Deregulation of apoptotic volume decrease and ionic movements in multidrug-resistant tumor cells: role of chloride channels


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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 298: C14–C25, 2010. First published October 21, 2009; doi:10.1152/ajpcell.00654.2008.—Changes in cell volume and ion gradients across the plasma membrane play a pivotal role in the initiation of apoptosis. Here we explore the kinetics of apoptotic volume decrease (AVD) and ion content dynamics in wild-type (WT) and multidrug-resistant (MDR) Ehrlich ascites tumor cells (EATC). In WT EATC, induction of apoptosis with cisplatin (5 μM) leads to three distinctive AVD stages: an early AVD1 (4–12 h), associated with a 30% cell water loss; a transition stage AVD2 (~12 to 32 h), where cell volume is partly recovered; and a secondary AVD2 (past 32 h), where cell volume was further reduced. AVD1 and AVD2 were coupled to net loss of Cl−, K+, Na+, and amino acids (ninhydrin-positive substances), whereas during AVD2, Na+ and Cl− were accumulated. MDR EATC was resistant to cisplatin, showing increased viability and less caspase 3 activation. Compared with WT EATC, MDR EATC underwent a less pronounced AVD1, an augmented AVD2, and a delay in induction of AVD2. Changes in AVD2 were associated with inhibition of Cl− loss during AVD2, augmented NaCl uptake during AVD2, and a delay of Cl− loss during AVD2. Application of the anion channel inhibitor NS3728 inhibited AVD and completely abolished the differences in AVD, ionic movements, and caspase 3 activation between WT and MDR EATC. Finally, the maximal capacity of volume-regulated anion channel was found to be strongly repressed in MDR EATC. Together, these data suggest that impairment of AVD, primarily via modulation of NaCl movements, contribute to protection against apoptosis in MDR EATC.

AVD is coupled to net loss of monovalent ions and osmotically obliged water due to changes in the plasma membrane permeability to cations and anions (2, 26, 40). The importance of cation channels in apoptosis has been widely documented (22), whereas less is known about the importance of anion channels. Activation of outwardly rectifying anion channels, including the volume-regulated anion channel (VRAC), following various apoptotic stimuli has, however, been demonstrated in different cell systems as has the protective effect of anion channel regulators against apoptosis (40). While the inhibition of K+ channels may prevent caspase activity via stabilization of intracellular K+ concentration (17, 22, 48), it is unclear how inhibition of the Cl− permeability protects against apoptosis. Apoptosis in most cells requires the reduction in cell volume (8, 34, 43), and Cl− channel inhibition has been shown to block AVD (34, 47); however, there are situations where apoptosis occurs without AVD (16, 49). Although AVD is a reasonably well-described phenomenon, a drawback of many studies is that they usually only provide information on one species of ion and do not give a detailed description of the sequential water, ion, and amino acid movements throughout the apoptotic process.

Chemotherapy regimens, for the treatment of cancer, target tumor cells disseminated throughout the body. Although drug treatment may initially be successful, cancer cells tend to acquire tolerance to drug treatment, a phenomenon termed multidrug resistance (MDR). MDR is usually characterized by cross-resistance to a wide range of structurally unrelated classes of anticancer compounds, and MDR is in most cases associated with upregulation of ATP-binding drug efflux transporters. Resistance may also involve decreased drug uptake, regulation of drug-target expression, detoxification, increased DNA repair, and deregulation and impairment of apoptotic cell death signaling (24, 32, 46).

Little is known about the involvement of AVD, ionic movements, and ion permeabilities in the development of MDR. The K+ ionophore amphotericin B has been shown to modulate cisplatin resistance (1, 38), and amphotericin B in combination with the Na-K-2Cl cotransporter (NKCC) blocker bumetanide was found to augment cisplatin-induced caspase 3 activation and nucleosome formation (36, 37). In addition, high-conductance K+ channels (BK) are activated following cisplatin exposure, and BK channel blockade inhibits cisplatin-induced apoptosis (28, 45). Other studies have shown alterations in Cl− conductivity in different models of MDR (10, 21, 25). In cardiomyocytes, the anticancer drug doxorubicin induces AVD and an anion current with electrophysiological characteristics similar to VRAC (4, 5). Okada and coworkers also demonstrated that cisplatin-induced apoptosis is associated with an increase in a VRAC current (19). Furthermore, cisplatin-resistant human epidermoid cancer KCP-4 cells exhibited a down-

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MOST ANTICANCER DRUGS KILL cancer cells by inducing programmed cell death (PCD). PCD or apoptosis is a fundamental biological mechanism characterized by programmed activation of specific biochemical pathways leading to an organized elimination of unwanted or damaged cells from a tissue. Apoptosis is traditionally divided into two phases: an initial phase during which the cell commits to apoptosis and an execution phase involving controlled breakdown of cellular constituents. Several important pathways of extra- and intracellular signaling involved in apoptotic suicide programs have recently been elucidated (7, 12).

An important aspect of apoptosis is the redistribution of intracellular ions which takes place during both the initial and execution phases of apoptosis (2). This ionic imbalance accounts for one of the classical hallmarks of apoptosis, the cell shrinkage, also known as “apoptotic volume decrease” (AVD).
regulated VRAC current which, when partially restored with the histone deacetylase inhibitor TSA, led to reestablishment of cisplatin-induced apoptosis (27). These studies indicate a role for cation and anion channels in controlling the progress of chemotherapy-induced apoptosis and also in the adaptation of cancer cells to prolonged chemotherapy exposure.

