Original Research

Dysfunction of volume-sensitive chloride channels contributes to cisplatin resistance in human lung adenocarcinoma cells

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Abstract

Cisplatin-based chemotherapy is the standard therapy used to treat non-small-cell lung cancer. However, its efficacy is largely limited due to the development of drug resistance. The exact mechanism in which cancer cells develop resistance to the drug is not yet fully understood. The purpose of the present study is to test the role of volume-sensitive Cl^- channels in cisplatin resistance in human lung adenocarcinoma cells (A549 cells) using patch-clamp recording, cell volume measurement and apoptosis assay. The results showed that cisplatin treatment induced an apoptotic volume decrease (AVD) and activated a Cl^- current that showed properties similar to the volume-sensitive outward rectifying (VSOR) Cl^- current in wild-type A549 cells. Both the AVD process and VSOR Cl^- current were blocked by the chloride channel blocker 4,4′-diisothiocyanostilbene-2,2′ disulfonic acid. However, the A549/CDDP cells, a model of acquired cisplatin resistance cells, on the other hand, had almost no AVD process and VSOR Cl^- current when treated with cisplatin. Treatment of A549/CDDP cells with trichostatin A (TSA), a drug that inhibits histone deacetylases, partially restored the VSOR Cl^- current and increased cisplatin-induced cell apoptosis rate. These results suggest that impaired activity of VSOR Cl^- channels contributes to the cisplatin resistance in A549/CDDP cells.

Keywords: A549 cells, A549/CDDP cells, cisplatin, apoptotic volume decrease, volume-sensitive Cl^- channels

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Introduction

Lung cancer is one of the leading causes of cancer-related death worldwide, representing about 29% of all such cases, while fewer than 15% of patients are still living after five years.[1,2] Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases.[3] The most promising therapy for NSCLC is complete lung resection. However, the survival rate after complete lung resection for NSCLC is far from satisfactory and most patients are offered chemotherapy as an alternative, in particular cisplatin (*cis*-diamminedichloroplatinum II)-based chemotherapy.[4]

Cisplatin has been used clinically for approximately 30 y and is effective against a variety of cancers. It is primarily effective through the formation of adducts with DNA, causing induction of apoptosis.[5] However, the efficacy of cisplatin-based chemotherapy is limited due to the development of multidrug resistance (MDR) of the cancer cells. Despite the recent increase in the understanding of the signals contributing to drug resistance, cisplatin resistance remains a major problem that severely limits the usefulness of this chemotherapeutic agent. Thus, elucidating the mechanism of drug resistance will be beneficial for the development of more effective methods to combat cisplatin resistance for lung cancer.

Recently, multiple studies have pointed to the involvement of plasma membrane ion channels in the cell's apoptotic response to chemotherapy. A growing body of evidence implicates the VSOR Cl^- channels as an important modulator of apoptosis. It has been reported that both mitochondrion-mediated intrinsic and death receptor-mediated extrinsic apoptotic stimuli rapidly activate volume-sensitive outward rectifying (VSOR) Cl^- conductance in a wide variety of cell types,[6–11] crucial to the occurrence of apoptosis by inducing apoptotic volume decrease (AVD), which is a major hallmark of cell apoptosis and is an early prerequisite to apoptotic events. AVD is an upstream event to biochemical apoptotic events such as cytochrome C release, caspase-3 activation and DNA laddering. Efflux of K^+ and Cl^- from the cell through K^+ and Cl^- channels is primarily responsible for AVD.

Following prevention of AVD in a variety of cell types, subsequent apoptotic biochemical and morphological events are also prevented and cells are rescued from death.[7,12–14]

Our previous study has also found that activation of VSOR Cl^- channels is involved in carboplatin-induced apoptosis in A549 cells by inducing AVD.[6] Blockage of VSOR Cl^- channels can eliminate the chemotherapeutic agent-induced AVD process and apoptosis rate in A549 cells. These results, along with the finding that VSOR Cl^- current activation is essential to the progression of apoptosis in various cell lines,[7–9] suggest that the alteration in VSOR Cl^- channel functional expression might contribute to cisplatin resistance in A549 cells.

In the present study, A549/CDDP cells, established from wild-type A549 cells via stepwise dose escalation of cisplatin, were used to represent a model of acquired drug resistance. The results showed that cisplatin treatment induced the AVD process and activated a Cl^- current that showed properties similar to the VSOR Cl^- current in wild-type A549 cells. Both the cisplatin-induced AVD process and VSOR Cl^- current were blocked by the chloride channel blockers 4,4′-diisothiocyanostilbene-2,2′ disulfonic acid (DIDS). However, the cisplatin-resistant A549/CDDP cells, on the other hand, exhibited no obvious AVD process and VSOR Cl^- currents when treated with cisplatin. Trichostatin A (TSA), a histone deacetylase inhibitor, not only partially restored VSOR Cl^- channel activity but also decreased the cisplatin resistance in A549/CDDP cells. The results suggest that functional expression of VSOR channels was virtually absent in A549/CDDP cells and that impaired activity of VSOR Cl^- channels contribute to the cisplatin resistance in A549/CDDP cells.

Materials and methods

Cell culture

The cisplatin-resistant human lung adenocarcinoma cell line, A549/CDDP cells (Cancer Institute and Hospital, Chinese Academy of Medical Sciences, Beijing, China) were cultured as a monolayer in RPMI Media 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA). Cisplatin (2 μg/mL) was added to the medium to maintain drug resistance. Wild-type human lung adenocarcinoma cells (WT A549 cells; Peking Union Medical College, Beijing, China) were cultured in identical medium without cisplatin. For patch-clamp recordings and volume measurements, cells were detached from the plastic substrate and suspended, as described previously.[15] Cell suspension samples (0.2 mL) were placed in a chamber and, after attachment to the glass bottom, perfused with bath solution at a flow rate of approximately 2 mL/min. To restore the activity of VSOR Cl^- channels, A549/CDDP cells in the log-growing phase were incubated with TSA (400 nmol/L) for 24 h, as described previously.[7]

Cell volume measurement

Cell volume was measured at room temperature (22–26°C) using the methods described previously.[6,15–19] Briefly, suspended cells in dishes were mounted onto an inverted microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan) with a high-resolution electronic camera (Spot RF/SE, Diagnostic Instruments Inc, Sterling Heights, MI, USA). Cell images were captured and analyzed with a medical digital image analysis system (HMIAS-2000, WuHan Champion Image Technology Co, Ltd, WuHan, China). As the cells maintained the spherical shape during the measurements, the relative cell volume was calculated as follows with each cell used as its own control:

$$vol_t/vol_c = (4/3\pi r_t^3)/(4/3\pi r_c^3)$$

where t is the apoptosis inducer or/and blocker group and c is the control.

For volume measurement, isotonic (310 mosmol/kg-H_2O) bathing solution contained (in mmol/L) 90 NaCl, 4.5 KCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 110 mannitol and 10 N-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) (pH 7.4).

