controls, Serum control (lyophilized for trace elements, level 1, range 7.1-11µg $Pt \cdot L^{-1}$ (Recipe, Munich, Germany). All other compounds were from Sigma-Aldrich (St Louis, MO, U.S.). The following stock solutions were prepared: Cisplatin (3.33 mM in RPMI medium, ultra sound), ortho-phtalaldehyde (OPA, 81,92 mM, 1 ml methanol, 125 µl K-borat buffer, 12.5 µl 3-Mercaptopropionic acid). Buthionine sulfoximine (BSO,10 mM, ddH_2O), N-Acetyl Cysteine (NAC, 25 mM, in RPMI medium).

Inorganic Media

Phosphate buffered saline (PBS) contained 137 mM NaCl, 2.6 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. Isotonic standard NaCl medium contained 143 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid. pH was in all media adjusted to 7.4.

Cell cultures

Non-adherent Ehrlich ascites mouse tumor cells (EATC) and adherent Ehrlich Lettre ascites mouse tumor cells (ELA, ATCC, USA) were grown in Cellstar© T75 flasks (75 cm^2) in RPMI-1640 medium supplemented with 10% fetal bovine serum plus 100U/ml penicillin and 0.1 mg/ml streptomycin. Cell lines were kept at 37°C/5% CO_2/100% humidity. EATC were maintained by dilution of the cell suspension in fresh RPMI-1640 medium every 3-4 days. ELA were passed on every 3-4 days using 0.5% trypsin in PBS to detach the cells. Only passages 6-30 were used for experiments.

Construction of and transfection with TauT miRNA

Plasmids for microRNA (miRNA) knock-down of TauT was generated using BLOCK-iT™ Pol II miR RNAi Expression Vectors (Invitrogen, Taastrup, Denmark). miRNA targeting mouse TauT were designed using Invitrogens on-line design tool generating sense and antisense single stranded DNA strings (ssDNA). Two miRNA were designed as follows:

miR-TauT-1-sense: 5'-TGC TGT TCT GAT GTG TAC TGG CCT ATG TTT TGG CCA CTG ACT GAC ATA GGC CAA CAC ATC AGA A -3',

miR-TauT-1-antisense: 5'- CCT GTT CTG ATG TGT TGG CCT ATG TCA GTC AGT GGC CAA AAC ATA GGC CAG TAC ACA TCA GAA C -3',

miR-TauT-2-sense: 5'- TGC TGT AAA GAT CAA CCA AGG ATG TGG TTT TGG CCA CTG ACT GAC CAC ATC CTG TTG ATC TTT A -3',

miR-TauT-2-antisense: 5'- CCT GTA AAG ATC AAC AGG ATG TGG TCA GTC AGT GGC CAA AAC CAC ATC CTT GGT TGA TCT TTA C -3'.

The ssDNAs were annealed in anneal buffer generating a dsDNA, which were then ligated into pcDNA™6.2-GW/EmGFP-miR generating miR-TauT-1 and -2. Control vector was pcDNA™6.2-GW/EmGFP-miR-neg. Plasmids were transformed into omnimax T-1 E.coli (Invitrogen, Taastrup, Denmark) and selected using spectinomycin as antibiotic (50 µg/µl). Plasmid inserts were confirmed by sequencing. The plasmids were purified using the Nucleobond AX Plasmid DNA purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. EATC and ELA were transfected with the purified plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 12 µg vector and 30 µl Lipofectamine 2000 was used per T75 tissue flask containing approximately $6 \cdot 10^5$ cells. Transfections were performed 48 hours prior to the experiments. Transfected/transformed cells were used for estimations of taurine influx, caspase-3 activity and cell volume in TauT knock down cells.

Western blotting

1-2 · 10^6 cells were washed in ice-cold PBS and lysed in lysis buffer (1% SDS, 150mM NaCl, 20 mM Hepes, 1 mM EDTA, 0,5% Triton x-100, 1 mM NaVO3 and 1% protease inhibitor mix). Protein content was estimated (DC assay, BioRad), equalized, and lysates mixed with sample buffer (10% SDS, 1% bromophenol blue, 10% glycerol, 5 mM Tris–Cl pH 6.8). SDS-PAGE gel electrophoresis was carried out in NuPAGE precast 10% Bis–Tris gels (Invitrogen) in NOVEX chambers under reducing and denaturing conditions (NuPAGE MOPS SDS running buffer, Invitrogen), using BenchMark protein ladder (Invitrogen). Proteins were transferred to nitrocellulose membranes using NuPAGE transfer buffer (Invitrogen). Membranes were Ponceau S stained to verify protein transfer, blocked for 2 h at 37° C in TBST (0.01 M Tris–HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk, and incubated overnight at 4° C with primary antibodies against TauT (TauT antibody from Yorkshire Bioscience;) or histone (loading control, histone antibody from Santa Cruz Biotechnology). Membranes were washed in TBST, incubated with secondary antibodies for 1 h, washed in TBST, and

developed using BCIP/NBT (KPL, Gaithersburg, MD, USA). Membranes were scanned and bands quantified using UN-SCAN-IT (Silk Scientific).

Glutathione content

EATC and ELA were grown in T75 tissue flasks at a density of 2–5·10^5 per ml. The cells were incubated for 20 hours with/without buthionine sulfoximine (BSO) and N-acetyl cysteine (NAC), and with/without 10 µM cisplatin for 18 hours. EATC cells were centrifuged. Cell pellets were washed once in PBS and resuspended in 1.2 ml MES buffer (0.4 M morpholino ethanesulfonic acid (MES), 0.1 M Na_2HPO_4, 2 mM ethylenediaminetetraacetic acid). ELA were washed once with PBS, MES buffer was added and the cells scraped off (rubber police man) the tissue flask. EATC and ELA cell homogenates were sonicated and 100 µl used for estimation of the protein content (Lowry procedure, bovine serum albumine (BSA) as protein standard) [30]. The residual homogenate was centrifuged (10,000 g, 15 min, 4°C) and the supernatant used for estimation of the total and oxidised glutathione content according to the manufacturer's protocol (Kinetic method, Caymans Glutathione Assay Kit) using a microplate reader (Bmg LabTechnologies, Offenburg, Germany). The cellular glutathione (µmol·g protein^{-1}) was estimated from the glutathione and the protein content.

Caspase-3 activity assay – apoptosis

EATC and ELA were grown in 75 cm^2 flasks at a density of 2–5·10^6. Following the incubation period with/without cisplatin, ELA were trypsinized and both EATC and ELA were centrifuged at 1,000 g for 6 min at 4°C. The pellets were washed once in PBS and lysed in ice-cold lysisbuffer, thoroughly resuspended and stored at -80°C over night. The cell lysates were subjected to 3 freeze-and-thaw cycles and 2 · 10 sec sonication to fully disrupt the cells and disperse cell debris. The cell lysates were then centrifuged 20,000 g for 5 min and the supernatants transferred to new eppendorf tubes. Protein contents in the supernatants were determined and adjusted with lysis buffer to equal concentration for activity measurements (4 µg/µl). Caspase-3 activity in cell lysates was estimated in 96-well plates using the ApoTarget™Caspase-3/CPP32 Colorimetric assay (Protease BioSource International,) according to the manufacturer's protocol by measuring protease activity towards the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) and estimating the p-nitroanilide (pNA) production. Absorbance was measured at 405 nm using a microplate reader (Bmg LabTechnologies, Offenburg, Germany). Experiments were performed in triplicate.