In this study we use a cell culture model for anthracycline resistance (MDR EATC) to study AVD, ionic movements, and the chloride permeability in cisplatin-induced apoptosis. We give a detailed description of the dynamic changes in cell volume and net ionic movements throughout apoptosis and demonstrate that cisplatin-induced AVD is a complex process consisting of three distinguishable stages defined by sequential efflux/influx of different ions, amino acids, and water. We also show that MDR in EATC is associated with stage-specific alterations in AVD and ionic movements that are coupled to functional downregulation of VRAC expression.

EXPERIMENTAL PROCEDURES

Materials. The standard isotonic medium (310 mosM) contained (in mM) 143 NaCl, 5 KCl, 1 MgSO4, 1 Na2HPO4, 1 CaCl2, 3.3 MOPS, 3.3 TES, and 5 HEPES, pH 7.4. Unless otherwise stated, reagents were analytical grade and obtained from Sigma (St. Louis, MO). All culture media were purchased from Invitrogen/Life technologies. Daunorubicin (DNR) was from Avensis Pharma.

Cell cultures and selection for drug resistance. Two different strains of EATC were used: The wild type, drug-sensitive EATC grown in the abdominal cavity of female NMRI mice (Naval Medical Research Institute) was approved by “Dyre-forsøgsstilsynet” 2007/561-1313. Estimation of cell volume. Cell volume was determined essentially as described previously (14) by electronic cell sizing using a Coulter Multisizer II (Coulter, Luton, UK). Final cell density was ~90,000 cells/ml, which is equivalent to a cytocr of ~0.008%. Cell volume was determined as the median of the cell volume distribution curves after calibration with latex beads (14.89-μm diameter; Coulter) and was analyzed using Coulter Multisizer AccuComp version 1.19 software. Media used for cell volume measurements were filtered (Millipore filters, 0.45 μm) before initiation of the experiments.

Estimation of cell water, ionic and amino acid content. Cell water, Na+, K+, Cl−, and nonprotein, ninhydrin-positive substances (NPS) were estimated essentially as described previously. In brief, ionic and water content were measured on 1-ml samples of cell suspension (2% cytocr), separated from the incubation medium by centrifuging (14,000 g), lysed in double-distilled H2O (ddH2O) and deproteinized with perchloric acid as previously described in detail (15, 44). K+ and Na+ were measured by atomic flame photometry (model 2380, Perkin Elmer Atomic Absorption Spectrophotometer), whereas Cl− content was determined by coulometric titration (CMT 10 chloride titrator, Radiometer). NPS were estimated using ninhydrin reagent as a substrate and glycin as standard as described by the manufacturer (Sigma). Cell water was determined by reweighing of cell pellets after 48 h of drying at 90°C. All values are corrected for trapped extracellular medium using [3H]insulin as marker (15).

Electrophysiology. Cells were transferred to 25-mm poly-L-lysine (0.1 mg/ml, Sigma)-coated glass coverslips and were mounted on a temperature-controlled chamber installed on an inverted microscope (Zeiss Axiosvert 10, Carl Zeiss). The cells were superfused at 1.5–2 ml/min with the experimental solution in a 500-μl volume chamber at 37°C. The extracellular isotonic solution was of the following composition (in mM): 125 NaCl, 2.5 CaCl2, 2.5 MgCl2, 10 HEPES, and 75 D-mannitol, pH 7.2 (adjusted with NaOH). To reduce extracellular osmolarity, D-mannitol was omitted. The pipette solution was (in mM) 150 CsCl2, 5 MgCl2, 10 HEPES, 10 EGTA, 2 ATP, and 0.1 GTP, pH 7.2 (adjusted with CsOH). The pipette solution was slightly hypotonic compared with the extracellular isotonic solution to prevent spontaneous activation of volume-regulated chloride current (ICl,vol). The pipettes were made from VITrex glass capillary tubing with an outside diameter of 1.7 mm (Modulohm, Herlev, Denmark) using a Narishige PP-830 puller (Tokyo, Japan) and had resistances of 4 MΩ. After a GΩ seal was obtained between the pipette and the cell membrane, the patch was ruptured by applying negative pressure. Currents were measured in the whole cell configuration of the patch-clamp technique using an Axopatch 200B (Axon Instruments) amplifier, and all current measurements were filtered at 2 kHz. A Digidata 1200 Interface board and pClamp7 software (Axon Instruments) were used to generate voltage-clamp command voltages and to digitize data. The holding potential was −40 mV. The following voltage protocol was used in most experiments and was applied every 10 s: a 500-ms step to −60 mV, a 200-ms step to −80 mV, followed by a 2.6-s linear voltage ramp to +80 mV. The whole cell capacitance was read from the compensation dial of the patch-clamp amplifier and was used as an estimate of cell surface area, and hence, cell size. Corrections for junction potential were calculated using pClamp software.