Patch-clamp recordings

Volume-sensitive Cl^- currents were recorded using whole-cell patch-clamp recordings at room temperature.[6,15] Patch pipettes were pulled from borosilicate thin-walled glass capillaries using a micropipette puller (PC-10: Sutter instruments, Novato, CA, USA) and had a resistance of 3–5 MΩ when filled with electrode solution. Data were acquired using an EPC-10 amplifier and Pulse software (HEKA Electroniks, Lambrecht, Germany). Current signals were low-pass filtered at 2.9 kHz using a four-pole Bessel filter. Sampled data were analyzed using an original software application called Pulsefit and Origin 6.1 (Origin Lab, Northampton, MA, USA). In all experiments, a grounded Ag-AgCl pellet electrode was placed in the perfusion solution. For whole cell recordings the series resistance (<10 MΩ) was compensated 70%. In order to monitor the voltage dependence of the current, step pulses were applied using a prepotential of −100 mV to test potentials ranging from −100 to +100 mV in 20 mV increments. To eliminate K^+ currents, a Cs-rich solution was employed. The pipette solution consisted of (in mmol/L): 110 CsCl, 2 Na_2ATP, 10 HEPES, 2 $MgSO_4$, 1 EGTA and 50 mannitol (295 mosmol (kg-H_2O)$^{-1}$, pH 7.3). Isotonic (330 mosmol (kg-H_2O)$^{-1}$) or hypotonic (250 mosmol (kg-H_2O)$^{-1}$) bathing solution consisted of (in mmol/L) 110 CsCl, 10 HEPES, 2 $MgSO_4$ and 80 or without mannitol (pH 7.4). To prevent spontaneous cell swelling after attaining the whole-cell mode,[15] the osmolality of the pipette solution was set lower than that of the isotonic bathing solution.

RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted from the cells using TRIzol reagent according to the manufacturer's instructions, as previously described.[20,21] Specific primers for human ClC-3 (forward primer: 5′-TATGATGCGTGGTCAGGATG-3′, reverse primer: 5′-AACCATCACATTAGTCCTGGCT-3′), MDR1 (forward primer: 5′-TCGTAGGAGTGTCCGTGGAT-3′, reverse primer:

5′-CAAGGGCTAGAAACAATAGTGAAA-3′), pIcln (forward primer: 5′-TATGGCACTTACTGATGTGGATGA-3′, reverse primer: 5′-GGAGCCGTCTGTCCGTGTAG-3′) were used to detect the expression of these three genes. The primers for β-actin (forward primer: 5′-GTGGGGCGCCCCAGGCACCA -3′, reverse primer: 5′-CTTCCTTAATGTCACGCA CGA TTTC-3′) were used as control for RNA integrity. Polymerase chain reaction (PCR) was performed by an Icycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: the PCR reaction mixture was denatured at 94°C (0.5 min), annealed at 55°C (0.5 min), and extended at 72°C (0.5 min) for 30 cycles. This was followed by a final extension at 72°C (5 min) to ensure complete product extension. Amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. PCR product bands were visualized by ultraviolet light, and the intensity values were measured by densitometry with the Image J software and normalized to the intensity values of β-actin for quantitative comparisons. The amplified products were sequenced, respectively.

Cell counting and viability assay

Cells were seeded in 24-well plates at a density of 2×10^4 cells/well for cell counting and 96-well plates at a density of 5×10^3 cells/well for cell viability determination. To observe whether TSA treatment affects the function of Cl^- channels and cisplatin resistance, A549/CDDP cells were exposed to the medium containing cisplatin (15 μmol/L) with or without TSA (400 nmol/L) for 24 h. Cell counting and viability were measured by directly counting the cell number and using the MTT assay, as described previously.[20,22]

Apoptosis rate assay

To measure the rate of apoptosis, cells were seeded at a density of 1×10^5 cells/well in six-well plates and exposed to medium containing cisplatin (15 μmol/L), with or without TSA (400 nmol/L) and DIDS (100 μmol/L) for 24 h. The MEBSTAIN Apoptosis TUNEL Kit Direct (MBL, Nagoya, Japan) was used and analysis was performed using a FACScan flow cytometer (Beckman Coulter, Fullerton, CA, USA). Four parallel samples were measured and over 1×10^4 cells were tested in each sample.[6]

Drugs and reagents

Cisplatin and TSA, as well as the Cl^- channel blockers, DIDS, 5-nitro-2-(3-phenyl propylamino)- benzoate (NPPB) and 4-(2-butyl-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) were obtained from Sigma-Aldrich (Shanghai, China). Stock solutions of DIDS, NPPB, DCPIB and TSA were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the experimental solutions was <0.1%.

Statistical analysis

Data are presented as the mean ± SEM of the number of observations. Statistical differences in the data were evaluated by the Student's paired or unpaired *t*-test and a $P < 0.05$ was considered statistically significant.

Results

Absence of the AVD process in A549/CDDP cells

Treatment of WT A549 cells with cisplatin (15 μmol/L) for two, four, six and eight hours, resulted in an obvious time-dependent reduction in cell volume (Figure 1). This normotonic cell shrinkage caused by apoptotic inducer is known as AVD and reported to be an early prerequisite for apoptotic cell death. This AVD process was blocked by the Cl^- channel blocker, DIDS. However, cisplatin-induced AVD was almost absent in the cisplatin-resistant A549/CDDP cells. Treatment of A549/CDDP cells with TSA partially restored the cisplatin-induced AVD process. These results suggest that A549/CDDP cells could escape from the cisplatin-induced AVD process that potentially contributes to protection against apoptosis in the cell line similar to the reported MDR Ehrlich ascites tumor cells.[23]

Dysfunction of VSOR Cl^- channels in A549/CDDP cells

It has been suggested that the AVD process is accomplished by activation of VSOR Cl^- channels in human epithelial HeLa, lymphoid U937, rodent neuronal PC12, NG108-15 cells and A549 cells.[7,9,24] Hence, we chose to compare the functional expression of VSOR Cl^- channels in A549/CDDP cells with WT A549 cells.