MTT assay – cell viability

The MTT calorimetric assay estimates the ability of cells to convert the yellow soluble tetrazolium salt 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble blue formazan precipitate. EATC and ELA were seeded in 96-well microplates at a density of 16·10^3 in 200 µl medium and incubated (37°C, 5% CO_2) overnight. After cisplatin treatment (10 µM) for 18 hours the growth medium was discarded and replaced with 100 µl fresh medium. The MTT solution (5 mg/ml sterilized PBS) was added to each well (25 µl/well) and the plate was incubated (37°C, 5% CO_2) for 3 hours. 100 µl of detergent SDS-HCl solution (5 ml 0.01 M HCl, 0.5 g SDS) was added to each well and mixed to lyse the cells and solubilize the colored formazan crystals. The samples were measured at 570 nm using a FLUOstar OPTIMA 96-well microplate plate reader (Bmg LabTechnologies, Offenburg, Germany). Data obtained were reported in terms of relative cell viability compared to the nonstimulated control, the absorbance values assumed to be directly proportional to the number of viable cells. Each experiment was performed in triplicate.

Cell volume

Cell volumes were estimated essentially as described previously [14] by electronic cell sizing. In brief, ELA were trypsinized, centrifuged (45 sec at 600 g), resuspended in 5 ml standard NaCl medium and incubated for 5 min at 37°C. Absolute cell volumes were estimated after 10 fold dilution (final cell density ~ 90,000 cells/ml) in standard medium by electronic cell sizing in a Beckmann Multisizer III (Coulter, Luton, UK), calibrated with latex beads (diameter 14.89 µm, Coulter). The median of the cell volume distribution curves were analyzed using Coulter Multisizer AccuComp version 1.19 software. Media used for cell volume measurements were filtered (Millipore filters, 0.45 µm) before the experiments.

K^+ and Na^+ content

Na^+ and K^+ were estimated essentially as described previously [18]. Briefly, ionic content was measured on 1 ml samples of cell suspension (2 % cytocrit), separated from the incubation medium by centrifugation (14,000 g), lysed in ddH_2O and deproteinized with perchloric acid. K^+ and Na^+ were measured by atomic flame photometry (Perkin Elmer Atomic Absorption Spectrophotometer, model 2380). All values are corrected for trapped extracellular medium.

Taurine content

EATC and ELA were lysed by addition of 1.8 ml of 4% sulfonsalicylic acid. The ELA lysate was scraped of the flask with a rubber policeman. EATC and ELA were homogenized by several passages through a syringe needle (1.2 mm diameter). Aliquots were denaturated with NaOH and proceeded for estimation of the protein content (Lowry procedure, BSA used as protein standard). The residual homogenate was centrifuged (20,000g, 10 min) and the supernatant filtered (Milex-GV, 0.22 µm). Samples and standards were OPA-derivatized, separated and quantified by reversed phase high pressure liquid chromatography (Gilson: 322-Pump, 234-Autoinjector, 155-UV/VIS) using a Nucleosil column (Macherey-Nagel, C18, 250/4, 5 µM), an acetonitrile/phosphate (12.5 mM, pH 7.2) buffer gradient, and UV detection (330 nm). A taurine standard (1.0 mM) was used for quantification and the cellular amino acid content (µmol·g protein^{-1}) was estimated from the amino acid and the protein content.

Taurine influx

EATC, grown in T75 flasks, were pelleted by centrifugation and resuspended in 8 ml isotonic NaCl medium. Taurine influx was initiated by addition of ^{3}H-taurine ($2.4 \cdot 10^4$ Bq/ml, 24 nM taurine). Taurine influx was terminated after 1, 5, 10, 15, and 20 min incubation by transferring 1 ml to an Eppendorff tube, centrifugation for 1 min (16,000 g) and rapid aspiration of the supernatant. 200 µl 1M NaOH was added to the pellet and left over night for hydrolysis. Samples were vortexed and 20 µl used for estimation of the protein content by the Lowry procedure (BSA used as protein standard) and 150 µl used for estimation of cellular ^{3}H-labelled taurine radioactivity (β-scintillation counting, Ultima Gold TM). ELA, grown in six-well polyethylene dishes (80-90% confluence, 9.6 cm^2 per well), were washed three times by gentle aspiration/addition of 1 ml experimental solution. Cells in wells 1 to 5 was used for estimation of taurine influx, whereas cells in well 6 were used for estimation of the average protein content (mg protein per well) by the Lowry procedure (BSA used as protein standard). Taurine influx was estimated by exposing the cell to isotonic NaCl medium containing ^{3}H-taurine ($2.4 \cdot 10^4$ Bq/ml, 24 nM taurine) for 3, 6, 9, 12 and 15 minutes. Taurine influx was terminated by aspiration of the extracellular medium followed by addition and aspiration of 1 ml ice cold $MgCl_2$ (100 mM). Cells were lyzed by addition of 200 µl 96% ethanol, the ethanol was evaporated and the cellular ^{3}H-taurine radioactivity subsequently extracted by addition of 1 ml ddH_2O (1 h). Water extracts plus two additional washouts from well 1 to 5 were transferred to a scintillation vial for estimation of ^{3}H-labelled taurine radioactivity as for EATC cells. Total ^{3}H taurine radioactivity taken up by the cells (EATC: cpm per 150 µl sample; ELA: cpm per well) was plotted versus time and the taurine influx (nmol·mg protein^{-1}·min^{-1}) was subsequently estimated from the slope (cpm·min^{-1} per well), the protein content (mg protein per well), the extra-cellular specific activity (nmol/cpm) and the counting efficiency for ^{3}H.

Nuclear and cytosolic fractionation

EATC and ELA were incubated with cisplatin for 18 hours. After the incubation EATC and ELA (trypsinized) were transferred to centrifuge tubes and centrifuged at 700 g, 45 sec at room temperature. The cells were washed three times with PBS by successive centrifugation. Cells were lysed with 2 mL ice-cold lysis buffer and a 10 µL subsample was transferred to a new tube for later estimation of protein concentration (DC Protein Assay, Bio-Rad). The lysates were transferred to micro centrifuge tubes and centrifuged for 5 min at 600 g, 4°C. These pellets constituted the nuclear fractions. The supernatants were transferred to new microcentrifuge tubes and centrifuged at 5500 g for 15 min 4°C. The supernatants, constituting cytosolic fractions, were collected in a new tube for determination of Pt. The remaining pellets were discarded. The nuclear pellets were washed three times with NaCl/KCl (30 mM/120 mM, 600 g, 4°C, 5 min) and the pellet was used for determination of platinum by inductively coupled plasma mass spectrometry (ICP-MS). For total Pt estimation in nucleus the pellets were dissolved in 100 µL 65% HNO_3 and left to dissolve for at least 24 hours at room temperature and vortexed carefully several times.