Taurine efflux measurements. EATC were adjusted to a cytocr of 6% in isotonic medium and were incubated for 30 min at 37°C. Cell suspensions, equilibrated with [3H]taurine (0.18 μCi/ml) and [14C]polyethylene glycol [0.1 μCi/ml, for trapped volume measurements determined in separate experiments (n = 8)] for 1 h, were packed by centrifuging and resuspended in isotonic medium. Samples for estimation of trapped volume and total cellular and extracellular tracer-activity were taken before initiation of the experiments. Unlabeled taurine (10 mM) was added to the medium to inhibit [14C]taurine reuptake via the active, Na+-dependent taurine cotransporter. [14C]Taurine release was measured by serially isolating extracellular...
medium by centrifugation (15,000 g, 20 s) of 500-μl cell suspension through a silicone oil phase (300 μl 5:1 wt/wt AR200, 200 and DC 200/20). 1H- and 14C-activities in cell pellets and extracellular medium were measured in a liquid scintillation spectrometer (Packard 2000CA Tri-Carb Liquid Scintillation Analyser) in channels ranging from 2.0 to 18.6 keV and 19 to 500 keV, respectively, using ULTIMA GOLD (Packard) as scintillation fluid and a 14C-standard for correction of 14C-activity in the 2.0–18.6 keV window. 14C-activity was plotted as total fraction of [14C]taurine released at time t (counts per minutes per mg dry wt); y' = (a_m - a_m0)/a_m0, where a_m and a_m0 are the 14C-activity in the efflux media at time t and time 0, respectively, and a_m0 is the total 14C-activity in the cells at time 0. The volume-sensitive taurine efflux follows a single exponential function of the type y = yo + a1(e−bt).

Rate constants (b) at single time points were estimated as the highest value of the slope (dy/dt)/(1 − y'). 14C-activity was plotted at total fraction of [14C]taurine remaining in the cells at time t. The slope of the plot at (1 − y') vs. time. dy/dt is the slope of the exponential plot. y' = b × a × e−bt. 1 − y' is the fraction of [14C]taurine remaining in the cells at time t. All experiments were performed in pairs (WT vs. MDR EATC).

Estimation of the free NS3728 concentration. NS3728 binds strongly to serum protein so the actual free concentration of NS3728 in the incubation medium was determined using Centrifree YM-30 microparticition devices (Millipore, Bedford, MA) and 14C-labeled NS3728 (Neurosearch, Ballerup, Denmark) essentially as recommended by the manufacturer. In brief, NS3728 in concentrations ranging from 5 μM to 500 μM (5 μM [14C]NS3728 + additional unlabeled NS3728) was incubated in 1 ml RPMI 1640 containing 10% serum for 18 h at 37°C. To correct for NS3728 binding to the Centrifree membrane, parallel control incubations with [14C]NS3728 were performed in the absence of serum. The concentrations were calculated by multiplying the total NS3728 concentration in the control incubations with [14C]NS3728 were made in ddH2O. After incubation, 500-μl samples were transferred to the Centrifree YM-30 sample reservoir and the microparticition device spun 1,500 g for 10 min at 37°C in a swinging bucket-type centrifuge (Jouan CT4.22, St-Herblain, France). The ultratritrate (250 μl) and the initial incubation medium (250 μl) were transferred to separate vials, and 3.5 ml ULTIMA GOLD(Packard) scintillation fluid was added. 14C-activities were measured in a liquid scintillation spectrometer (Packard 2000CA Tri-Carb Liquid Scintillation Analyser). The percentage of unbound NS3728 was calculated as the fractional activity in the ultratritrate corrected for the percentage of NS3728 retained by the Centrifree membrane in the control incubations. The concentrations were calculated by multiplying the total NS3728 concentration in the initial incubations with the percentage of unbound NS3728.

Cell viability assay. Cells were seeded in 96-well microplates at a density of 16 × 10^4 in 100 μl medium and incubated (37°C, 5% CO2) overnight. After cisplatin treatment (2–5 μM) for 18 h, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. In brief, MTT solution (12 mM) was added to each well (10 μl/well) and the plate was incubated (37°C, 5% CO2) for 2 h; 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 reader (Bmg LabTechnologies, Offenburg, Germany). Data obtained were reported in terms of relative cell viability compared with control (nonstimulated), the absorbance values assumed to be directly proportional to the number of viable cells. Each experiment was performed in at least triplicate.

Necrosis assay. Necrotic cell death was evaluated as glucose-6-phosphate-dehydrogenase (G6PD) release from Ehrlich ascites tumor cell cultures, using a Vybrant Cytotoxicity Assay kit (Invitrogen), following the manufacturer’s instructions. The assay quantifies plasma membrane integrity as released G6PD, based on the G6PD-dependent conversion of NADP+ to NADPH and subsequent reduction of resazurin to resorufin, which emits fluorescence at 590 nm after excitation at 530 nm. Briefly, cells seeded at a density of 10^4 cells/50 μl in 96-well plates were incubated for 18 h with or without 5 μM cisplatin followed by addition of 50 μl 2× reaction mixture containing 30 μM resazurin and 25 min incubation at 37°C. Fluorescence was measured in a FLUOstar OPTIMA 96-well plate reader with excitation at 544 nm and emission at 590 nm. All values were corrected for background fluorescence, and data are presented as the relative cytotoxicity of treated cells (corrected fluorescence/corrected fluorescence of fully lysed cells) normalized to relative cytotoxicity of untreated cells.

Caspase 3 activity assay. Cells were grown in 75-cm² flasks at a density of 2–5 × 10^5/ml. Following treatment, cells were centrifuged at 1,000 g for 6 min at 4°C. The pellet was washed once in PBS and lysed in ice-cold lysis buffer, thoroughly resuspended, and stored at −80°C overnight. The cell lysates were subjected to three freeze-and-thaw cycles and 2 × 10-s sonication to fully disrupt the cells and disperse cell debris. The cell lysate was then centrifuged 20,000 g for 5 min and the supernatant transferred to new Eppendorf tubes. Protein content in supernatants was determined and concentration was adjusted for activity measurements (1–4 μg/μl). Caspase 3 activity in cell lysates was estimated in 96-well plates using the ApoTargetCaspase 3/CP32 Colorimetric assay (Invitrogen, Taastrup, Denmark) according to the manufacturer’s protocol by measuring protease activity toward 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). Experiments were performed in triplicate.