As shown in Figure 2, hypotonic solution induced an obvious Cl^- current in WT A549 cells that exhibited moderate outward rectification and time-dependent inactivation kinetics at a large positive potential. Addition of a volume-sensitive Cl^- channel blocker, NPPB (100 μmol/L) and

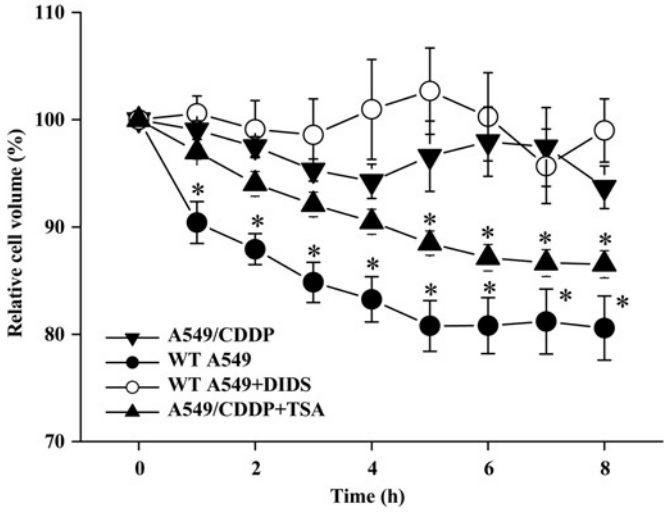

Figure 1 Cisplatin (CDDP)-induced AVD in WT A549 and A549/CDDP cells. Cisplatin (15 μmol/L) was used to induce AVD process. The blocking effect of DIDS (100 μmol/L) and restoration effect of TSA were observed. Each symbol represents the mean ± SEM (vertical bar). *$P < 0.05$ WT A549 versus other groups ($n = 25$). AVD, apoptotic volume decrease; DIDS, 4,4′-diisothiocyanostilbene-2,2′ disulfonic acid; TSA, Trichostatin A; SEM, standard error of mean

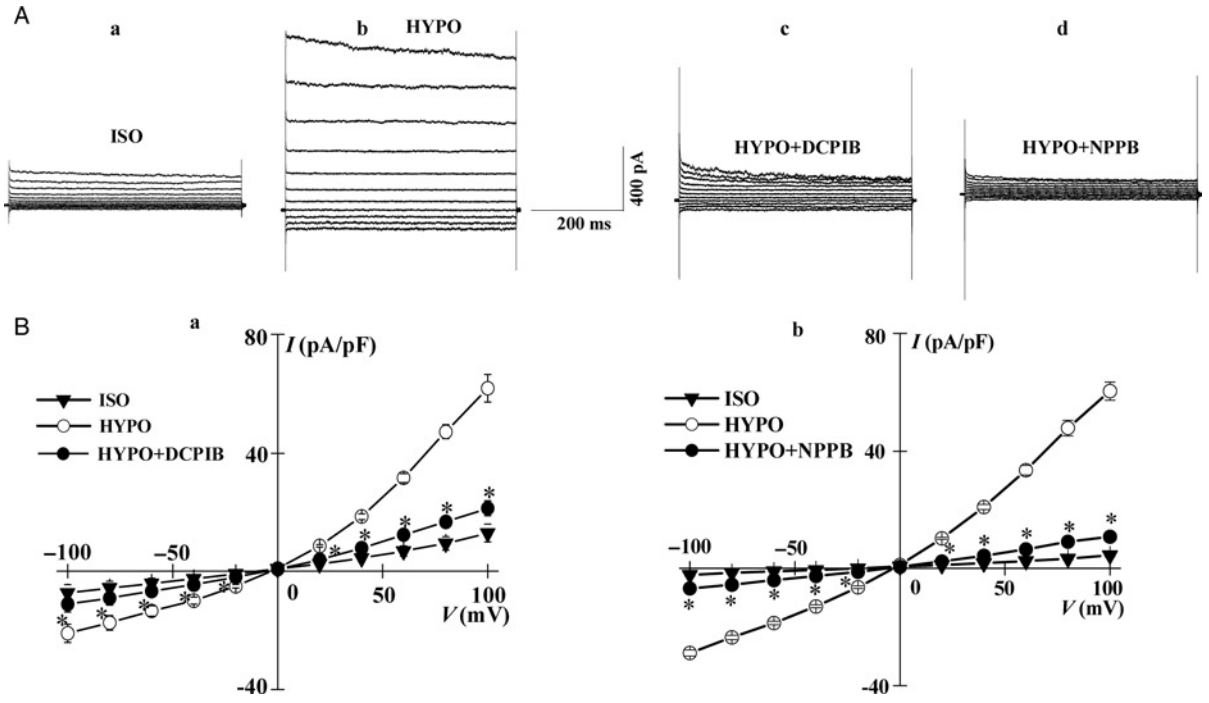

Figure 2 Hypotonic-activated VSOR Cl^- currents in WT A549 cells. (A-a) Basal currents in WT A549 cells under isotonic solution. (A-b) Hypotonic-activated Cl^- currents. (A-c, d) Blockade effect of DCPIB and NPPB on Cl^- currents. (B) I/V relationships of the volume-sensitive Cl^- currents in the presence of isotonic, hypotonic and hypotonic solutions containing DCPIB (20 μmol/L) or NPPB (100 μmol/L). Each symbol represents the mean $\pm$ SEM (vertical bar). $^*P < 0.05$ versus hypotonic groups ($n = 5$). VSOR, volume-sensitive outward rectifying; DCPIB, 4-(2-butyl-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid; NPPB, 5-nitro-2-(3-phenyl propylamino)-benzoate; SEM, standard error of mean

DCPIB (20 μmol/L), to the bath solution inhibited hypotonic-activated current at both the outward and inward directions. All of these properties are the hallmark of VSOR Cl^- channel current.

Perfusion of WT A549 cells with cisplatin in an isotonic solution also activated a VSOR-like current, which exhibited outward rectification, time-dependent inactivation kinetics at a large positive potential and sensitivity to DCPIB (Figure 3).

However, in cisplatin-resistant A549/CDDP cells, hypotonic-activated Cl^- currents were virtually undetectable despite the cells exhibiting osmotic swelling to nearly the same extent under hypotonic conditions. As shown in Figure 4, the currents were almost completely absent in A549/CDDP cells responding to the hypotonic challenge as well as cisplatin. This is a surprising finding because VSOR Cl^- currents have been detected in nearly all cell types to date.[6,15] Figure 5 depicts a comparison of Cl^- currents in A549 and A549/CDDP cells during hypotonic and cisplatin treatment.

TSA treatment restored VSOR currents in A549/CDDP cells

The above results implied that VSOR Cl^- channel currents were involved in the AVD process in WT A549 cells, and that impaired activity of VSOR Cl^- channels may be responsible for the absence of the AVD process in A549/CDDP cells. In order to demonstrate this further, we need to restore the activity of VSOR Cl^- channels. Currently, no specific opener for VSOR Cl^- channels has been determined. It has been reported that TSA, a drug that inhibits histone deacetylases, by removing the gene inhibition, could partially restore VSOR Cl^- currents in KCP-4 cells.[8] Thus, we treated A549/CDDP cells with TSA (400 nmol/L) for 24 h. As shown in Figure 6, hypotonic- and cisplatin-induced Cl^- currents were partially restored after the TSA treatment. The restored currents exhibited properties similar to VSOR Cl^- currents, sensitive to DCPIB as well as NPPB and DIDS (data not shown).

TSA treatment decreased cisplatin resistance in A549/CDDP cells

Cell number and viability were measured to determine whether TSA treatment exhibits an effect on the cisplatin resistance in A549/CDDP cells. The results showed that there was a significant decrease in A549/CDDP cell number (Figure 7A) and viability (Figure 7B) after treatment with cisplatin (15 μmol/L) plus TSA (400 nmol/L) for 24 h, in comparison with cisplatin alone, suggesting that the cell's sensitivity to cisplatin is increased after TSA treatment. This effect was inhibited by DIDS (100 μmol/L).

