

Administration of isothiocyanate (E-4IB) and cisplatin leads to altered signalling and lysosomal export in human ovarian carcinoma sensitive- and cisplatin-resistant cells

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The aim of this study was to compare the effect of a new synthetic isothiocyanate derivative, ethyl 4-isothiocyanatobutanoate (E-4IB) and cisplatin (CDDP) in CDDP-sensitive human ovarian carcinoma cell line (A2780) and its resistant subline (A2780/CP). In parental cells, in comparison to untreated cells, sequential administration of both compounds led to higher exosomal dye (LysoTracker Green DND-26) retention and to alterations of mitogen-activated protein kinases (MAPKs), JNK, ERK and p38, or Akt kinase accompanied by changes in several anti- and pro-apoptotic molecules and lysosomal protein LAMP-1, as detected by Western blotting. On the contrary, variant A2780/CP cells were resistant to CDDP- or to combined sensitizer (E-4IB)/inducer (CDDP)-related apoptosis induction and exerted minor changes in the levels of these molecules.

Key words: ovarian carcinoma cells, isothiocyanate E-4IB, cisplatin (CDDP), cisplatin (CDDP) resistance, apoptosis, lysosomes/exosomes

Cisplatin (cis-diamminedichloroplatinum(II), CDDP) has broad antitumor activity in ovarian tumors, lung cancer, advanced bladder cancer and many other solid tumors [1]. Repeated exposure of cancer cells to CDDP results in the development of resistance to metaloids, including CDDP, and this resistance is associated with alterations in DNA repair, drug accumulation [2, 3] and linked with the expression of several transporters, particularly the copper protein Ctr1, metallochaperons and P-type ATP-ase transporters ATP7A and ATP7B [4, 5]. In various cells CDDP can activate multiple signaling pathways, including those involving p53, Bcl-2 family, caspases, cyclins, CDKs, pRb, PKC, PI3K/Akt and MAPKs (JNK, p38 and ERK), and other signal transduction pathways [6]. In cell response to CDDP, MAPKs activation appeared as major phenomenon deciding the cell fate in several cancer cell lines [7, 8]. In sensitive cells, inhibition of CDDP-induced JNK and p38 activation blocked CDDP-induced apoptosis and persistent activation of JNK resulted in hyperphosphorylation

of the c-Jun transcription factor, which, in turn, stimulated the transcription of an immediate downstream target, the death inducer Fas ligand (FasL) [9].

In human ovarian tumor cells CDDP accumulates in a variety of vesicular structures, including lysosomes [10]. CDDP-resistant cells release large amounts of lysosomal/exosomal enzymes and membrane proteins into their environment [11]. Lysosomal compartment in CDDP-resistant cells has been shown markedly abnormal in comparison with that in CDDP-sensitive cells and also participate in apoptotic and necrotic cell death pathways [12, 13]. Several transporters, predominantly ATP7A, ATP7B and MRP2, found in exosomes, are believed to be involved in the movement of CDDP across vesicle membranes and their levels were higher in the exosomes released from CDDP-resistant cells than those released from sensitive counterparts [14].

Isothiocyanates (ITCs), naturally occurring in abundance in cruciferous vegetables, may play a significant role in affording the cancer chemopreventive activity. ITCs perturb several steps in the carcinogenic process by inhibiting cell growth due to cell cycle arrest and removing premalignant and malignant cells through the activation of apoptosis [15,

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16, 17]. During the course of apoptosis induction by ITCs, multiply signaling pathways including MAPKs and apoptosis-related molecules are activated [18, 19].

In the present study we show that E-4IB- or E-4IB/CDDP treatments in human ovarian sensitive cell line and its CDDP-resistant variant were associated with exosomal pathways that participate in CDDP export and these resulted in various alterations of several signaling molecules linked with the changes in pro- and anti-apoptotic molecules.