Gel electrophoresis and Western blot analysis. Cells were grown in 75-cm² flasks at a density of 2–5 × 10^5/ml. Following stimulation, cells were centrifuged at 1,000 g for 6 min at 4°C. The pellet was washed once in PBS and lysed in 1% SDS, 10 mM Tris pH 7.4, 1 μM NaVO₄, and protease-inhibitor cocktail (Complete Mini, Roche). Cell lysates were homogenized through a 21-gauge syringe, spun for 5 min, 20,000 g, and supernatants were used for protein determination (DC Protein Assay, Bio-Rad). Protein content in supernatants was adjusted and diluted in LDS NuPAGE sample buffer (Invitrogen) with 1 mM DTT, boiled for 5 min, separated by SDS-PAGE on 10% NuPAGE Bis-Tris gels in NOVEX electrophoresis chambers (Invitrogen), and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C in blocking buffer (120 mM NaCl, 10 mM Tris pH 7.5, 0.1% Tween 20, and 5% nonfat dry milk), followed by 2 h at room temperature with primary antibody [rabbit monoclonal caspase 3 antibody (Cell Signaling, Beverly, MA)] diluted in blocking buffer, washed in TBST (120 mM NaCl, 10 mM Tris, pH 7.5, and 0.1% Tween 20), and incubated with the appropriate alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch). Caspase 3 was detected by BCIP/NBT
(KPL). The final blots were scanned and densitometric analysis was performed using UN-SCAN-IT software (Silk Scientific).

Statistical evaluation. The data are expressed as means ± SE. Where nothing else is indicated, statistical comparisons were made using paired, two-tailed Student’s t-test. One-way ANOVA with a Tukey-Kramer multiple-comparison post test was used as indicated in the figures. The confidence level was set at 95%.

RESULTS

Changes in cell water and cell volume during cisplatin exposure. Exposure of WT EATC to 5 μM cisplatin resulted in dynamic changes in cell water (Fig. 1A). These changes fall into three sub-stages: an early AVD stage (AVD1), a transition stage (AVD2), and a late AVD stage (AVD3). AVD1 resulted in ~30% loss of cell water between 4 and 10 h, whereas cell water was partially recovered between 12 and 24 h as the cells moved through AVD3. AVD2 was initiated around 32 h and led to ~50% loss of cell water after 48 h. Similar results were obtained when measuring cell volume using electronic cell sizing (Coulter Counter). Figure 1B shows the development in cell volume for the initial 18 h following cisplatin exposure. Consistent with the water content measurements, 5 μM cisplatin also elicits a significant loss of cell volume between 5 and 8 h during the AVD1 stage followed by a recovery stage (AVD3). However, loss of cell volume during AVD1 only amounts to ~7% relative to the initial value, much less compared with the ~30% loss of cell water. This is partly due to a relatively high fraction of dry matter which does not decrease during the water loss (see DISCUSSION for further explanation). AVD1 is then followed by the cell volume recovery phase (AVD3) where cell volume is significantly increased compared with the cell volume during AVD1. In contrast to the partial cell water recovery during AVD1 between 12 and 24 h, cell volume is completely restored to initial values after 12 h.

Time-dependent changes in ionic content and ionic concentrations following cisplatin exposure. To get a direct estimate of net ionic movements throughout AVD, we measured the changes in ionic content and amino acids (estimated as NPS) in μmol/g dry wt during 48 h of cisplatin exposure. Loss of cellular K+, Cl−, and NPS from WT EATC appeared concomitant with the water loss that defined AVD1 (Fig. 2). Significant net loss of K+, Cl−, and NPS was seen after 4, 6 and 2 h of cisplatin treatment, respectively (Fig. 2, A, B, and D). Consistent with earlier studies of regulatory volume decrease (RVD) in EATC (31), net loss of cations exceeded the loss of anions during AVD1 (values are taken 8 h after addition of cisplatin, Fig. 2E). After 12 h, as the cells progressed through AVD3, they continued to lose K+ and NPS but began to take up Cl− (~46 and 143 μmol/g dry wt between 8 and 18 h, respectively). The late AVD3 was characterized by maintained, although limited, K+ and NPS loss and reintroduction of Cl− and Na+ loss. The earliest significant changes in the cellular concentrations were seen after 8, 10, 3, and 12 h for K+, Cl−, NPS, and Na+, respectively, the cellular concentration of K+ and NPS being decreased and Cl− and Na+ being increased (Fig. 3).

MDR EAT cells are resistant to cisplatin-induced apoptosis. The MDR EATC used in this study were originally developed to study resistance to the anthracycline daunorubicin and display the classical phenotype of MDR with high expression of the mdr1 gene product P-glycoprotein (PGP) (6, 29). Additional sources of resistance, which we here speculate may be related to changes in ionic homeostasis, have, however, developed in these cells (29). To study these PGP-independent mechanisms, we have used the non-PGP transport substrate, the platinum-based anticancer drug cisplatin, to induce apoptosis.