In order to elucidate whether the TSA-induced cell viability decrease was due, at least in part, to cell apoptosis, flow cytometry was performed to assess the cell apoptosis rate. As shown in Figure 7C, cells treated with TSA and cisplatin had a significantly enhanced apoptosis rate in comparison with cells treated with TSA or cisplatin alone. This enhancement was similarly blocked by the addition of DIDS

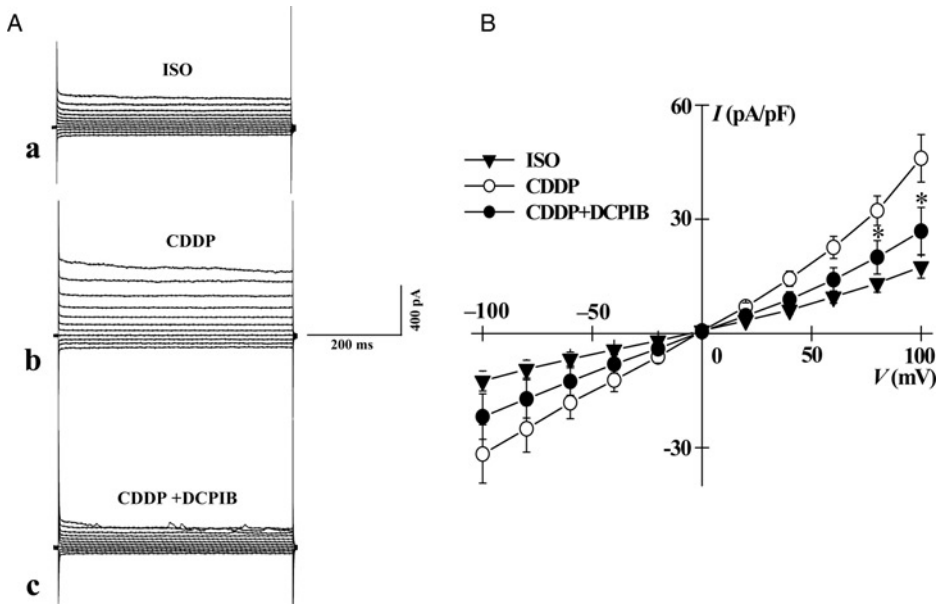

Figure 3 Cisplatin (CDDP)-activated VSOR-like Cl^- channel current in WT A549 cells. (A-a) Basal currents in WT A549 cells. (A-b) CDDP-activated Cl^- channel currents in WT A549 cells. (A-c) Blockade effect of DCPIB. (B) I/V relationships of CDDP-activated Cl^- currents under control, CDDP and CDDP + DCPIB conditions. Each symbol represents the mean ± SEM (vertical bar). $^*P < 0.05$ compared with blocker groups. CDDP: 15 μmol/L; DCPIB: 20 μmol/L, $n = 5$. DCPIB, 4-(2-butyl-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid; SEM, standard error of mean

(100 μmol/L) to the medium. These results further confirmed that the decrease in cisplatin resistance in A549/CDDP cells follows restoration of VSOR chloride channel activity by TSA, suggesting that the activity of volume-sensitive Cl^- channels is an important factor in determining the sensitivity of cancer cells to cisplatin.

TSA treatment did not alter MDR1, pIcln and ClC-3 gene expression level

TSA is a kind of a drug that inhibits histone deacetylases. It can cause the increase in gene expression or downregulation of certain gene expression by inhibiting histone deacetylase (HDAC). In order to investigate whether TSA restored the

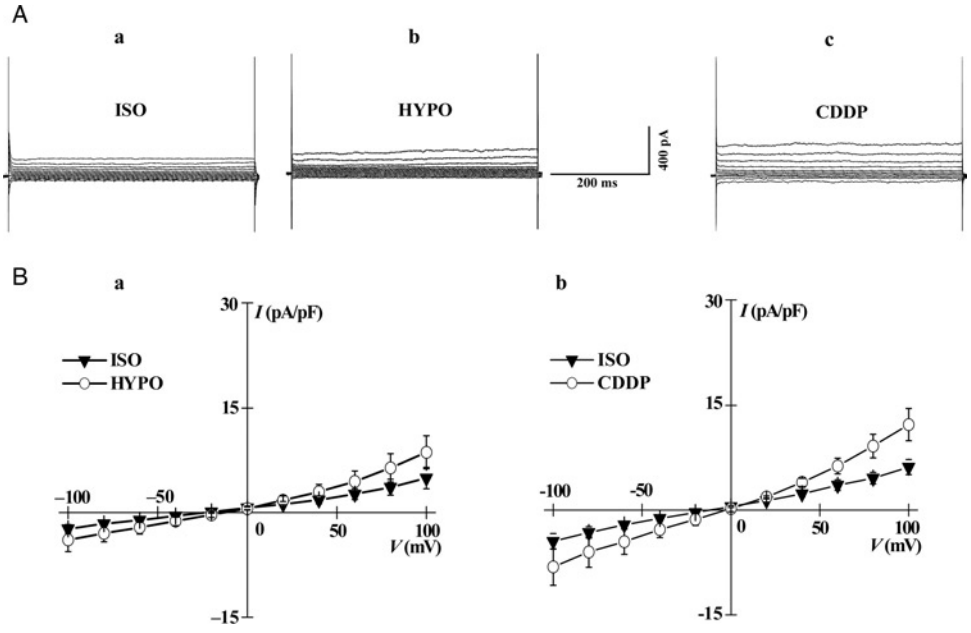

Figure 4 Hypotonic- and cisplatin-activated VSOR Cl^- currents in A549/CDDP cells. (A-a) Basal currents in A549/CDDP cells under isotonic condition. (A-b) Hypotonic-activated Cl^- channel currents in A549/CDDP cells. (A-c) CDDP-activated Cl^- channel currents in A549/CDDP cells. (B) I/V relationships of hypotonic- (a) and CDDP- (b) activated Cl^- currents. Each symbol represents the mean ± SEM (vertical bar) ($n = 5$). VSOR, volume-sensitive outward rectifying; SEM, standard error of mean