Materials and methods

Compounds. Propidium iodide (PI) and cisplatin were from Sigma. E-4IB was synthesized as described [20]. Protein inhibitor set was purchased from Calbiochem and LysoTracker Green DND-26 (LTR) from Invitrogen. Polyclonal/monoclonal antibodies against PARP, Bax, Bcl-2, Bcl-x_L, survivin, c-IAP-1, p53, NF-κB, JNK-1, p-JNK, p38, p-p38, ERK-1, p-ERK, c-Jun, p-c-Jun, Akt-1, p-Akt, LAMP-1, and β-actin IgGs, as well as goat anti-rabbit/goat anti mouse IgG HRP and the ECL chemiluminescence reagent were purchased from Santa Cruz Biotechnology, CA.

Cells. Human ovarian carcinoma cell line A2780 and its CDDP-resistant subline A2780/CP were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100 μg/ml penicillin and 50 μg/ml streptomycin. 0.5 x 10⁶ cells/ml were cultured in 96-, 24- or six well plates (Greiner, Germany).

LysoTracker Green DND-26 (LTR) cell staining for flow cytometry. A2780 cells were stained by the technique described in Current Protocols in Cytometry UNIT 9.4. Briefly, the cells were plated in 12-well culture plates (0.5 - 1.0 x 10⁶ cells/ml) and next day treated with E-4IB (0.5 μM) and/or CDDP (2.5- or 5 μM). For drug combination, E-4IB pretreatment (for 3 h) was followed by concomitant adding of the respective concentrations of CDDP. 24 hours after CDDP treatments the media were aspirated and 1 ml of serum-free RPMI medium was added to the cells. After 5 min at 37 °C, 10 μl of LTR Green-DND-26 (0.1 μM) was added, cells were shaken and incubated for 25 min at 37°C in the dark. Then cells designed for LTR accumulation measurements were harvested, washed twice in PBS at room temperature and co-stained with propidium iodide (5 μM final concentration) to exclude dead cells. Finally, cells were analyzed using a Coulter Epics Altra Flow Cytometer (Beckman-Coulter). Remaining cells were incubated another 24 hours to allow the efflux of the drugs, then harvested, washed, co-stained and measured as described.

Flow cytometry measurements and data analysis. Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser and fluorescence emission was measured using bandpass filters (LTR Green/FL1-525 nm, PI/FL2-575 nm) required for utilized fluorochromes. Forward/side light scatter characteristic and/or PI positivity was used to exclude cell debris and dead cells from the analysis. For all measurements, at least 1 x 10⁴ cells were collected in a list mode collected.

Data were analyzed with WinMDI software using Coulter Epics Altra Flow Cytometer (Beckman-Coulter).

Western blot analysis. Cells were sonicated by the two low amplitude cycles in a buffer containing 50 mM Tris (pH 7,5), 150 mM NaCl, 1 mM EDTA and 1 mM EGTA, 2 mM sodium orthovanadate, 2 mM sodium fluoride and a mixture of protease inhibitors (1 mM PMSF, 1 mM pefabloc, 1 μM leupeptin, 1 μM pepstatin and 0.3 μM aprotinin, Calbiochem). After two cycles of short-time sonication (10 s), cell lysates were equilibrated with NP-40 and SDS, to a final concentrations of detergents 1 % and 0.02 %, respectively. The cell lysates were then collected by microcentrifugation at 10.000 x g, at 2 °C for 10 min and used for SDS-PAGE. Equal amounts of lysate protein (75 μg/lane) were run in 12.5% SDS/PAGE gels and transferred to nitrocellulose. Blots were incubated with the corresponding polyclonal/monoclonal rabbit/mouse IgGs and antibody reaction with anti- rabbit/mouse IgG-HRP was visualized by chemiluminescence reagent (ECL). β-actin was used to control equal gel loading.

Results

We previously referred relatively low cytotoxic activity of synthetic E-4IB in intact human ovarian A2780 cells and tested it in combination with CDDP. Flameless atomic absorption spectrophotometry revealed that in sensitive cells E-4IB increased CDDP accumulation by more than 3-fold in comparison with a single CDDP [21]. To study experimental conditions we assessed cell viability and combinatory indices (CI) for sensitive (A2780)- and CDDP resistant cells (A2780/CP) and fixed working concentrations and time for both drugs as 0.5- or 1.0 μM for E-4IB, 5- or 10 μM for CDDP (for intact cells) and 0.5- or 1.0 μM for E-4IB and 10- or 25 μM CDDP (for resistant cells) [22]. We also indicated that sequential pre-treatment with E-4IB (3 h) followed by CDDP (24 h) yielded in sensitive cells more apoptotic cells than co-treatment with both compounds simultaneously (data not shown).