Fig. 1. Time-dependent changes in the cellular water content and cell volume characterization of cisplatin-induced apoptotic volume decrease (AVD) in wild-type (WT) Ehrlich ascites tumor cells (EATC). A: WT EATC were incubated with 5 μM cisplatin and samples were taken for cell water content measurements throughout a period of 48 h following. Cell water measurements (presented as ml/g dry wt) were normalized to values obtained at time 0 and are given as mean values ± SE of 8 sets of experiments. *Significantly different from the initial value obtained at time 0 tested using ANOVA with Tukey-Kramer multiple-comparison test. B: cell volume (in fl) measured by electronic cell sizing (Coulter Counter) during the initial exposure to 5 μM cisplatin (0–18 h). Values are given relative to the initial cell volume and represent mean values ± SE of 11 experiments. *Significantly different from the initial value obtained at time 0, **significantly different from AVD1 times 5, 6, and 8 h, P < 0.05. Data were tested for significance using repeated-measures ANOVA with Tukey-Kramer multiple-comparison test. As outlined in the figure, cell water content and cell volume changes during cisplatin-induced apoptosis were divided into three distinguishable stages on the basis of time-dependent changes in cell water and volume and designated AVD1, AVD2 (as transition), and AVD3.
until after 18-h cisplatin treatment compared with 10 h in WT EATC (Fig. 4C). Furthermore, caspase activity in MDR cells was after 18 h only about one third of the activity in WT EATC (Fig. 4C). From Fig. 4D it is seen that initiation of caspase 3 activity in WT EATC was associated with significant loss of cell water (ml/g dry wt) of ~30% after 8-h cisplatin exposure concomitant with the reduction in the cellular Cl\(^{-}\) content (µmol/g dry wt) by 14%. In comparison, water content in MDR EATC was only reduced by ~13% after 8 h while Cl\(^{-}\) content was unchanged (Fig. 4D). Thus, although selected for resistance to daunorubicin, MDR EATC also possesses increased tolerance to cisplatin.

Changes in cell volume, ionic content, and concentrations in MDR EATC. From Table 1 it can be seen that, apart from a 10 mM reduction in K\(^{+}\) concentration in MDR EATC, steady-state ion concentrations, water content, and cell volume are...
Cell volume, fl 1,631/H11006
Cell water, ml/g dry wt 3.59/H11006

is significantly larger. Compared with MDR EATC, water
that cell volume in MDR EATC after 48-h cisplatin treatment

tumor cells at 0-h and 10-h cisplatin (5
Cl
NPS, mM 87.1
Na
gives the cellular Na
EATC was not significantly different from WT EATC. Table 1

). The reduced AVD1 observed in MDR EATC (Fig.

in WT EATC to 13% and 26% in MDR EATC, respectively

AVD1, apoptotic volume decrease, initial stage. Significance levels (P)
are indicated.

Values are means ± SE of 5 independent experiments, except for Cl
measurements for which 8 experiments were performed. The K
Na
Cl
ninhydrin-positive substance (NPS) concentrations were estimated as described in EXPERIMENTAL PROCEDURES and are presented as μmol/ml cell water (in mM). Water content was measured in ml/g dry weight and cell volume in fl as determined by Coulter Counter measurements. WT, wild type; MDR, multidrug resistant; AVD1, apoptotic volume decrease, initial stage. Significance levels (P) are indicated.

similar in WT and MDR EATC. Moreover, it was previously
shown that the intracellular pH is identical in the two cell lines (30). Figure 5 compares cell volume changes and water and net
ion movements in WT and MDR EATC following cisplatin

loss during AVD1 and AVD2 was reduced from 30% and 50%

changes in Cl

addition of cisplatin, which is right before the first significant
increase in caspase 3 activity, is observed in the two strains. During AVD1 the increase in cellular Cl
and Na
content was enhanced in MDR EATC compared with WT EATC (Fig.

Cl
NaCl

time 0
mosM) and hyposmotic medium (2/3 of the isotonic value)

activity, we measured the maximal VRAC current in WT and

movements. To investigate whether the altered
Cl
homeostasis in MDR EATC involved changes in VRAC
activity, we measured the maximal VRAC current in WT and

was enhanced in MDR EATC compared with WT EATC (Fig.

K
Na
K
and Cl

loss (Fig. 5
D
), which explains the observed cell swelling seen in

and MDR EATC using whole cell patch clamp. We also estimated
the swelling regulated release of the organic osmolyte taurine
using a radioactive tracer technique.

Figure 6, A and C, shows representative current traces from
WT and MDR EATC, respectively, under isosomotic (310
mosM) and hyposmotic medium (2/3 of the isotonic value)
using a step protocol. Figure 6, B and D, shows current-voltage

Fig. 4. Cisplatin sensitivity of WT and multidrug-resistant (MDR) EATC. A: relative cell viability mea-
sured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-
rrozolium bromide (MTT) cell viability assay in WT
EATC (black bars) and MDR EATC (gray bars) after
18-h incubation with 2 and 5 μM cisplatin (Cis; n = 5). B: release of glucose 6-phosphate dehydroge-

nase (G6PD) from WT EATC (black bars) and MDR
EATC (gray bars) after incubation with 5 μM cisplatin. Values are normalized to untreated control
cells (black bars).