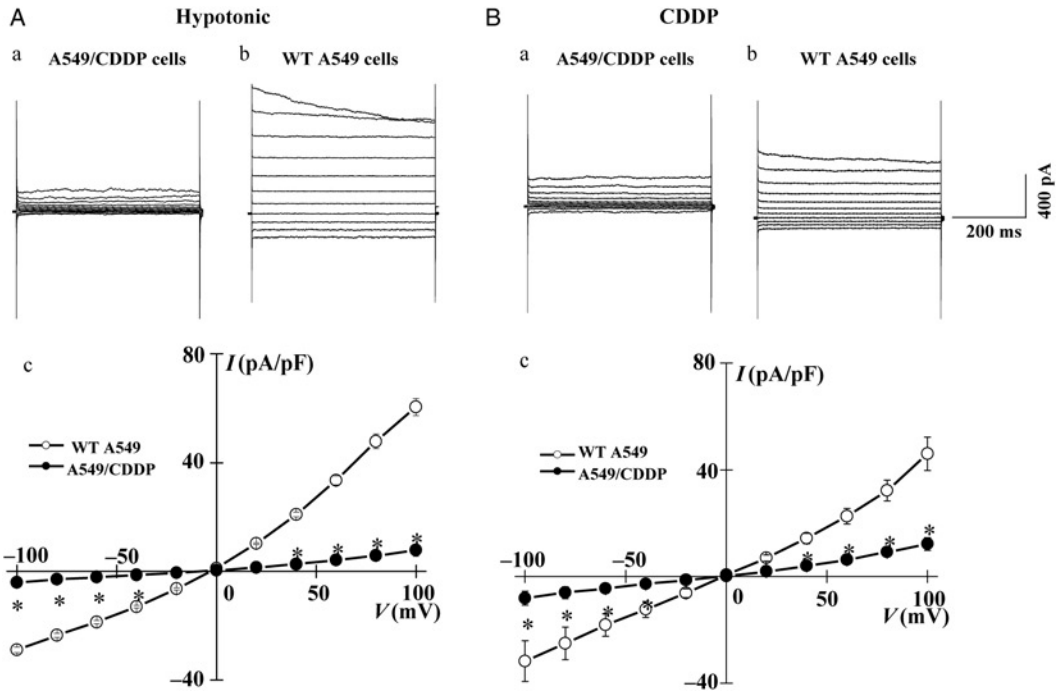

Figure 5 Comparison of VSOR Cl^- currents between WT A549 and A549/CDDP cells. (A) Hypotonic-activated Cl^- currents in A549/CDDP cells (a) and WT A549 (b). (A-c) I/V relationships of hypotonic-activated Cl^- currents in WT A549 and A549/CDDP cells. (B) CDDP-activated Cl^- currents in A549/CDDP cells (a) and WT A549 (b). (B-c) I/V relationships of CDDP-activated Cl^- currents in WT A549 and A549/CDDP cells. Each symbol represents the mean $\pm$ SEM (vertical bar). $^*P < 0.05$ compared with A549 group ($n = 5$). VSOR, volume-sensitive outward rectifying; SEM, standard error of mean

VSOR Cl^- channel current and if decreased cisplatin resistance in A549/CDDP cells is related to the gene expression changes, the level of mRNA transcript expression of three molecular candidates reported previously for the VSOR Cl^- channel was detected by semi-quantitative reverse transcriptase (RT)-PCR.[15,25] First, the expression level of MDR1 (gene encoding P-glycoprotein), pIcln and ClC-3, was compared in both WT A549 and A549/CDDP cells. As shown in

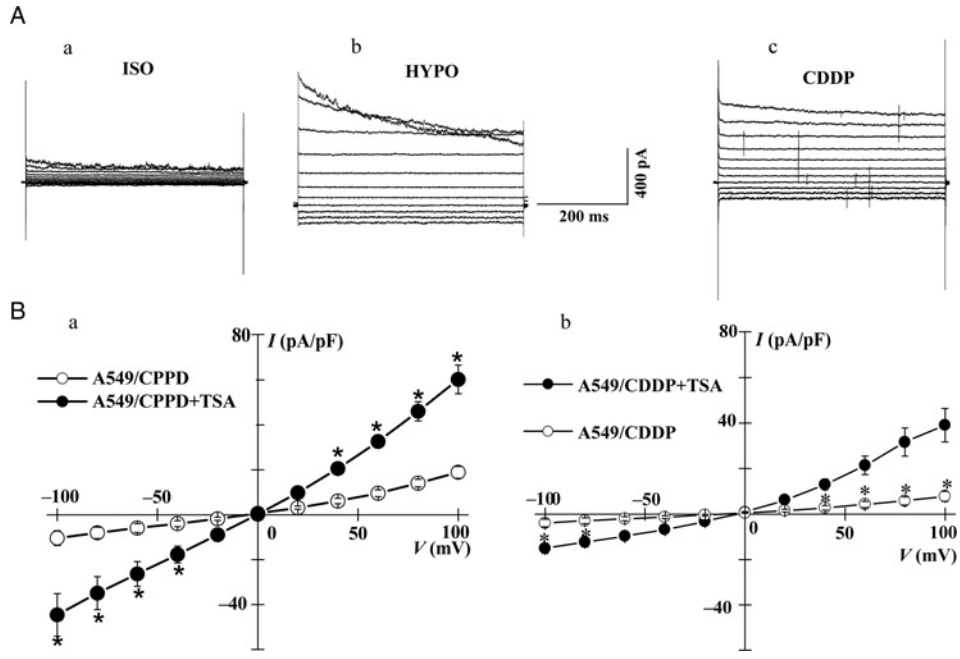

Figure 6 Restoration of VSOR Cl^- channel activity by TSA treatment in A549/CDDP cells. (A-a) Basal currents in TSA-treated A549/CDDP cells under isotonic solution. (A-b) Hypotonic-activated Cl^- currents in TSA treated A549/CDDP cells. (A-c) CDDP-activated Cl^- currents in TSA-treated A549/CDDP cells. (B) Comparison of hypotonic solution- (a) and cisplatin- (b) induced VSOR Cl^- currents in A549/CDDP cells before and after TSA treatment. Each symbol represents the mean $\pm$ SEM. $^*P < 0.05$ versus blocker groups (B-a, b). NPPB: 100 μmol/L; DCPIB: 20 μmol/L, $n = 5$. TSA, trichostatin A; VSOR volume-sensitive outward rectifying; SEM, standard error of mean

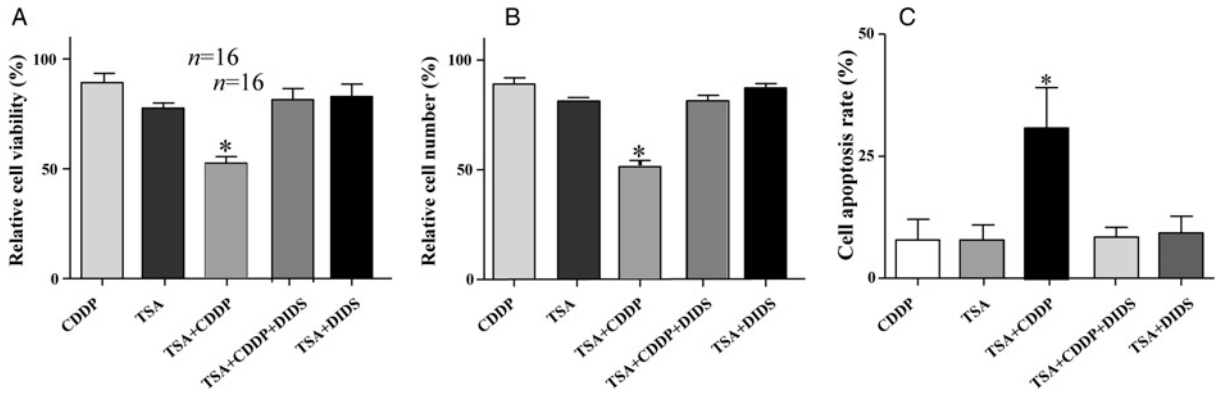

Figure 7 TSA treatment decreased cisplatin resistance in A549/CDDP cells. Cisplatin (CDDP)-induced A549/CDDP cell number, cell viability and apoptosis rate after treated with cisplatin (15 μmol/L), TSA (400 nM) or together for 24 h with or without DIDS (100 μmol/L). (A) Relative cell number was determined by cell counting. (B) Relative viability of the cells was measured by MTT. (C) CDDP-induced apoptosis rate assessed by flow cytometry analysis. Each symbol represents the mean $\pm$ SEM (vertical bar), $^{*}P < 0.05$ TSA + CDDP versus other groups ($n = 6$). TSA, trichostatin A; DIDS, 4,4′-diisothiocyanostilbene-2,2′ disulfonic acid; SEM, standard error of mean

