Inhibition of the EGFR/STAT3/CEBPD axis reverses cisplatin cross-resistance with paclitaxel in the urothelial carcinoma of the urinary bladder

Wei-Jan Wang¹, Chien-Feng Li⁶, Yu-Yi Chu⁴, Yu-Hui Wang⁴, Tzyh-Chyuan Hour⁸, Chia-Jui Yen⁵, Wen-Chang Chang², ⁷ and Ju-Ming Wang², ³, ⁴, ⁷*

¹Institute of Basic Medical Science, ²Infectious Disease and Signaling Research Center, ³Center of molecular inflammation, and ⁴Institute of Bioinformatics and Biosignal Transduction, ⁵Division of Hematology/Oncology, Department of Internal Medicine, National Cheng Kung University Hospital, National Cheng Kung University, Tainan 701, ⁶Department of Pathology, Chi-Mei Medical Center, Tainan 701, ⁷Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, ⁸Department of Biochemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan R. O. C.

*To whom all correspondence should be addressed:
Ju-Ming Wang
Institute of Bioinformatics and Biosignal Transduction
College of Bioscience and Biotechnology
National Cheng Kung University
Tainan 701, Taiwan
Phone:
Fax:
E-mail:

CONFLICT OF INTEREST
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RUNNING TITLE:
Targeting CEBPD prevents drug resistance in UCUB
Abstract

**Purpose:** Cisplatin (CDDP) is frequently used in combination chemotherapy with paclitaxel (PTX) for treating urothelial carcinoma of the urinary bladder (UCUB). CDDP cross-resistance has been suggested to develop with PTX, thus hindering successful UCUB treatment. Therefore, elucidating the mechanisms underlying CDDP-induced anticancer drug resistance is imperative and may provide an insight in developing novel therapeutic strategy.

**Experimental Design:** Loss-of-function assays were performed to elucidate the role of the epidermal growth factor receptor (EGFR) and transducer and activator of transcription 3 (STAT3) in CDDP-induced CCAAT/enhancer-binding protein delta (CEBPD) expression in UCUB cells. Reporter and in vivo DNA-binding assays were employed to determine whether CEBPD directly regulates ATP binding cassette subfamily B member 1 (ABCB1) and ATP binding cassette subfamily C member 2 (ABCC2) activation. Finally, a xenograft animal assay was used to examine the abilities of gefitinib and S3I-201 (a STAT3 inhibitor) to reverse CDDP and PTX sensitivity.

**Results:** CEBPD expression was maintained in postoperative chemotherapy patients, and this expression was induced by CDDP even in CDDP-resistant UCUB cells. Upon CDDP treatment, CEBPD activated ABCB1 and ABCC2. Furthermore, the EGFR/STAT3 pathway contributed to CDDP-induced CEBPD expression in UCUB cells. Gefitinib and S3I-201 treatment significantly reduced the expression of CEBPD and enhanced the sensitivity of CDDP-resistant UCUB cells to CDDP and PTX.

**Conclusions:** Our results revealed the risk of CEBPD activation in CDDP-resistant UCUB cells and suggested a therapeutic strategy for patients with
UCUB or UCUB resisted to CDDP and PTX by combination with either gefitinib or S3I-201.
Translational Relevance

Despite extensive investigation, urothelial carcinoma of the urinary bladder (UCUB) remains a relatively understudied disease, and nonsurgical treatments have undergone little development in recent decades. We demonstrated that epidermal growth factor receptor (EGFR)-induced CEBPD promotes cisplatin (CDDP) and paclitaxel (PTX) cross-resistance in CDDP-resistant UCUB by elevating ATP binding cassette subfamily B member 1 (ABCB1) and ATP binding cassette subfamily C member 2 (ABCC2) levels, respectively. In addition, our results validated suppression of CEBPD by gefitinib or S3I-201 to enhance the apoptotic effects in UCUB and CDDP-resistant UCUB cells in vitro and in vivo. The study also describes the first preclinical evaluation of S3I-201 in UCUB and supports that CEBPD inhibition can reduce drug resistance and promote therapeutic efficiency in CDDP-treated UCUB.
Introduction

UCUB is the fourth and tenth most common malignancy in men and women, respectively. At present, CDDP is the most common agent for treating locally advanced and metastatic UCUB. However, the intrinsic and acquired CDDP resistance of UCUB is a major clinical problem, which eventually results in treatment failure (1). In addition, PTX, a microtubule dissociation inhibitor, is a widely accepted combination agent for use in CDDP in UCUB therapy. However, CDDP-resistant tumors (including UCUB) are not completely responsive to salvage combination chemotherapy involving PTX (2). Therefore, novel therapeutic combinations or treatments are required to reverse CDDP-induced PTX cross-resistance.

Approximately 50% of bladder tumors exhibit strong immunohistochemical staining for EGFR, which is also associated with muscle invasion and poor tumor differentiation (3, 4). In addition, a subgroup of muscle-invasive bladder carcinoma displaying a basal-like phenotype is sensitive to EGFR inhibitor-erlotinib (5). However, the underlying mechanisms remained unknown. Gefitinib, also known as ZD1839, selective EGFR tyrosine kinase antagonist that inhibits tumor cell growth (6), attenuates UCUB cell proliferation and enhances apoptosis (7). However, the detailed mechanisms underlying the effects of EGFR in UCUB cells drug resistance, remain largely unknown.