Values are normalized to the levels in untreated control
cells. Caspase 3 activity was measured using a calori-
metric assay (n = 4). D: water (light gray bars) and
Cl
(dark gray bars) content in WT and MDR EATC
after 8-h cisplatin exposure measured as ml/g dry wt and μmol/g dry wt, respectively. Values are normal-
ized to their respective time 0 control cells (black bars). Error bars represent SE of 8 experiments. *Signifi-
cantly different from control. + WT and MDR EATC significantly different, P < 0.05.
Effect of the Cl⁻ channel inhibitor NS3728 on caspase 3 activity. To further understand the role of chloride channels in apoptosis and drug resistance, we investigated the effect of the acidic di-aryl urea NS3728, a high-affinity inhibitor of both VRAC and Ca²⁺-activated Cl⁻ channel (CaCC; IC₅₀ = 0.40 μM and 1.5 μM, respectively) (11, 23), on caspase 3 activity and pro-caspase 3 cleavage in both EATC strains. Although the IC₅₀ of NS3728 was shown to be in the nanomolar range, micromolar concentrations were used in the long-term experiments due to the presence of serum in the incubation medium (see experimental procedures). The concentrations of NS3728 indicated are thus the determined free concentrations of NS3728 in the incubation medium containing 10% serum. The presence of NS3728 in the incubation media decreased cisplatin-stimulated caspase 3 activity (Fig. 7A) as well as pro-caspase 3 cleavage (Fig. 7B) after 18-h cisplatin exposure in a dose-dependent manner in WT EATC. In MDR EATC, NS3728 also decreased the already low caspase 3 activity dose-dependently (Fig. 7, A and B). Neither WT EATC nor MDR EATC showed caspase 3 activation at a concentration of 17 μM free NS3728 (Fig. 7, A and B).

Effect of NS3728 on AVD and ionic content. Treatment of WT EATC with 50 μM NS3728 (equivalent to 11 μM free NS3728) resulted in inhibition of water loss and cell shrinkage during AVD₁, AVD₇, and AVD₂ (Fig. 8, A and B). As seen from Fig. 8B, 96-h cisplatin treatment is required to obtain significant reduction in cell volume in the presence of NS3728.
Fig. 6. The capacity of the volume-regulated Cl− current and taurine efflux is downregulated in MDR EATC. A: representative current traces from WT EATC under isosmotic (ISO; 310 mosM) and hyposmotic (HYPO) medium (2/3 of the isotonic value) using a step protocol (see EXPERIMENTAL PROCEDURES). B: current-voltage (I/V) curves of the current in WT EATC obtained under isosmotic and hyposmotic conditions when the cells have reached steady-state level, using a linear voltage ramp (see EXPERIMENTAL PROCEDURES). C: current traces from MDR EATC under isosmotic and hyposmotic conditions (step protocol). D: I/V curve of MDR EATC under isosmotic and hyposmotic conditions. E: [3H]taurine efflux in WT and MDR EATC after exposure to hyposmotic medium (190 mosM). Data are shown as the fractional release of [3H]taurine as a function of time in WT (open symbols) and MDR EATC (filled symbols). The curve is representative of 21 sets of experiments. F: chloride current (I_{Cl}) in pA/pF at +80 mV in WT EATC (open bar) and MDR EATC (filled bar). Value for MDR is shown relative to the WT values. Error bars represent SE of 21 experiments. *P < 0.05, ***P < 0.001.

Fig. 8C shows that when WT and MDR EATC are treated with cisplatin and NS3728 combined, AVD becomes virtually identical in the two cell lines. In agreement with this, Cl−, K+, Na+, and NPS movements in cisplatin and NS3728-treated WT and MDR EATC were identical throughout the 48 h measured (Fig. 9, A–D). These results are taken to indicate that apoptotic resistance in MDR EATC is coupled to the impairment of AVD via alterations in chloride movements, likely the downregulation of VRAC.

Effect of the TASK-2 channel inhibitor clofilium on caspase 3 activity. TASK-2 was previously shown to be the swelling-activated K+ channel in EATC and to be sensitive to clofilium (13). Clofilium was found to inhibit AVD1 (Fig. 10A) as well as caspase activation (Fig. 10B) in a manner similar to NS3728. Thus inhibition of either the volume-sensitive Cl− channel or alternatively the volume-sensitive K+ channel both impaired cisplatin-induced apoptosis.

DISCUSSION

Water loss (AVD) is intimately associated with apoptosis and is driven by net changes in cellular content of ions and organic osmolytes. Movement of monovalent cations and anions is important for both the commitment to and the execution of apoptosis (2, 3), suggesting that resistance to apoptosis, as experienced in many MDR tumor cells, may involve deregulation or impairment of AVD.

AVD consists of three phases: AVD1, AVDε, and AVD2. The present study describes the AVD response in EATC to the platinum-based anticancer drug cisplatin in terms of three distinct stages defined by changes in cell water/volume and the content of cations and anions as well as amino acids. AVD1 in WT EATC is associated with a loss of ~30% cell water due to a reduction in cellular cations and anions as well as amino acids. Although changes in cell volume did follow the same dynamics, the magnitude of cell volume loss during AVD1 was much smaller than water loss (7% compared with 30%). Part of this difference is likely due to a relatively high fraction of dry matter which does not decrease during the water loss. Moreover, it is well known that osmotic shrinkage is associated with a net increase in actin polymerization which to a certain degree counteracts cell shrinkage (13). The loss of cations exceeds the loss of anions during AVD1 as previously reported during RVD in these cells (31). EATC undergoing RVD lose K+ and Cl−, but due to fast anion exchange, part of the Cl− loss is transformed into a HCO3−-loss resulting in cytoplasmic acidification. The acidification results in an activation of the Na+/H+ exchanger (31). Intracellular acidification and activation of the Na+/H+ exchanger may be a general response of cells undergoing RVD and likewise in the early stages of apoptosis. Early intracellular acidification during apoptosis appears to be a general feature (see Ref. 26 for review).