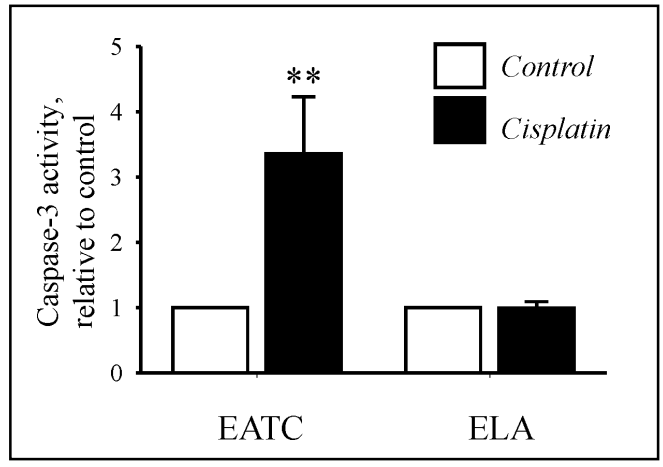

Fig. 1. Caspase-3 activity in non-adherent EATC and adherent ELA following cisplatin exposure. Caspase-3 activity was estimated in EATC and ELA after 18 h exposure to 10 µM cisplatin, using a colorimetric assay. Values for cisplatin treated cells (black bars), given relative to non-treated cells (open bars), represent means ± SEM of 7 (EATC) and 3 (ELA) sets of independent experiments. **significantly increased compared to untreated control ($p<0.01$).

Cisplatin content - inductively coupled plasma mass spectrometry

The content of cisplatin was estimated by estimating the total platinum content using ICP-MS (Elan 6000, Perkin Elmer Sciex, Concord, ON, Canada). As platinum is one of the rarest elements, occurrence is less than 0.003 ppb in the Earth's crust, the detected platinum in the samples is taken to originate from the cisplatin exposure. All samples, nuclear and cytosolic, were diluted with 0.1% HCl and 0.65% HNO_3 to a concentration within the calibration curve, 0.1 – 10 µg Pt·l^{-1}. Accuracy of the method was investigated by analyzing a serum control sample according to the manufacturer's protocol.

DNA purification – estimation of DNA bound cisplatin

Cellular genomic DNA from EATC and ELA was purified using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany). $2 \cdot 10^6$ cultured cells (ELA, trypsinized prior to the purification) were washed 3 times in PBS and lysed in 300 µl cell lysis solution. Proteins were precipitated using 100 µl protein precipitation solution. The supernatant was transferred to a tube containing 300 µl isopropanol. After centrifugation (16,000 g, 5 sec.) the supernatant was discarded and the DNA pellet washed in 300 µl 70% ethanol. The supernatant was discarded and the DNA pellet dissolved in 50 µl DNA hydration solution, incubated at 65°C for 1 hour in order to fully dissolve the DNA. The samples incubated at room temperature overnight with gentle shaking. The A_{260}/A_{280} ratio was measured the following day using a spectrophotometer and the DNA concentration is given in µg/µl. The sample was evaporated, treated with 65 % HNO_3 and the amount of Pt was determined by ICP-MS.

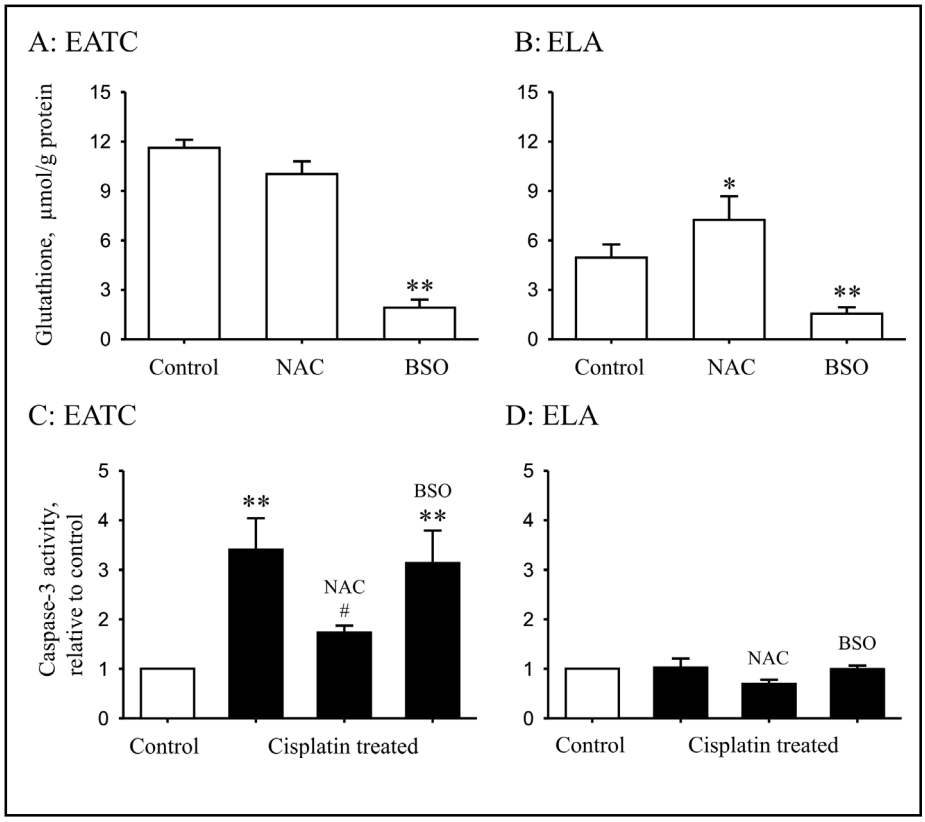

Fig. 2. Cellular glutathione content and its impact on cell death (apoptosis) in EATC and ELA following cisplatin exposure. Glutathione content in EATC (Panels A, C) and ELA (Panels B, D) was increased or reduced by 20 hours preincubation with 2.5 mM NAC or 0.01 mM BSO, respectively. Cisplatin treated cells were exposed to 10 µM cisplatin (18 h, black bars). Control cells (white bars) were exposed to neither NAC/BSO nor cisplatin. Cell homogenates were prepared and the protein content, glutathione content and caspase activity estimated in the homogenates. Glutathione content (µmol·g protein^{-1}) and caspase-3 activity (relative to control cells not treated with cisplatin) are given as mean values ± SEM of 3 (EATC and ELA) sets of individual experiments for each cell line and experimental setup. Asterix indicates significantly increased compared to untreated control (*: $p<0.05$; **: $p<0.01$). # significantly reduced compared to cisplatin treated cells ($p<0.05$).

Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM) of at least three independent sets of experiments. Statistical significance was estimated by Student's t-test (one-tailed) or ANOVA, Tukey test. Level of significance is indicated by * ($p < 0.05$) and ** ($p< 0.01$).