Figure 8A, there was no obvious difference in the expression level of pIcln and ClC-3, but MDR1 expression decreased in A549/CDDP cells compared with WT A549 cells. TSA treatment had no obvious effect on the expression level of the three molecular candidates, as summarized in Figure 8B. These data indicate that ClC-3, MDR1 and pIcln may not be responsible for TSA-induced restoration of VSOR Cl^- channel activity and decrease in cisplatin resistance in A549/CDDP cells. The mechanism for TSA-induced VSOR Cl^- channel activity restoration needs to be further explored.

Discussion

In this study we demonstrated that impaired activity of volume-sensitive Cl^- channels was involved in cisplatin resistance in A549/CDDP cells. These results further implicated the close relationship between VSOR Cl^- channel activity and drug sensitivity in this cell line. The evidence comprises: (a) VSOR Cl^- channel activity is responsible for the AVD and apoptosis process induced by cisplatin in WT A549 cells; (b) VSOR Cl^- channel currents are almost absent in A549/CDDP cells; (c) A549/CDDP cells lack the cisplatin-induced AVD process; and (d) restoration of

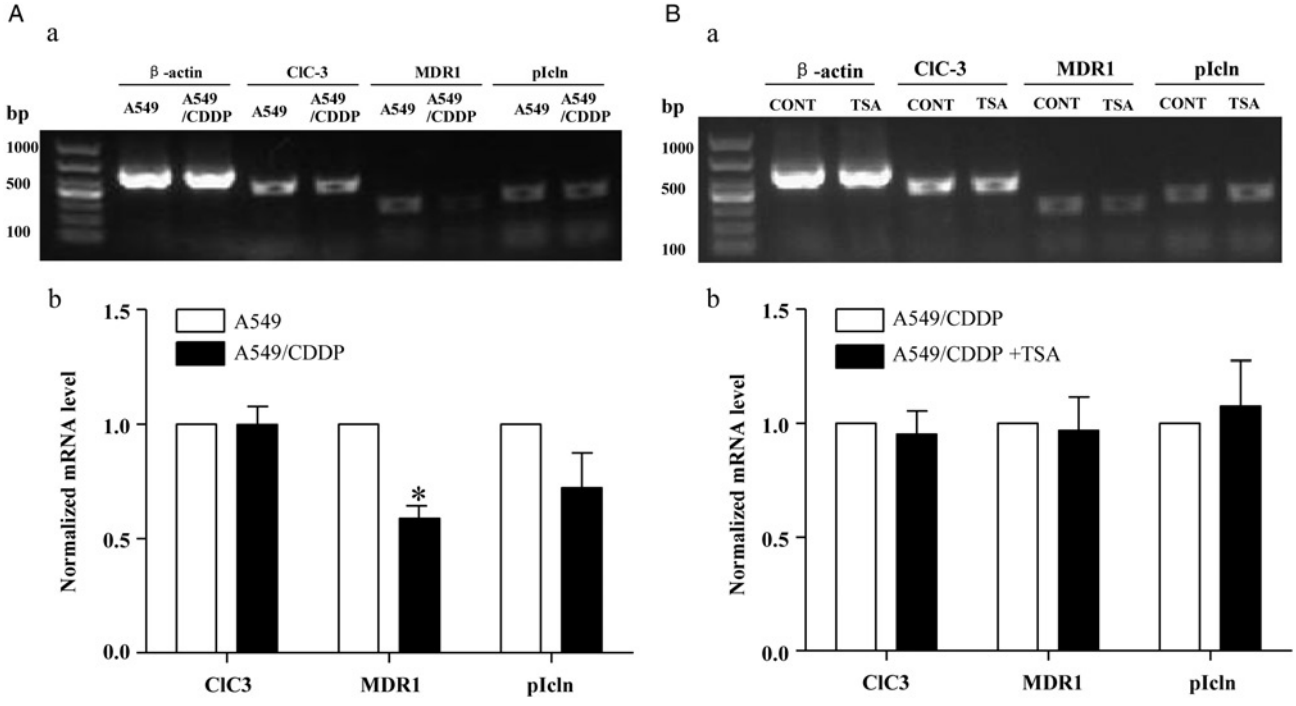

Figure 8 The mRNA expression levels of three candidate genes for volume-sensitive Cl^- channels. (A-a) PCR-amplified products in WT A549 cells and A549/CDDP cells. (A-b) Data normalized to the amount of β-actin ($n = 4$). $^{*}P < 0.05$ versus A549 ($n = 4$). (B-a) PCR-amplified products of A549/CDDP and TSA-treated A549/CDDP cells. (B-b) Data normalized to the amount of β-actin ($n = 4$). PCR, polymerase chain reaction; TSA, Trichostatin A

VSOR Cl^- channel activity in A549/CDDP cells increases cisplatin sensitivity.

The occurrence of drug resistance is a common drawback of cancer chemotherapy, and cisplatin is no exception. It is clear that cisplatin resistance of cancer cells is a complicated phenomenon that occurs through a variety of mechanisms, including reduction in platinum accumulation or uptake, increase of thiol-containing biomolecules such as glutathione and metallothioneins, alteration of expression of oncogenes that limit the formation of cisplatin-DNA adducts and activation of anti-apoptotic pathways, enhancement of the DNA damage repair and mutation of p53 and other genes.[5]

Recently, VSOR Cl^- channel blockers were found to inhibit apoptotic cell death in cancer cells treated with cisplatin,[26] in cardiomyocytes subjected to ischemia–reperfusion[27] and in hippocampal neurons subjected to ischemia–reperfusion *in vivo*.[28] In the present study, the cisplatin-induced AVD process in WT A549 cells was also prevented by VSOR Cl^- blocker DIDS. Thus, the results suggest that the VSOR Cl^- channel activity may be a prerequisite for the occurrence of cisplatin-induced apoptosis. This would explain why cisplatin-treated WT A549 cells exhibited an increased VSOR Cl^- current and A549/CDDP cells, with impaired activity of VSOR Cl^- channels, did not undergo AVD during exposure to cisplatin.