AVD1 in EATC is succeeded by AVDε during which gradient reversal of Na+/K+ as well as a Cl− influx takes place leading to net uptake of Na+ and Cl− and partial recovery of cell volume. This stage is likely to replicate a regulatory volume increase (RVI) process which is known to involve activation of the Na+/H+ exchanger mentioned above as well as the other transporters involved in RVI, i.e., NKCc1 and nonselective cation channels (13). A characteristic difference between shrinkage-induced RVI and cisplatin-induced AVDε is that uptake of Na+ during RVI is not seen unless the Na+/K+-ATPase is inhibited because of immediate exchange of Na+ for K+ by the Na+/K+-ATPase (20). Significant uptake of Na+ during AVDε and almost complete reversal of
Na\(^{+}\)-K\(^{+}\) concentrations to outside levels thus indicates that the Na\(^{+}\)-K\(^{+}\)-ATPase is inhibited at this stage. The important role of Na\(^{+}\)-K\(^{+}\)-ATPase inhibition in the apoptotic process has previously been reviewed (41). Thus, it is suggested that the RVI-like AVDT plays an important role in the apoptotic response. Inhibition of the RVI capabilities during apoptosis has previously been shown by Maeno et al. (35), suggesting that the strongly amplified AVDT in MDR EATC may involve RVI deregulation. Major loss of cell volume occurs during AVD2, leading to a ~60% loss of cell volume within 96 h. This is primarily due to loss of NaCl, but a minor loss of K\(^{+}\) and NPS is also seen. At 18-h cisplatin treatment, no increase in G6PD release is observed, indicating that the membrane is intact and that the cells are not necrotic. However, during AVD2 we cannot exclude that inhibition of the pump is combined with increased ion leakiness.

The phased nature of AVD has in part been described by Bortner and coworkers (3), who identified an early AVD characterized by 20–40% cell volume loss and K\(^{+}\)-Na\(^{+}\) gradient reversal and a late cytoskeleton-dependent AVD defined by severe cell shrinkage and K\(^{+}\) and Na\(^{+}\) loss. Although anion and amino acid movements were not investigated, the early stage may well correspond to AVD\(_{1}\) plus AVD\(_{T}\) identified here and the late cytoskeleton-dependent stage are comparable to AVD\(_{2}\). Transport systems in EATC that are involved in RVD as well as RVI are strongly dependent on the cytoskeleton (13). The dependence of late-stage AVD on an intact cytoskeleton (3) is clearly in agreement with these observations, although the precise roles of the separate transport systems in AVD\(_{T}\) and AVD\(_{2}\) and their dependence on the cytoskeleton have yet to be clarified.

Reductions in the concentrations of intracellular ions, specifically potassium, have previously been suggested to promote caspase activation (2). In WT EATC, caspase 3 activity is induced during AVD\(_{1}\) where the K\(^{+}\) and NPS concentrations

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**Fig. 7.** Effect of NS3728 on cisplatin-induced caspase 3 activity and pro-caspase 3 cleavage. A: caspase 3 activity, measured by calorimetry, was estimated in WT EATC (black bars) and MDR EATC (gray bars) under control conditions and 18-h treatment with 5 μM cisplatin and 0, 10, 25, 50, or 75 μM NS3728. The concentrations of NS3728 shown are the levels of free NS3728 in the serum containing incubation medium estimated in separate experiments (see EXPERIMENTAL PROCEDURES). B: Western blot of cells treated as described above using an antibody against the cleaved and activated form of caspase 3. The blot shown is representative of 3. Cell line and treatment are indicated below the image. Significantly different from control, *P < 0.05 and **P < 0.01. +Significantly different from WT EATC, P < 0.05.

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**Fig. 8.** Effect of the Cl\(^{-}\) channel inhibitor NS3728 on cisplatin-induced AVD. A: comparison of changes in cell water content during cisplatin-induced apoptosis in WT EATC in the absence (black bars) and presence (gray bars) of 11 μM free NS3728. Data are presented as means ± SE of 8 experiments. B: effect of 11 μM NS3728 on cell volume during cisplatin-induced AVD in WT EATC: untreated WT EATC (black bars) and treated with NS3728 (gray bars). C: comparison of AVD in WT (light gray bars) and MDR EATC (white bars) in the presence of 11 μM free NS3728. Data were normalized to initial time 0 values, and error bars represent SE of 5 experiments. *P < 0.05 compared with control. +Significantly different from respective controls, +significantly different from WT EATC, P < 0.05.
are reduced by 23 mM and 35 mM, respectively, and the Na$^+$ and Cl$^-$/H$^+$ concentrations are increased by 35 mM and 8 mM (Fig. 3) (see Table 1 and Figs. 1 and 3). Caspase activity is further augmented during AVD$_T$ concomitant with a major decrease in K$^+$ concentration and increase in Na$^+$ concentration.

Protection of MDR EATC against apoptosis. Protection of MDR EATC against apoptosis involves attenuation of water loss during AVD$_1$ likely via the reduction in Cl$^-$/H$^+$ loss. The importance of the Cl$^-$/H$^+$ permeability in MDR is supported by the fact that water loss, cell shrinkage, and caspase 3 activation in WT EATC is repressed by the Cl$^-$/H$^+$ channel blocker NS3728 to a similarly low level as seen in untreated MDR EATC. As previously mentioned, the effect of NS3728 may indicate involvement of either VRAC and/or CaCC. However, the observed impairment of VRAC in MDR EATC (Fig. 6) suggests that this channel contributes to the decrease in Cl$^-$/H$^+$ loss. NS3728 also inhibits cisplatin-induced NPS loss at 3–8 h, and volume-sensitive taurine release is also strongly reduced in MDR EATC, suggesting that inhibition of NS3728-sensitive amino acid-permeable channels may also be involved in the protection.