Results

It has previously been shown that exposure to cisplatin induces caspase-3 activity, i.e., apoptosis, in non-adherent EATC [18]. This is confirmed in Fig. 1 where it is seen that 18 hours exposure to 10 µM cisplatin increases the caspase-3 activity significantly in EATC. Adherent ELA, on the other hand, shows no significant increase in caspase-3 activity within 18 hours treatment with 10 µM cisplatin (Fig. 1). Apart from inducing apoptotic cell death cisplatin is reported to induce necrosis [31, 32]. However, chromatin condensation assay (Hoechst staining, $2.5 \cdot 10^{-4}$ mg Hoechst 33258/ml for 20 min) indicated that 6% of the EATC were stained following exposure to 10 µM cisplatin for 18 hours, which is to be compared with 0.6% in control cells. These data support our conclusion that EATC enters apoptosis following cisplatin treatment. To evaluate whether cisplatin induces cell death in ELA we used a MTT cell death assay, which revealed that 83±5% (n = 3) of ELA survived 18 hours exposure to 10 µM cisplatin. This is to be compared with less than 40% viability in EATC following 18 hours exposure to 5 µM cisplatin [18]. Hence, 10 µM cisplatin induces only minor caspase-3 independent cell death in ELA within 18 hours.

Does cellular glutathione regulate cisplatin-induced apoptosis?

Glutathione binds cisplatin [33] possibly inactivating the drug and an elevated cellular glutathione content has been demonstrated in cisplatin resistant cells [7]. To test whether increased cellular glutathione content could account for the lack of caspase-3 activity in cisplatin treated ELA we estimated the glutathione content in EATC and ELA. From Fig. 2 (A, B) it is seen that cellular gluta thione content is more than two fold higher in EATC (11.6 ± 0.5 µmol·g protein^{-1}) compared to ELA (5.0 ± 0.8 µmol·g protein^{-1}). Oxidized gluthatione was estimated at 2.0 ± 0.3 µmol·g protein^{-1} (n = 4) and 1.2 ± 0.6 µmol·g protein^{-1} (n = 7) in EATC and ELA, respectively. Hence, 83% and 75% of the cellular glutathione is in a reduced form in EATC and ELA cells, respectively. Preincubating the cells with N-acetyl cysteine (NAC), i.e., the precursor for glutathione synthesis, had no effect on the glutathione content in EATC, which was already high,

	EATC Non-adherent	ELA Adherent
Taurine content, μmol·g protein^{-1}	22 ± 10 (19)	62 ± 10 (4)*
Taurine influx, nmol·g protein^{-1}·min^{-1}	0.015 ± 0.001 (3) 0.003 μM extracellular taurine	1.13 ± 0.45 (4) 0.037 μM extracellular taurine
K_m for taurine, μM	42	20
V_{max}, nmol·g protein^{-1}·min^{-1}	206	611

Table 1. Taurine content and TauT kinetics in EATC and ELA. Taurine content (μmol·g protein^{-1}) was estimated by HPLC technique. Taurine influx was estimated by tracer technique at an extracellular taurine concentration of 0.003 μM (EATC) and 0.037 μM (ELA). Number of experiments is indicated in brackets. K_m values, indicating the extracellular taurine concentration required to obtain 50% of the maximal taurine uptake (V_{max}), are previously published values, i.e., EATC [26] and ELA [40]. V_{max} is estimated from the Michaelis Menten equation, i.e., taurine influx = (V_{max}·[taurine])/(K_m+[taurine]) using the taurine influx and K_m values. * indicates significantly higher compared to EATC.

whereas it increased the glutathione content in ELA (Fig. 2A, B). Preincubating the cells with buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase and hence the synthesis of glutathione, on the other hand, reduced the cellular glutathione content significantly in EATC and ELA (Fig. 2A, B). From Fig. 2C it is seen that NAC, even though it does not affect glutathione content, still reduces cisplatin-induced caspase-3 activity in EATC significantly. BSO, on the other hand, has no effect on the cisplatin-induced caspase-3 activity in EATC (Fig. 2C). Neither NAC nor BSO treatment affected the cisplatin-induced caspase-3 activity in ELA (Fig. 2D). As NAC had no detectable effect on glutathione content but reduced caspase-3 activity it seems reasonable to assume that NAC traps and reduces the effect of cisplatin. From Fig. 2 it is also seen that even though the glutathione content in BSO treated EATC and ELA is reduced to the same level, the caspase-3 activity is still significant in EATC and three times higher in EATC compared to ELA. Hence, the reduced effect of cisplatin in ELA compared to EATC (Fig. 1) does not correlate with the cellular glutathione content (Fig. 2).

Does cellular taurine modulate cisplatin-induced apoptosis?

A high cellular taurine content has previously been suggested to protect cells against apoptosis [20, 21, 24, 25]. The cellular taurine content is significantly higher in ELA compared to EATC (Table 1, Figs. 3A, B). To test whether an increased taurine content could save EATC from cisplatin-induced apoptosis we estimated the caspase-3 activity in EATC preincubated with high extracellular taurine. From Fig. 3A it is seen that 24 hours exposure to 20 mM and 40 mM extracellular taurine increased the cellular taurine content significantly, i.e., 10 fold and 20 fold, respectively. On the other hand, preincubation with β-alanine (5 mM, competitive TauT inhibitor) reduced the cellular taurine content in EATC by 90% (Fig. 3A). Similarly, preincubating ELA with 20 mM and 40 mM extracellular taurine resulted in an 8 fold and 10 fold increase in the cellular taurine content, respectively (Fig. 3B). However, modulation of the cellular taurine content has no significant effect on the cisplatin-induced caspase-3 activity in either EATC (Fig. 3C) or ELA (Fig. 3D). Thus, an altered cellular taurine content does not seem to protect EATC against or sensitize ELA to cisplatin-induced apoptosis.

Does TauT activity modulate cisplatin-induced apoptosis?

Chesney and Han recently suggested that it is the TauT activity *per se*, rather than the cellular taurine content, which protects kidney cells from cisplatin-induced apoptosis [29]. We have estimated the maximal taurine uptake (V_{max}) in EATC and ELA and from Table 1 it is seen that V_{max} is approximately 3 times higher in ELA compared to EATC. Furthermore, Western Blot analysis indicates that the expression of TauT is significantly higher in ELA compared to EATC (1.6 fold, $p < 0.05$), i.e., the TauT (70 kDa band) to histone protein ratio is 0.98 ± 0.24 (n=3) and 0.61 ± 0.11 (n=3) in ELA and EATC, respectively. Thus, the higher V_{max}/TauT expression could explain the increased resistance against cisplatin in ELA. From Fig. 4 it is seen that TauT knock down (miRNA) reduces taurine influx, i.e., TauT activity, in ELA by 50% (Fig. 4A) and that exposure to cisplatin induced a