Several experimental data suggest that one of mechanisms by which CDDP is exported from the cells is *via* sequestration into vesicles of the secretory pathway [14, 23]. In this context it was of interest to compare LTR-stained vesicles accumulated in- and released by the parental cells following the drug treatment. We determined the amount of vesicles at the end of 27-h incubation with E-4IB and/or CDDP (E-4IB pretreatment for 3 h) and at the end of subsequent 24-h period of CDDP efflux. LTR staining showed slightly increased dye accumulation in the cells at the 24 h after E-4IB (0.5 μM), CDDP (5 μM) and after their combination (Fig 1A), but significant dye retention was noticed only after combinatory drug treatments after another 24-h incubation (Fig. 1B).

To gain a deeper insight into the molecular mechanism(s) of a complex intracellular apoptosis- and/or signaling network that ultimately regulates gene expression in response to E-4IB and/or E-4IB/CDDP we used Western blotting (Fig. 2) to analyze some critical components associated with these events.

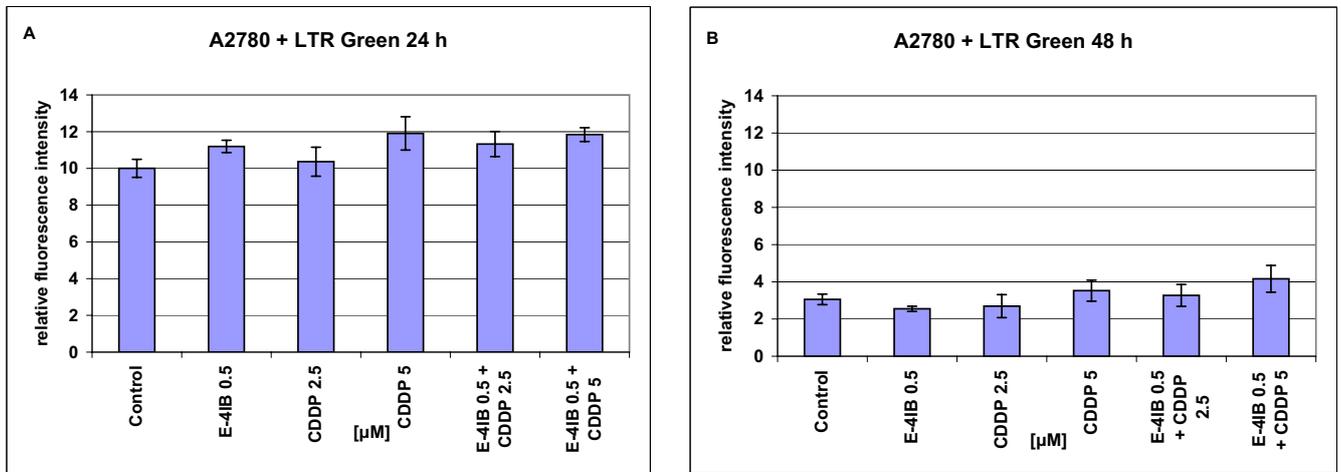


Figure 1. LysoTracker Green-DND 26 staining for 24h (A) and 48h (B) after CDDP treatment in intact A2780 cell line. Cells were treated with E-4IB and/or CDDP as described in Material and Methods. Relative fluorescence intensity (RFI) is expressed as multiple of fluorescence intensity of control samples. Averages of at least three independent experiments \pm S.D. are shown.