According to the results of the present experiments, it can be concluded that the absence of VSOR Cl^- channel activity in A549/CDDP cells may be a mechanism to protect the cells from cisplatin-induced cell death by at least partially inhibiting the AVD process. To investigate the reason for the loss of VSOR Cl^- channel activity in A549/CDDP cells, semi-quantitative RT-PCR was used to check whether the level of transcript expression was different in WT A549 as compared with A549/CDDP cells, for genes that have previously been suggested to encode the VSOR Cl^- channel (MDR1, pIcln and ClC-3).[8] Of the three genes, only the level of MDR1 expression was significantly decreased in A549/CDDP cells (Figure 8A); however, no clear differences were found before and after TSA pretreatment (Figure 8B). These results suggested that these three genes have no clear relationship with the activity changes induced by TSA in A549/CDDP cells.

TSA is known to be a HDAC inhibitor and, along with histone acetyltransferase, regulates the transcriptional status of chromatin by determining the level of histone acetylation.[8,29] However, the exact mechanism for restoration of the VSOR Cl^- channel by TSA treatment is unknown. It might be related to the upregulation of the gene encoding the VSOR Cl^- channel, the alteration of the expression for a regulator gene of the channel, or related to changes in the expression level of multiple genes that affect the VSOR Cl^- channel activity.

MDR1 is the gene encoding P-glycoprotein. P-glycoprotein is an important drug efflux protein. The changes of the MDR1 expression level in drug resistance cells are controversial. In NSCLC cell lines, it has been reported that increased MDR1 mRNA and/or protein expression levels were associated with resistance to anthracyclines,[30,31] vinca alkaloids,[32] etoposide[33] and taxanes.[34] However, some other reports indicated that the MDR1/P-gp expression neither correlate significantly with sensitivity to cisplatin or carboplatin, nor with intracellular or intranuclear cisplatin accumulation, and cisplatin and carboplatin actually increase cellular concentrations of some other agents by inhibiting P-gp.[35–40] The results of this experiment showed that MDR1 expression decreased in A549/CDDP cells. But we could not yet reach any conclusion with regards to what the decreased MDR1 expression in A549/CDDP cells really means. In this experiment, the A549/CDDP cells were cultured in the medium with 2 μg/mL cisplatin to maintain the cell's resistance to cisplatin. Maybe we can attribute the MDR1 expression reduction in A549/CDDP cells to the cisplatin in the medium because it has been reported that platinum drugs could inhibit the expression of MDR1. In addition, it should be noted that P-glycoprotein is a known regulator of the VSOR Cl^- channel,[41,42] and thus it is possible that an alteration in this protein could affect Cl^- channel activation. But TSA-induced restoration of the Cl^- channel activity is independent of MDR1 because TSA does not change the expression level of it.

The molecular mechanism for the loss of activity of VSOR Cl^- channels in A549/CDDP cells is not clear. It may involve a disruption or lack of the protein(s) comprising the channel *per se*; alternatively, the alteration of channel regulators may be also involved. There may be other intracellular signals involved in the impairment of VSOR Cl^- channel activity in cisplatin-resistant cells.

Similar results have been reported in a cisplatin-resistant KCP-4 human epidermoid cancer cell line.[26] It is possible that lack of VSOR Cl^- channel activity is specific to cisplatin-resistant cells as activity of the channel was observed in other types of drug-resistant cells. Thus, the results of this experiment imply the importance of further investigation into the signaling pathways of the channel activation and the molecular orientation that may shed light on the mechanism of cisplatin resistance and lead to more effective cancer therapies.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. X-jM, WH, JL and BH conducted the experiments; JW, HL and S-cH designed the experiment and wrote the manuscript.

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REFERENCES

1 Herbst RS, O'Neill VJ, Fehrenbacher L, Belani CP, Bonomi PD, Hart L, Melnyk O, Ramies D, Lin M, Sandler A. Phase II study of efficacy and safety of bevacizumab in combination with chemotherapy or erlotinib