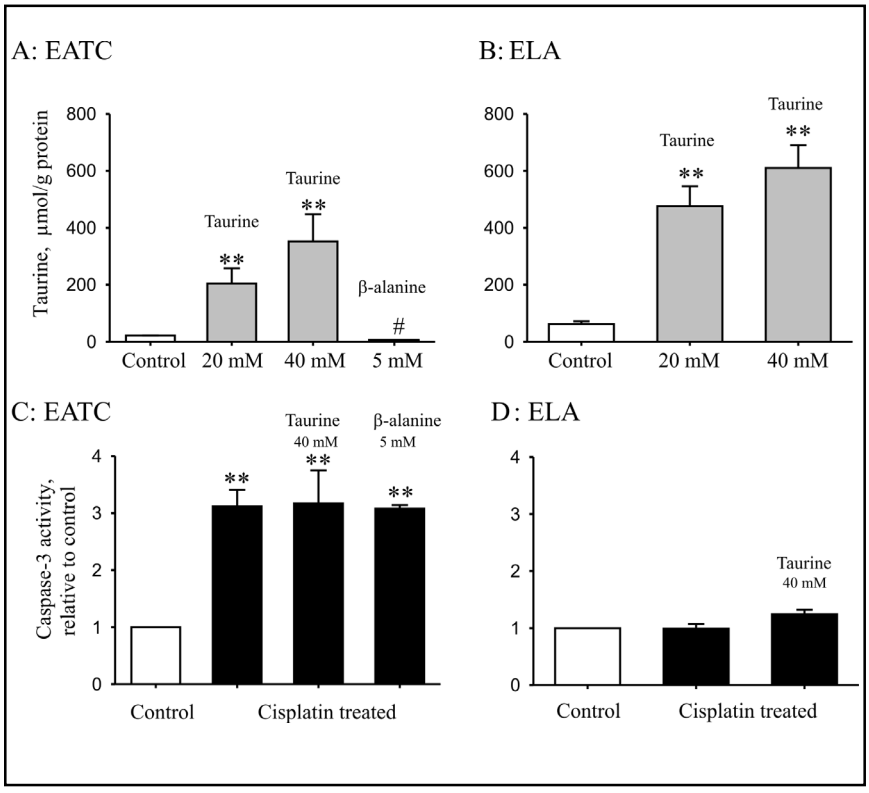

Fig. 3. Cellular taurine content and its impact on cell death (apoptosis) in EATC and ELA following cisplatin exposure. Cellular taurine content in EATC (Panels A, C) and ELA (Panels B, D) was increased or reduced by 24 hours preincubation with 20 to 40 mM taurine or 5 mM β-alanine (grey bars), respectively. Cisplatin (10 µM, black bars), when present, was added and the cells incubated for another 18 hours. Control cells (white bars) were exposed to neither amino acids nor cisplatin. Cell homogenates were prepared and the protein content, taurine content and caspase activity estimated in the homogenates. Taurine content (µmol·g protein^{-1}) is given as mean values ± SEM of EATC 19 (control), 4 (20 and 40 mM taurine), 3 (5 mM β-alanine) and for ELA 4 (control), 5 (20 and 40 mM taurine) sets of individual experiments. Caspase-3 activity (relative to control cells, not treated with cisplatin) represents mean values ± SEM of 7 (EATC) and 3 (ELA) individual experiments.*significantly increased compared to untreated control. #significantly reduced compared to untreated control cells.

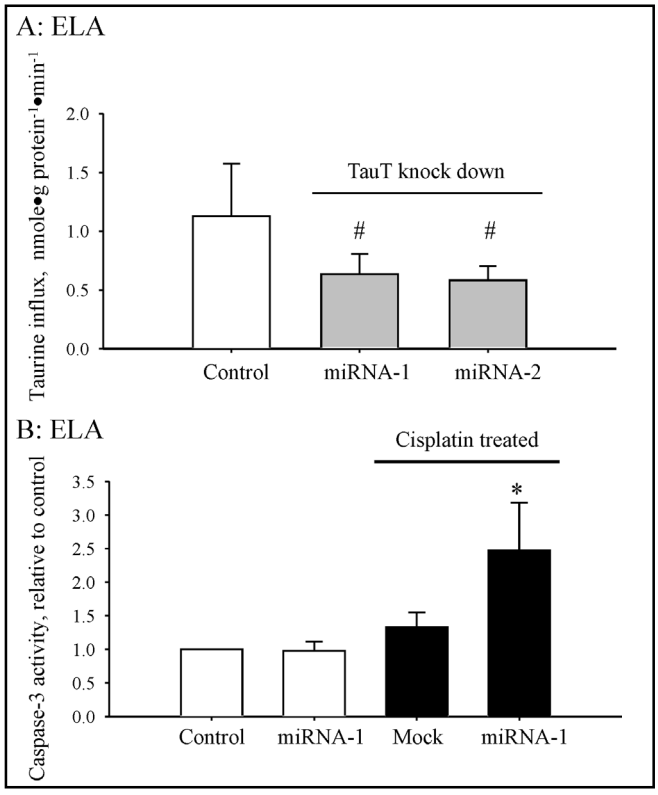

Fig. 4. Effect of TauT knock down on taurine influx and cell death in ELA following cisplatin exposure. TauT in ELA was knocked down by miRNA transfection (miRNA-1/miRNA-2). Mock indicates cells treated Mock miRNA. Cisplatin (10 µM), was added and the cells incubated for another 18 hours. Control cells (white bars) were exposed to neither miRNA nor cisplatin. Taurin influx (nmol·g protein^{-1}·min^{-1}, Panel A) and caspase activity (relative to control cells not treated with cisplatin) were estimated and given as mean values ± SEM of 4 (taurine influx) and 5 (caspase) sets of experiments. *significantly increased compared to untreated control. #significantly reduced compared to untreated control cells.

significant increase in caspase-3 activity in the knock down cells (Fig. 4B). Western Blot analysis indicates that the expression of TauT is significantly reduced by 19 % in ELA by the TauT knock down ($p < 0.05$), i.e., the TauT (70 kDa band) to histone protein ratio is 0.81 ± 0.11 (n=3) and 0.65 ± 0.07 (n=3) in control and knock down cells, respectively. Taken together these data indicate a correlation between TauT expression/activity and protection against cisplatin-induced caspase-3 activity.

Are cell volume and ion content affected in ELA upon cisplatin exposure?

Cell shrinkage, i.e., apoptotic volume decrease (AVD) is an initial event in cisplatin-induced apoptosis in EATC cells and it has been estimated that the cellular water content is reduced by 30% within 12 hours following exposure to 5 µM cisplatin [18]. As attenuation of this