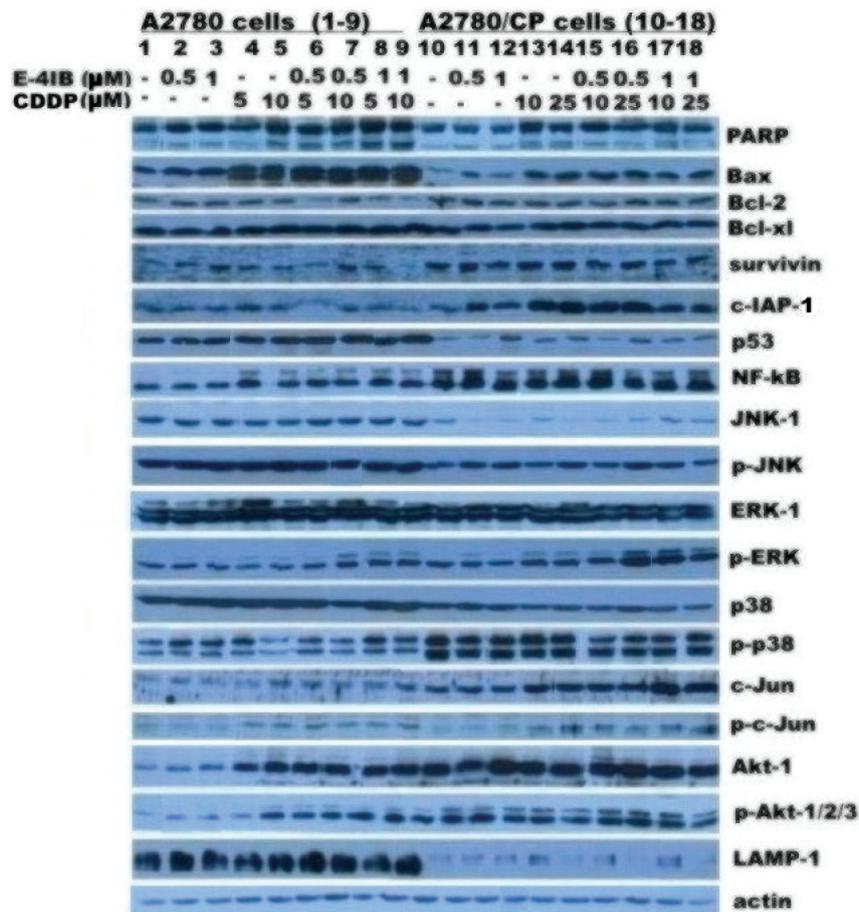


Figure 2. Western blotting analysis of human ovarian parental A2780- and CDDP resistant A2780/CP cells. Cell cultures were treated with E-4IB (0.5- or 1 μ M) and CDDP (5-, 10-, and 25 μ M, respectively) or in combined applications for 48 h as shown. Cell lysates were prepared and handled for immunoblotting with corresponded antibodies as described in Material and methods. Rabbit polyclonal anti- β actin IgG was used as reference antibody.

For combined drug effects we used sequential administration of E-4IB and/or CDDP for both cell variants. No cleavage of poly-ADP-ribosyl- polymerase (PARP) after single treatment with E-4IB in both cell lines was determined, however PARP cleavage appeared in parental cells in response to CDDP or combined treatment with both drugs in dose-dependent manner. These events were in parental cells associated with up-regulation (phosphorylation) of apoptotic Bax, whereas the levels of this protein in resistant cells were markedly decreased, however, slightly increased in response to CDDP or combined drug treatments, but lacking phosphorylation. Contrarily, no changes of anti-apoptotic Bcl-2 were observed in both cell variants regardless of drug treatments used, however, resistant cells exerted increased basal levels of Bcl-2. Anti-apoptotic Bcl-xl remained on its similar basal levels in both cell sublines. Further, parental cells expressed low basal levels of apoptosis inhibitory proteins c-IAP-1 and survivin, which were however, were up-regulated (predominantly c-IAP-1) in resistant cells. No effect of single drugs or in combination was observed on expression of these inhibitory molecules. Furthermore, E-4IB- and/or CDDP administrations led to dose-dependent increased p53 but this protein was markedly down-regulated in resistant cells regardless of the doses of the compounds used. The levels of transcription factors NF- κ B and c-Jun, as well as c-Jun activated form (p-c-Jun) substantially increased in resistant cells, but, contrary to NF- κ B, c-Jun/p-c-Jun increased in dose-dependent manner of the drugs administered.