Protection of MDR EATC also appears to involve prevention of secondary cell shrinkage via increased accumulation of NaCl during AVD$_T$ and inhibition of NaCl loss during AVD$_2$. This in turn leads to augmented cell volume recovery during AVD$_T$ and preservation of cell volume during AVD$_2$ (Fig. 5). This likely delays DNA degradation and apoptotic body formation, typical features of late AVD (3), and consequently proper execution of apoptosis. The difference in AVD$_T$ and AVD$_2$ between WT EATC and MDR EATC may reflect the downregulation of VRAC in MDR EATC since incubation with the Cl$^-$ channel inhibitor NS3728 results in an identical volume and ionic behavior in the two strains (Figs. 8 and 9). The importance of downregulation of VRAC in MDR is supported by similar observations in cisplatin-resistant KCP-4 cells (27) and MES-SA cells (33). In addition, unpublished data from our lab show that MCF-7 cells selected for resistance to mitoxantrone also have a significant reduced VRAC current. There are two obvious mechanisms by which decreased Cl$^-$ channel activity potentially protects against apoptosis in MDR.

Fig. 10. Effect of clofilium on AVD and caspase 3 activity. A: effect of 25 μM clofilium on cell volume during 24-h cisplatin treatment in WT EATC. Note that samples were taken primarily during AVD$_1$ exposure. B: effect of 25 μM clofilium on caspase 3 activity after 18-h cisplatin exposure (5 μM). Data in A and B were normalized to time 0 values, and error bars represent SE of 4 experiments. *Significantly different from control without clofilium, P < 0.05.
EATC, i.e., stabilization of the intracellular Cl\(^-\) and/or K\(^+\) concentrations or inhibition of cell shrinkage during AVD. Stabilization of intracellular Cl\(^-\) and K\(^+\) concentrations appear unlikely because, although net loss of both Cl\(^-\) and K\(^+\) is slightly repressed in MDR EATC, the concentrations of Cl\(^-\) and K\(^+\) are not significantly different between WT and MDR EATC before and parallel with caspase 3 activation (Table 1). Thus, it appears that the cation environment required for activation of caspases is present after ~10 h cisplatin treatment in both WT and MDR EATC; however, it is only in WT EATC that caspase 3 is actually activated. The early cell shrinkage during AVD has previously been suggested to be an inducer of apoptosis (8, 9). Ernest and coworkers (8) recently suggested that a certain degree of cytoplasmic condensation for a certain amount of time is required and sufficient for induction of the intrinsic pathway of apoptosis. This threshold was estimated at ~30% shrinkage maintained for 3 h and could be prevented by Cl\(^-\) efflux inhibition. This correlates well with our observations of ~30% water loss during AVD\(_1\) (which is sustained for ~6 h) in WT EATC before caspase 3 activity was induced. AVD\(_1\) in MDR EATC was not delayed but water loss was reduced to 10%, cell shrinkage was absent, and caspase 3 activation was significantly delayed. This indicates that reduced cell shrinkage during AVD\(_1\) may elicit a weaker response which delays caspase activation and AVD\(_2\). Exposing MDR EATC (after 4-h cisplatin exposure) to a moderate hyperosmotic medium (470 mosM, isotonic medium supplemented with sucrose), which induces initial cell shrinkage of ~30% and thus partly simulates the cell shrinkage seen in WT EATC during AVD\(_1\), did, however, only enhance the activation of caspase 3 slightly (data not shown). Sucrose addition results in cell shrinkage without a concomitant loss of ions, i.e., the intracellular ion concentrations are significantly higher after sucrose supplementation compared with after cisplatin addition. Alterations in cisplatin transport in MDR EATC could potentially contribute to the observed cisplatin resistance. However, preliminary experiments (n = 3, data not shown) indicate that the accumulation of cisplatin in the cytosol and nuclei after 18-h exposure (5 \(\mu\)M) is not significantly different in WT and MDR EATC [isolated by differential centrifugation and measured by atomic absorption spec- trometry (Stefan Stürup, Charlotte Møller, Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark)]. This is not unexpected because MDR EATC are selected for resistance to the anthracycline daunorubicin and have not developed specific resistance to cisplatin. In addition, PGP, which is highly overexpressed in MDR EATC, does not transport cisplatin (18). Of other transporters proposed to be involved in cisplatin transport, multidrug resistance-associated protein 1 (MRP-1), which is the only MRP expressed in EATC, and the copper transporters Slc31a1 and 2 (50) are expressed at equal levels in WT and MDR EATC (Dr. T. Litman, unpublished observation). Thus, although it cannot be completely ruled out, alterations to cisplatin transport in MDR EATC seems unlikely.

In conclusion, these results indicate that apoptotic resistance in MDR EATC involves impairment of AVD. This occurs via inhibition of AVD\(_1\) and the subsequent caspase 3 activation, via a more pronounced RVI process during AVD\(_2\), and finally via inhibition and delay of AVD\(_2\) preventing or delaying the execution of apoptosis. This alteration of AVD is, at least in part, controlled by functional impairment of NS3728-sensitive chloride channels, likely VRAC. Increasing the Cl\(^-\) permeability by a channel opener or by stimulation of channel synthesis and insertion in the membrane may be considered as an attractive therapeutic approach, not only to circumvent MDR, but also in other conditions where sensitivity to apopto- tic stimuli is desired. Conversely, VRAC inhibitors could be valuable tools in cases where unwanted apoptosis should be prevented.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