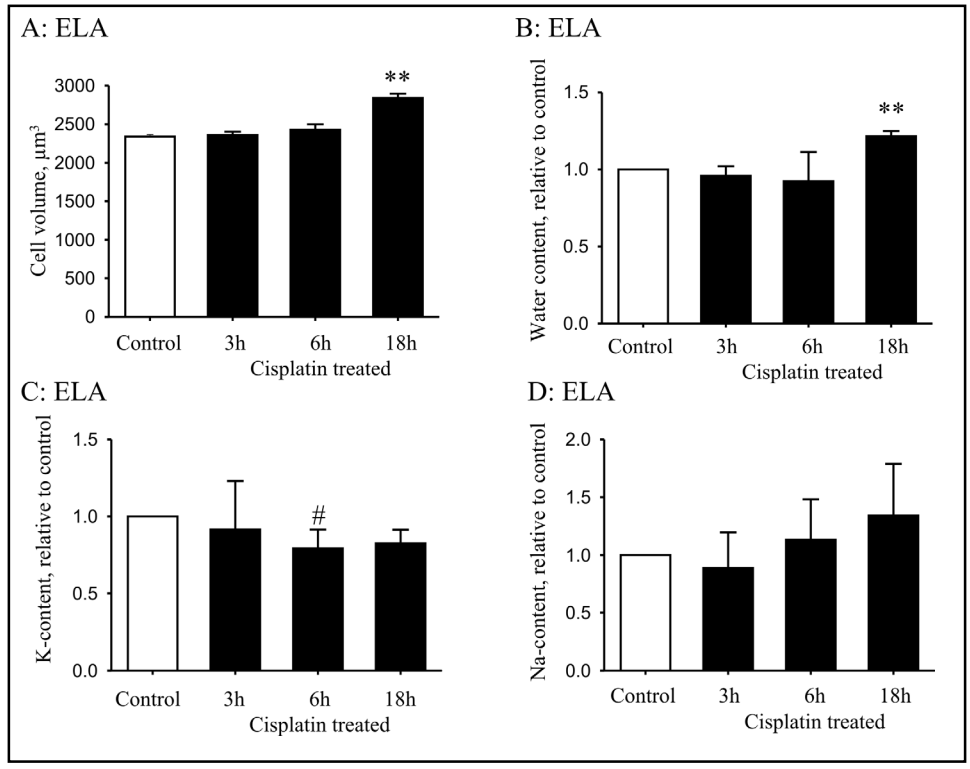

Fig. 5. Effect of cisplatin on cell volume, water and K^+/Na^+ content in ELA. ELA were exposed to 10 µM cisplatin for 3, 6 and 18 hours (black bars). Control cells (white bars) were not exposed to cisplatin. Cell volume was estimated using Coulter Counter, K^+ and Na^+ content was estimated using flame photometry and water content was estimated from the ionic measurements. Values are given in absolute values (μm^3, Panel A) and relative values ± SEM of 3 sets of individual experiments (Control Panel B: 6.7 ± 1.5 ml cell water·g dry wt^{-1}; Control Panel C: 99±16 µmol·g $protein^{-1}$; Control Panel D: 54±11 µmol·g $protein^{-1}$). #significantly decreased compared to untreated control. **significantly increased compared to untreated control ($p<0.01$).

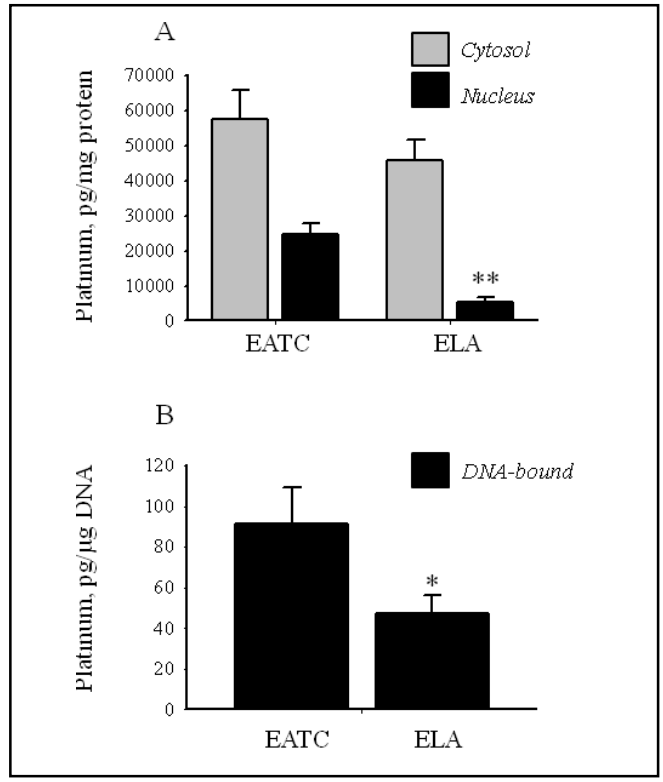

Fig. 6. Cytosolic and nuclear cisplatin content in EATC and ELA following cisplatin exposure. Cells, treated with 10 µM cisplatin (18h), were washed, lysed and protein content was estimated in the lysates. Cytosolic (grey bars) and nuclear (black bars) fractions were obtained by centrifugation and the cisplatin content in each fraction was estimated by ICP-MS. Cisplatin content (Panel A) is given relative to the original protein content and represents means ± SEM of 4 (EATC) and 4 (ELA) sets of experiments. Genomic DNA (Panel B) was purified using the Qiagen Gentra Puregene Cell Kit. The DNA was quantified and the DNA-bound cisplatin was estimated by ICP-MS. Cisplatin content is given relative to the DNA content and represent means ± SEM of 3 (EATC) and 3 (ELA) sets of experiments. **nuclear content in ELA significantly lower compared to EATC ($p<0.01$). *DNA bound cisplatin in ELA significantly lower compared to EATC ($p<0.05$).

water loss reduces the cisplatin-induced caspase-3 activity in EATC [18], we estimated the cell volume and water content in ELA exposed to 10 µM cisplatin for 3, 6 and 18 hours. From Fig. 5A it is seen that no cell shrinkage is detected in ELA after 3 and 6 hours, whereas as slight but significant increase in cell volume is seen after 18 hours exposure to cisplatin. The water content is similarly unaffected within the first 6 hours and increased significantly after 18 hours exposure to cisplatin (Fig. 5B).

AVD is associated with K^+ loss and Na^+ uptake [18] and to test whether the unaffected cell volume reflected a complete balance between K^+ loss and Na^+ uptake we estimated the K^+/Na^+ content in ELA following cisplatin

exposure (3, 6, 18 hours). From Figs. 5C and 5D it is seen that the cellular K^+ content is reduced significantly after 6 hours cisplatin exposure and that this loss is balanced by Na^+ uptake. It is emphasized that ion movements are quantitatively limited in ELA following cisplatin treatment compared to the ion movements seen in EATC [18].

Cisplatin accumulation in the nucleus and binding to DNA

The reduced sensitivity towards cisplatin in ELA compared to EATC could reflect differences in the nuclear uptake and DNA binding. From Fig. 6A it is seen that cisplatin is accumulated to almost the same extent in the cytosol in both cell lines. However, the nuclear cisplatin content in ELA is significantly lower compared to EATC ($p<0.001$). The lower nuclear cisplatin content is reflected in less DNA bound cisplatin (Fig. 6B). Thus, the reduced cisplatin sensitivity in ELA compared to EATC could reflect the reduced nuclear accumulation and DNA binding of cisplatin.

Discussion

Cisplatin resistance and accumulation

Cisplatin is used in the treatment of various solid tumours and it has turned out that cisplatin treatment, just like treatment with other anticancer agents, results in resistance towards the drug.