We further compared activation of JNK, p38 and ERK in sensitive and resistant cell types after treatment with E-4IB and/or CDDP. In both cell lines JNK remained on its basal levels (decreased in resistant cells) irrespective of CDDP- and/or E-4IB/CDDP treatments. Activated JNK (p-JNK) distribution paralleled its unphosphorylated form in both cell sublines. Notably, p38 levels paralleled JNK and p-JNK distribution, but, in resistant cells, activated p38 (p-p38) remained on their augmented basal levels regardless of the drug treatments. On the other hand, ERK levels were comparable in both cells types irrespective of the drug administration. Interestingly, in comparison to parental cells, phosphorylate form (p-ERK) increased in resistant cells in drug dependent manner.

Several lines of evidence showed that alterations in apoptotic pathways are accompanied by alterations in P-I3K/Akt signaling pathway [24, 25]. Here we determined that E-4IB used alone in parental cells had no effect on Akt kinase levels but sequential cooperation of the drugs led to increased levels of Akt paralleled by its activated form p-Akt in dose dependent manner. On the contrary, resistant cells exerted higher basal Akt- and p-Akt levels which were not regulated by the single drugs or those used in combination.

To compare lysosomal cell contents, postnuclear lysates of the both cell types were probed with monoclonal antibody to lysosomal marker protein LAMP-1 [14, 26]. The images in Fig. 2 (*bottom*) showed that marked decreased content of ly-

sosomal vesicles in CDDP-resistant cells and basal levels of LAMP-1 were not influenced by the drug treatments in both cell sublines.

Discussion

We have previously reported that a new synthetic isothiocyanate, E-4IB, appeared to be an effective modulator of cellular proliferation, inducer of apoptosis and a sensitizer of CDDP in sensitive (A2780)- and CDDP-resistant (A2780/CP) human ovarian cancer cells [21, 22].

Growing evidence indicates that CDDP is sequestered into intracellular vesicles, some of which belong to secretory pathways [10, 14, 23]. Therefore, we determined the efflux of the LTR dye following a single and/or combined effect of the drugs. Increased dye retention attests to the effective role of E-4IB to modulate platinum efflux in CDDP-sensitive ovarian cancer cells. In parallel, we analyzed the whole (postnuclear) cell lysates of drug treated intact- and resistant cells by Western blotting and probed them with the antibody to LAMP-1. This protein has been shown as a marker of lysosomal vesicles [26, 14]. The images presented in Fig. 2 (*bottom*) determined marked down-regulation of LAMP-1 in lysosomal compartments of CDDP-resistant cells which remained intact after a single E-4IB or sequential treatments by E-4IB/CDDP. These observations indicate that CDDP is in resistant cells also exported *via* lysosomal compartments. Similar results were documented in other CDDP-resistant ovarian carcinoma cell lines [14, 10] and indicate that besides the regulation of the drug resistance-related genes expression, lysosomal compartment of human ovarian carcinoma cells might be also selected for its stable resistance to CDDP.

By Western blotting (Fig. 2) we found further that single E-4IB, as well as E-4IB/CDDP, led in CDDP sensitive cells, in response to drug-dose administrations, to alterations in apoptosis-related proteins PARP, Bax (with extensive phosphorylation) and p53, whereas anti-apoptotic proteins Bcl-2, Bcl-xl, survivin and c-IAP-1 remained on their basal levels irrespective of the drugs and concentrations used. However, resistant subline exerted increased Bcl-2, survivin and c-IAP-1 levels accompanied by a marked decrease of p53 in all treatments. This corresponded to several findings, when Bcl-2 and Bcl-xl [27, 28, 29] protein over-expression is often, but not always [29], associated with CDDP resistance, and when Bcl-2 or Bcl-xl antagonists augmented CDDP efficacy [31, 32]. In parental human bladder cancer cells, in response to CDDP, up-regulation of Bax was time-dependent and resulted in apoptosis. However, in CDDP-resistant cells, over-expressed Bcl-2 inhibited CDDP-induced Bax translocation to the mitochondria, but down-regulation of Bcl-2 by RNAi potentiated the redistribution of Bax and reversed CDDP resistance [33]. Similarly, over-expression of c-IAP-1, c-IAP-2 and survivin in diverse cancer cells correlated with MDR1-associated- and CDDP-induced resistance [34, 35, 36] and, in other carcinoma cells, survivin antisense oligonucleotides augmented CDDP-