compared with chemotherapy alone for treatment of recurrent or refractory non small-cell lung cancer. *J Clin Oncol* 2007;**25**:4743–50
2 Park KS, Kim HK, Lee JH, Choi YB, Park SY, Yang SH, Kim SY, Hong KM. Transglutaminase 2 as a cisplatin resistance marker in non-small cell lung cancer. *J Cancer Res Clin Oncol* 2010;**136**:493–502
3 Seve P, Reiman T, Dumontet C. The role of beta III tubulin in predicting chemoresistance in non-small cell lung cancer. *Lung Cancer* 2010;**67**:136–43
4 Okuda K, Sasaki H, Hikosaka Y, Kawano O, Yukiue H, Yano M, Fujii Y. Excision repair cross complementation group 1 polymorphisms predict overall survival after platinum-based chemotherapy for completely resected non-small-cell lung cancer. *J Surg Res* 2010 (In press)]
5 Liu LZ, Zhou XD, Qian G, Shi X, Fang J, Jiang BH. AKT1 amplification regulates cisplatin resistance in human lung cancer cells through the mammalian target of rapamycin/p70S6K1 pathway. *Cancer Res* 2007;**67**:6325–32
6 He W, Li H, Min X, Liu J, Hu B, Hou S, Wang J. Activation of volume-sensitive Cl^- channel is involved in carboplatin-induced apoptosis in human lung adenocarcinoma cells. *Cancer Biol Ther* 2010;**9**:1–7
7 Lee EL, Shimizu T, Ise T, Numata T, Kohno K, Okada Y. Impaired activity of volume-sensitive Cl^- channel is involved in cisplatin resistance of cancer cells. *J Cell Physiol* 2007;**211**:513–21
8 Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y. Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci USA* 2000;**97**:9487–92
9 d'Anglemont de Tassigny A, Souktani R, Henry P, Ghaleb B, Berdeaux A. Volume-sensitive chloride channels (ICl, vol) mediate doxorubicin-induced apoptosis through apoptotic volume decrease in cardiomyocytes. *Fundam Clin Pharmacol* 2004;**18**:531–8
10 Szabo I, Lepple-Wienhues A, Kaba KN, Zoratti M, Gulbins E, Lang F. Tyrosine kinase-dependent activation of a chloride channel in CD95-induced apoptosis in T lymphocytes. *Proc Natl Acad Sci USA* 1998;**95**:6169–74
11 Okada Y, Sato K, Numata T. Pathophysiology and puzzles of the volume-sensitive outwardly rectifying anion channel. *J Physiol* 2009;**587**:2141–9
12 Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S. Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). *J Physiol* 2001;**532**:3–16
13 Okada Y, Maeno E. Apoptosis, cell volume regulation and volume-regulatory chloride channels. *Comp Biochem Physiol A Mol Integr Physiol* 2001;**130**:377–83
14 Bortner CD, Cidlowski JA. Cellular mechanisms for the repression of apoptosis. *Annu Rev Pharmacol Toxicol* 2002;**42**:259–81
15 Wang J, Morishima S, Okada Y. IK channels are involved in the regulatory volume decrease in human epithelial cells. *Am J Physiol Cell Physiol* 2003;**284**:C77–84
16 Zuo W, Zhu L, Bai Z, Zhang H, Mao J, Chen L, Wang L. Chloride channels involve in hydrogen peroxide-induced apoptosis of PC_{12} cells. *Biochem Biophys Res Commun* 2009;**387**:666–70
17 Roy J, Vantol B, Cowley EA, Blay J, Linsdell P. Pharmacological separation of hEAG and hERG K^+ channel function in the human mammary carcinoma cell line MCF-7. *Oncol Rep* 2008;**19**:1511–6
18 Mao J, Chen L, Xu B, Wang L, Li H, Guo J, Li W, Nie S, Jacob TJ, Wang L. Suppression of ClC-3 channel expression reduces migration of nasopharyngeal carcinoma cells. *Biochem Pharmacol* 2008;**75**:1706–16
19 Mao JW, Wang LW, Jacob T, Sun XR, Li H, Zhu LY, Li P, Zhong P, Nie SH, Chen LX. Involvement of regulatory volume decrease in the migration of nasopharyngeal carcinoma cells. *Cell Res* 2005;**15**:371–8
20 Wang C, Wang J, Zhao L, Wang Y, Liu J, Shi L, Wang C. Sildenafil inhibits human pulmonary artery smooth muscle cell proliferation by decreasing capacitative Ca^{2+} entry. *J Pharmacol Sci* 2008;**108**:71–8
21 Kim YJ, Huh JW, Kim DS, Bae MI, Lee JR, Ha HS, Ahn K, Kim TO, Song GA, Kim HS. Molecular characterization of the DYX1C1 gene and its application as a cancer biomarker. *J Cancer Res Clin Oncol* 2009;**135**:265–70
22 Wang C, Li JF, Zhao L, Liu J, Wan J, Wang YX, Wang J, Wang C. Inhibition of SOC/Ca/NFAT pathway is involved in the anti-proliferative effect of sildenafil on pulmonary artery smooth muscle cells. *Respir Res* 2009;**10**:123
23 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**:C14–25
24 Porcelli AM, Ghelli A, Zanna C, Valente P, Ferroni S, Rugolo M. Apoptosis induced by staurosporine in ECV304 cells requires cell shrinkage and upregulation of Cl^- conductance. *Cell Death Differ* 2004;**11**:655–62
25 Duan D, Winter C, Cowley S, Hume JR, Horowitz B. Molecular identification of a volume-regulated chloride channel. *Nature* 1997;**390**:417–21
26 Ise T, Shimizu T, Lee EL, Inoue H, Kohno K, Okada Y. Roles of volume-sensitive Cl^- channel in cisplatin-induced apoptosis in human epidermoid cancer cells. *J Membr Biol* 2005;**205**:139–45
27 Wang X, Takahashi N, Uramoto H, Okada Y. Chloride channel inhibition prevents ROS-dependent apoptosis induced by ischemia-reperfusion in mouse cardiomyocytes. *Cell Physiol Biochem* 2005;**16**:147–54
28 Inoue H, Ohtaki H, Nakamachi T, Shioda S, Okada Y. Anion channel blockers attenuate delayed neuronal cell death induced by transient forebrain ischemia. *J Neurosci Res* 2007;**85**:1427–35
29 Weidle UH, Grossmann A. Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. *Anticancer Res* 2000;**20**:1471–85
30 Yabuki N, Sakata K, Yamasaki T, Terashima H, Mio T, Miyazaki Y, Fujii T, Kitada K. Gene amplification and expression in lung cancer cells with acquired paclitaxel resistance. *Cancer Genet Cytogenet* 2007;**173**:1–9
31 Pesic M, Markovic JZ, Jankovic D, Kanazir S, Markovic ID, Rakic L, Ruzdijic S. Induced resistance in the human non small cell lung carcinoma (NCI-H460) cell line *in vitro* by anticancer drugs. *J Chemother* 2006;**18**:66–73
32 Bessho Y, Oguri T, Ozasa H, Uemura T, Sakamoto H, Miyazaki M, Maeno K, Sato S, Ueda R. ABCC10/MRP7 is associated with vinorelbine resistance in non-small cell lung cancer. *Oncol Rep* 2009;**21**:263–8
33 NicAmhlaoibh R, Heenan M, Cleary I, Touhey S, O'Loughlin C, Daly C, Nuñez G, Scanlon KJ, Clynes M. Altered expression of mRNAs for apoptosis-modulating proteins in a low level multidrug resistant variant of a human lung carcinoma cell line that also expresses mdr1 mRNA. *Int J Cancer* 1999;**82**:368–76
34 Kitazaki T, Oka M, Nakamura Y, Tsurutani J, Doi S, Yasunaga M, Takemura M, Yabuuchi H, Soda H, Kohno S. Gefitinib, an EGFR tyrosine kinase inhibitor, directly inhibits the function of P-glycoprotein in multidrug resistant cancer cells. *Lung Cancer* 2005;**49**:337–43
35 Stewart DJ. Tumor and host factors that may limit efficacy of chemotherapy in non-small cell and small cell lung cancer. *Crit Rev Oncol Hematol* 2010;**75**:173–234
36 Yabuki N, Sakata K, Yamasaki T, Terashima H, Mio T, Miyazaki Y, Fujii T, Kitada K. Gene amplification and expression in lung cancer cells with acquired paclitaxel resistance. *Cancer Genet Cytogenet* 2007;**173**:1–9
37 Kawai H, Kiura K, Tabata M, Yoshino T, Takata I, Hiraki A, Chikamori K, Ueoka H, Tanimoto M, Harada M. Characterization of non-small-cell lung cancer cell lines established before and after chemotherapy. *Lung Cancer* 2002;**35**:305–14
38 Ikuta K, Takemura K, Sasaki K, Kihara M, Nishimura M, Ueda N, Naito S, Lee E, Shimizu E, Yamauchi A. Expression of multidrug resistance proteins and accumulation of cisplatin in human non-small cell lung cancer cells. *Biol Pharm Bull* 2005;**28**:707–12
39 Bergman AM, Pinedo HM, Talianidis I, Veerman G, Loves WJ, van der Wilt CL, Peters JG. Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. *Br J Cancer* 2003;**88**:1963–70
40 Bogush TA, Konukhova AV, Ravcheeva AB, Zabotina TN, Kadagidze ZG, Bogush EA, Komov DV, Polotskiĭ BE, Laktionov KK, Davydov MI. Inhibition of ABC-transporter(s)′ function in non-small cell lung cancer cells by platinum drugs. *Antibiot Khimioter* 2003;**48**:11–5
41 Miwa A, Ueda K, Okada Y. Protein kinase C-independent correlation between P-glycoprotein expression and volume sensitivity of Cl^- channel. *J Membr Biol* 1997;**157**:63–9
42 Valverde MA, Diaz M, Sepulveda FV, Gill DR, Hyde SC, Higgins CF. Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. *Nature* 1992;**355**:830–3

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