We find that ELA, which is an adherent sub-line of EATC, do not enter apoptosis (Figs. 1 and 5) following exposure to 10 μM cisplatin (18 h). EATC, on the other hand enters apoptosis following 18 hours exposure to 10 μM and 5 μM cisplatin (Fig. 1; [18]). Cisplatin resistance may involve modulation of cisplatin uptake via the copper transporters (CTR1, cisplatin influx) and the organic cation transporters (OCT1-3, cisplatin influx) or elimination through P-type ATPases (7ATPA and 7ATPB, cisplatin efflux) [34]. Cisplatin release seems not to be associated with removal via the P-glycoprotein [10]. Micro-array data indicate that both OCT and ATP7B are expressed in EATC and ELA, whereas qPCR (CTR1-fw: 5'-ACC ACC TCA GCC TCA CAC-3', CTR1-rv: 5'-AGA CCC TCT CGG GCT AT-3´; Stratagene MX4000 Real Time Thermal cycler) indicated that CTR1 is present in both cell lines (unpublished data). As ELA are an adherent cell line the cells are polarized and many of the membrane bound transporters localized to either the basolateral and/or the apical plasma membrane. However, the spatial localization of the cisplatin transporting systems in ELA vs. EATC is not known. Data in Fig. 6 clearly indicates that cisplatin is accumulated in the cytosol almost to the same extent in both cell lines. However, cisplatin accumulation and binding to DNA in the nucleus are more profound in EATC compared to ELA (Fig. 6). The amount of cisplatin bound to DNA in EATC is sufficient to induce significant cell death (Figs. 1, 2 and 3; [18]), whereas the amount bound in ELA appears to be insufficient (Fig. 1). Hence, there seems to be a correlation between nuclear/DNA-bound cisplatin and caspase-3 activity. It can not be excluded that ELA have additional DNA-repair mechanisms to circumvent the cisplatin-induced damage.

Glutathione in cisplatin resistance

Cisplatin binds to reduced glutathione (GSH) and cell resistance towards cisplatin has been associated with an elevated level of cellular GSH [35]. The cellular glutathione content is not only higher in the cisplatin sensitive EATC cells but a larger fraction is found in its reduced form. Hence, the difference in cisplatin sensitivity in EATC and ELA seems not to represent trapping of cisplatin by glutathione. Furthermore, reducing the cellular content in ELA (BSO treatment) does not sensitize ELA towards cisplatin (Fig. 2). Treating the cells with NAC, which is a precursor for GSH, increased the GSH content in ELA (1.5 fold) significantly but had no effect on the GSH content in EATC. However, NAC treatment reduced the caspase-3 activity significantly in EATC compared to the untreated control. NAC binds anticancer drugs directly [33] and the observed effect of NAC treatment in the present investigation does not directly indicate an involvement of GSH in cisplatin trapping. It is noted that the cellular GSH content following BSO treatment in EATC and ELA is quantitatively similar, but even under these conditions only EATC enter apoptosis. Hence, the GSH depletion has no effect on cisplatin-induced apoptosis in EATC and ELA.

Taurine and TauT in cisplatin resistance

Taurine is previously reported to protect cells against apoptosis. This effect is manifested by attenuation of platinum (cisplatin) accumulation [24], suppression of the activation of caspase-3 [36], reduction in caspase-8 and caspase-9 expression [22], prevention of caspase-3/-9 cleavage and assembly of the apoptosome [20, 21], as well as inhibition of DNA fragmentation and apoptotic cell shrinkage [25]. We find that neither taurine enrichment nor taurine depletion have any effect on the cisplatin-induced caspase-3 activity, i.e., cellular taurine does not

rescue EATC from cisplatin-induced apoptosis. Han and Chesney recently demonstrated that forced overexpression of the taurine transporter TauT protects LLC-PK1 proximal tubular renal cells against cisplatin-induced apoptosis *in vitro* and they suggested that functional TauT plays a critical role in protecting against cisplatin-induced nephrotoxicity, possibly by attenuation of a p53-dependent pathway [29]. Cisplatin is shown to increase p38 activity [37] and p38 is known to be upstream of activation of p53 [38]. It is thus possible that the effect of TauT could involve an inhibition of p38. This has not yet been tested. ELA have an increased TauT expression and activity compared to EATC (Table 1) and knock down of TauT (miRNA) results in significantly reduced TauT activity which is accompanied by significantly increase in cisplatin-induced caspase-3 activity (Fig. 4). It is noted that long-term exposure to high extracellular concentrations of taurine down regulates TauT expression [19], i.e., a reduced TauT expression in EATC and ELA following pre-exposure to high taurine concentration could balance a putative beneficial effect of high intracellular taurine content.

Cisplatin resistance in adherent vs. non-adherent cancer cells

In the present work we demonstrate that the adherent ELA are less responsive towards cisplatin-induced apoptosis, i.e., they show minor cell death (MTT assay) but no significant increase in caspase-3 activity after 18 hours exposure, limited ion movements and no reduction in cell volume (AVD) compared to the parental non-adherent EATC (Figs. 1 and 5, [18]). Multidrug resistant (MDR) EATC are resistant to cisplatin as evidenced by increased viability and less caspase-3 activation compared to wild type EATC [18]. The reduced sensitivity to cisplatin in MDR EATC was correlated with a low activity of the volume-sensitive Cl^- channel [18]. Janson and coworkers have demonstrated that cisplatin resistance in malignant mesothelioma cells involves a marked reduction in the $Na^+,K^+,2Cl^-$ cotransport (NKCC1) activity [39]. However, the reduced NKCC1 activity was not important for the cisplatin resistance [39]. In agreement we find that NKCC activity counteracts ion and water loss during apoptosis [18]. It may be speculated that the lack of cisplatin-induced apoptosis in ELA involves reduced nuclear cisplatin accumulation, increased TauT expression and quantitatively limited anion movements. The latter could reflect low K^+,Cl^- cotransport (KCC) activity and/or low Cl^- channel activity in ELA compared to EATC. As we have recently demonstrated that exogenous H_2O_2 activates KCC cotransport in EATC but Cl^- channels in ELA and other adherent cells [28], and as cisplatin is known to increase the ROS production [27], it is suggested that the limited anion movement in ELA cells reflects the lack of cisplatin induced KCC transport activity. Comparing ELA, which in the present investigation is shown be cisplatin resistant, with cisplatin resistant MDR EATC, reveals similarities, i.e., restriction in ion movements, and differences in TauT expression, i.e., TauT is down regulated in MDR but upregulated in ELA.

Acknowledgements

The present work was supported by The Danish Council for Independent Research / Natural Sciences (Grant 272-07-0530, 272-08-0170, 272-07-0258), The Danish Council for Independent Research / Medical Sciences (Grant 271-08-0520). The technical assistance of Dorthe Nielsen and Søren Lek Johansen are gratefully acknowledged.

References

1 Todd RC, Lippard SJ: Inhibition of transcription by platinum antitumor compounds. Metallomics 2009;1:280-291.

2 Ciarimboli G, Ludwig T, Lang D, Pavenstadt H, Koepsell H, Piechota HJ, Haier J, Jaehde U, Zisowsky J, Schlatter E: Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2. Am J Pathol 2005;167:1477-1484.

3 Ishida S, Lee J, Thiele DJ, Herskowitz I: Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci U S A 2002;99:14298-14302.