induced apoptosis [30]. Furthermore, Bcl-2 blocked CDDP-induced apoptosis by suppression of p53 accumulation in neuroblastoma cells [37] and, p53 down-regulation may confer CDDP resistance, possibly by over-expression of the negative feedback p53 regulator Mdm2 in a variety of solid tumors [30].

Our findings further demonstrate no differential response to E-4IB and/or CDDP between sensitive- and resistant cells with regard to transcription factor NF- κ B, and showed markedly higher accumulation of this regulation molecule in CDDP-resistant cells. Several studies have indicated NF- κ B activities in diverse cancer cell types. Up-regulation of NF- κ B resulted in its anti-apoptotic role in CDDP-resistant human bladder cells [38], and NF- κ B inhibitors augmented CDDP activity against several cancer cell types, but not against non-malignant cells and some other cancer cell lines [39, 40, 41]. On the contrary, NF- κ B activation was required for CDDP-induced apoptosis in some cancer cell lines [42, 43].

To study apoptotic and signaling molecules, we further investigated the expression and activation of JNK, p38 and ERK in response to E-4IB and/or CDDP. Both cell variants showed JNK and its activated form (p-JNK) to be remained on their basal levels (markedly decreased in resistant cells), irrespective of the drug applied. Interestingly, JNK activity paralleled c-Jun phosphorylation in sensitive cells, but not in resistant subline, suggesting cooperation of this kinase and transcription factor c-Jun in cells lacking CDDP resistance. P38 levels corresponded to JNK distributions in intact cells, and they were accompanied with up-regulated activated p38 (p-p38) in resistant cells. ERK distribution was comparable in both cells types regardless of the drug administrations. P-ERK profiles in both cell lines appeared to be similar with regard to drugs and their concentrations used. A large body of evidence indicates that the MAPK family protein kinases JNK, p38 and ERK appear to be mediators of cell death and survival, and activated by diverse stressful stimuli including CDDP [7, 8]. While activation of JNK (along with p38) has rather pro-death role, the induction of ERK can have both, pro-survival or cell death consequence [9]. It has also been referred that attenuated MAPK signaling may be associated with CDDP resistance, possibly due to decreased activation of JNK and p38 kinase. CDDP-induced activation of JNK- and p38 kinase is significantly decreased in resistant cells, where inhibition of JNK attenuated CDDP-induced apoptosis. JNK activity paralleled phosphorylation c-Jun in intact cells, which was shown to be essential for cell death suggesting the enzyme plays an important role in regulating c-Jun transcriptional activity in apoptotic signaling, also leading to transcriptional activation of FasL [9]. Furthermore, JNK stimulation activated c-Jun and sensitized resistant cells to CDDP [44].

We further found that application of E-4IB in both cell lines caused no effect on Akt or p-Akt expression, while CDDP or E-4B/CDDP treatments in intact cells led to Akt kinase up-regulation in dose dependent manner. Resistant cells, in comparison to intact cells, exerted higher levels of

Akt and p-Akt, which were not regulated by administered drugs. Several results referred that alterations in apoptotic pathways are related to alterations in P-I3K/Akt signaling pathway. Cells expressing Akt1, Akt2 and Akt3 displayed CDDP resistance with threshold modulation for several apoptotic pathways including increased Bcl-x(L) expression and delayed p53 activation, when Akt knockout reduced CDDP resistance [25, 45].

The results here showed that modulation of apoptotic- and signaling pathways in human sensitive- or CDDP-resistant cells, in response to treatment E-4IB and/or CDDP, exerted various effects on the cells accompanied in sensitive cells by diverse CDDP efflux. Thus, this combined sensitizer (E-4IB)/inducer (CDDP) concept might be a novel strategy to enhance the efficacy of cancer therapy in a variety of human tumors.

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