4 Lin X, Okuda T, Holzer A, Howell SB: The copper transporter CTR1 regulates cisplatin uptake in Saccharomyces cerevisiae. Mol Pharmacol 2002;62:1154-1159.
5 Stavrovskaya AA: Cellular mechanisms of multidrug resistance of tumor cells. Biochemistry (Mosc) 2000;65:95-106.
6 Kasherman Y, Sturup S, Gibson D: Is glutathione the major cellular target of cisplatin? A study of the interactions of cisplatin with cancer cell extracts. J Med Chem 2009;52:4319-4328.
7 Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME: High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci U S A 1992;89:3070-3074.
8 Mestdagh N, Pommery N, Saucier JM, Hecquet B, Fournier C, Slomianny C, Teissier E, Henichart JP: Chemoresistance to doxorubicin and cisplatin in a murine cell line. Analysis of P-glycoprotein, topoisomerase II activity, glutathione and related enzymes. Anticancer Res 1994;14:869-874.
9 Gottesman MM, Pastan I: Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62:385-427.
10 Ren L, Xiao L, Hu J: MDR1 and MDR3 genes and drug resistance to cisplatin of ovarian cancer cells. J Huazhong Univ Sci Technolog Med Sci 2007;27:721-724.
11 Kuo MT, Chen HH, Song IS, Savaraj N, Ishikawa T: The roles of copper transporters in cisplatin resistance. Cancer Metastasis Rev 2007;26:71-83.
12 Krishna R, Mayer LD: Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 2000;11:265-283.
13 Lothstein L, Israel M, Sweatman TW: Anthracycline drug targeting: cytoplasmic versus nuclear-a fork in the road. Drug Resist Updat 2001;4:169-177.
14 Hoffmann EK, Lambert IH, Pedersen SF: Physiology of cell volume regulation in vertebrates. Physiol Rev 2009;89:193-277.
15 Lambert IH, Hoffmann EK, Pedersen SF: Cell volume regulation: Physiology and Pathophysiology. Acta Physiol Scand 2008;194:255-282.
16 Okada Y, Maeno E: Apoptosis, cell volume regulation and volume-regulatory chloride channels. Comp Biochem Physiol A Mol Integr Physiol 2001;130:377-383.
17 Yang J, Xu ZP, Huang Y, Hamrick HE, Duerksen-Hughes PJ, Yu YN: ATM and ATR: sensing DNA damage. World J Gastroenterol 2004;10:155-160.
18 Poulsen KA, Andersen EC, Hansen CF, Klausen TK, Hougaard C, Lambert IH, Hoffmann EK: Deregulation of apoptotic volume decrease and ionic movements in multidrug-resistant tumor cells: role of chloride channels. Am J Physiol Cell Physiol 2010;298:C14-C25.
19 Lambert IH: Regulation of the Cellular Content of the Organic Osmolyte Taurine in Mammalian Cells. Neurochem Res 2004;29:27-63.
20 Takatani T, Takahashi K, Uozumi Y, Shikata E, Yamamoto Y, Ito T, Matsuda T, Schaffer SW, Fujio Y, Azuma J: Taurine inhibits apoptosis by preventing formation of the Apaf-1/caspase-9 apoptosome. Am J Physiol Cell Physiol 2004;287:C949-C953.
21 Takatani T, Takahashi K, Uozumi Y, Matsuda T, Ito T, Schaffer SW, Fujio Y, Azuma J: Taurine prevents the ischemia-induced apoptosis in cultured neonatal rat cardiomyocytes through Akt/caspase-9 pathway. Biochem Biophys Res Commun 2004;316:484-489.
22 Taranukhin AG, Taranukhina EY, Saransaari P, Djatchkova IM, Pelto-Huikko M, Oja SS: Taurine reduces caspase-8 and caspase-9 expression induced by ischemia in the mouse hypothalamic nuclei. Amino Acids 2008;34:169-174.
23 Das J, Ghosh J, Manna P, Sinha M, Sil PC: Taurine protects rat testes against NaAsO(2)-induced oxidative stress and apoptosis via mitochondrial dependent and independent pathways. Toxicol Lett 2009;187:201-210.
24 Saad SY, Al-Rikabi AC: Protection effects of Taurine supplementation against cisplatin-induced nephrotoxicity in rats. Chemotherapy 2002;48:42-48.
25 Lang F, Madlung J, Siemen D, Ellory C, Lepple-Wienhues A, Gulbins E: The involvement of caspases in the CD95(Fas/Apo-1) but not swelling-induced cellular taurine release from Jurkat T-lymphocytes. Pflugers Arch 2000;440:93-99.
26 Poulsen KA, Litman T, Eriksen J, Mollerup J, Lambert IH: Downregulation of taurine uptake in multidrug resistant Ehrlich ascites tumor cells. Amino Acids 2002;22:333-350.
27 Kim HJ, Lee JH, Kim SJ, Oh GS, Moon HD, Kwon KB, Park C, Park BH, Lee HK, Chung SY, Park R, So HS: Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. J Neurosci 2010;30:3933-3946.
28 Lambert IH, Klausen TK, Bergdahl A, Hougaard C, Hoffmann EK: Reactive oxygen species activate KCl co-transport in non-adherent Ehrlich ascites cells but K+ and Cl- channels in adherent Ehrlich Lettre and NIH3T3 cells. Am J Physiol Cell Physiol 2009;297:C198-C206.
29 Han X, Yue J, Chesney RW: Functional TauT protects against acute kidney injury. J Am Soc Nephrol 2009;20:1323-1332.
30 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein Measurement with the Folin Phenol Reagent. J Biol Chem 1951;193:265-275.
31 Gonzalez VM, Fuertes MA, Alonso C, Perez JM: Is cisplatin-induced cell death always produced by apoptosis? Mol Pharmacol 2001;59:657-663.
32 Wang D, Lippard SJ: Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 2005;4:307-320.
33 Brozovic A, Ambriovic-Ristov A, Osmak M: The relationship between cisplatin-induced reactive oxygen species, glutathione, and BCL-2 and resistance to cisplatin. Crit Rev Toxicol 2010;40:347-359.
34 Kelland L: The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 2007;7:573-584.
35 Stewart JH, Tran TL, Levi N, Tsai WS, Schrump DS, Nguyen DM: The essential role of the mitochondria and reactive oxygen species in Cisplatin-mediated enhancement of fas ligand-induced apoptosis in malignant pleural mesothelioma. J Surg Res 2007;141:120-131.
36 Das J, Ghosh J, Manna P, Sinha M, Sil PC: Taurine protects rat testes against NaAsO(2)-induced oxidative stress and apoptosis via mitochondrial dependent and independent pathways. Toxicol Lett 2009;187:201-210.
37 Montiel M, Urso L, de la Blanca EP, Marsigliante S, Jimenez E: Cisplatin reduces endothelial cell migration via regulation of type 2-matrix metalloproteinase activity. Cell Physiol Biochem 2009;23:441-448.
38 Friis MB, Friborg C, Schneider L, Nielsen MB, Lambert IH, Christensen ST, Hoffmann EK: Cell shrinkage as a signal to apoptosis in NIH3T3 fibroblasts. J Physiol 2005;567:427-443.
39 Janson V, Andersson B, Behnam-Motlagh P, Engstrom KG, Henriksson R, Grankvist K: Acquisition of cisplatin-resistance in malignant mesothelioma cells abrogates Na+,K+,2Cl(-)-cotransport activity and cisplatin-induced early membrane blebbing. Cell Physiol Biochem 2008;22:45-56.
40 Villumsen KR, Duelund L, Lambert IH: Acute cholesterol depletion leads to net loss of the organic osmolyte Taurine in Ehrlich Lettré tumor cells. Amino Acids 2010;39:1521-1536.