Under normal physiological conditions, the expression levels of transcription factor CEBPD is relatively low, but the levels can be upregulated by various extracellular stimuli (8). CEBPD is also responsive to several anticancer drugs, including vitamin D3 (9) and 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDB) (10). Several studies have suggested that the p38/CREB (cAMP responsive element binding protein)
and JAK (Janus kinase)/STAT3 (signal transducer and activator of transcription 3) pathways play crucial roles in CEBPD transcriptional activation (10, 11). Previous studies have also suggested that CEBPD plays a vital role in inflammation and inflammatory disease processes (12, 13). However, unlike the inflammatory effectors nuclear factor-κB and STAT3 that are consistently activated in cancer cells, CEBPD inactivation has been observed in several types of cancer, including cervical and hepatocellular carcinoma (10, 14), breast (15), prostate cancer (9), and leukemia (16). In addition to acting as a tumor suppressor, several recent reports have suggested that CEBPD plays an oncogenic role under certain conditions (17, 18). Furthermore, CEBPD attenuation can sensitize CDDP-induced cell death in CDDP-resistant UCUB cells (19). However, the details remain largely unclear, and the improvement in CEBPD activation following CDDP and PTX cross-resistance remains uninvestigated.

Multidrug resistance (MDR) transporter proteins are most known for their contribution to chemoresistance through the cellular efflux of anticancer drugs. Several members of the ABC transporter family can induce MDR. To date, tumor tissue studies have consistently revealed that in most cancer cells, the major mechanism underlying MDR involves ABCB1, ABCC1, or ABCG2. ABCC2 endows tumor cells with resistance to various anticancer drugs, including CDDP (20). Furthermore, the ABC transporter family proteins have well-known specific chemotherapy substrates: ABCC2 effluxes CDDP and mitoxantrone, whereas ABCC1 effluxes mitoxantrone and PTX (21). Currently, most evidence for additional roles is correlative, and definitive studies are required to confirm the causality.

In this study, we showed that CDDP-resistant UCUB cells also exhibited considerable PTX resistance. CEBPD responded to CDDP treatment, and CEBPD
activation contributed to $ABCB1$ and $ABCC2$ expression in UCUB cells. Furthermore, CDDP-induced EGFR phosphorylation and gefitinib markedly inhibited CEBPD levels in UCUB cells. We observed that STAT3 activation was EGF responsive and sustained in CDDP-resistant NTUB1 (NTUB1/P) cells. Moreover, CEBPD expression correlated with pEGFR and pSTAT3 levels in specimens from patients with UCUB receiving postoperative chemotherapy. The ABC transporters $ABCB1$ and $ABCC2$ were upregulated by CDDP-induced CEBPD through transcriptional regulation and contributed to CDDP resistance and CDDP-induced PTX cross-resistance. Finally, CDDP administered in combination with either gefitinib or S3I-201 significantly inhibited viability of CDDP-resistant UCUB cells and tumor growth. Overall, our results suggest that gefitinib and S3I-201 are useful adjuvants for treating CDDP-resistant UCUB, particularly for patients who are administered these drugs in combination with CDDP or PTX.
Materials and Methods

Patients and Tumor Specimens

This study was approved by the Institutional Review Board (approval number IRB10302015) of the Chi Mei Medical Center, Tainan City, Taiwan. All samples were obtained from the Chi Mei Medical Center BioBank and were previously collected according to the official ethical guidelines. We retrieved UCUB cases (1996–2004) from the aforementioned medical center archives for immunohistochemical and survival analysis, as previously described (22). To evaluate the significance of CEBPD expression with respect to a CDDP-based chemotherapy response, we enrolled 79 UCUB cases with pT2 to pT4 tumors or with nodal involvement in which CDDP-based postoperative adjuvant chemotherapy was applied. To closely validate the significant findings, 60 independent cases were collected from Liou Ying Campus of Chi Mei Healthcare system. The clinicopathological evaluation criteria were essentially identical to those used in our previous study (23). An expert pathologist (C.F.L) reevaluated hematoxylin and eosin-stained sections from each case.

Cell Lines and Culture Conditions

J82, TSGH8301, and TCCSUP cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin. The J82 and TCCSUP cells were purchased from American Type Culture Collection. Human NTUB1 (24) and the TSGH8301 (25) cell lines were obtained from and authenticated by Dr. Tzyh-Chyuan Hour. The NTUB1 and nasopharyngeal cancer HONE1 cells were maintained in RPMI 1640 media supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL.
streptomycin. Stable CDDP-resistant NTUB1/P and HONE1 (HONE1/R) clones were maintained in the aforementioned media with 10 μM CDDP (Sigma).

Cell Viability and Cell Death Assays

Cell survival was measured using the Cell Counting Kit-8 (Sigma). Experimental cells were treated with either CDDP (20 μM; BioVision) or PTX (1 μM; BioVision) for 24 h. For the combination treatment, the cells were treated with CDDP or PTX plus either gefitinib (5 μM; BioVision) or S3I-201 (10 μM; BioVision) for 24 h. The percentages of cell viability and death were determined for each treatment through comparisons with the untreated control group. For cell death assays, the cells were plated and cultured in regular media as previously mentioned for 16 h and then treated with CDDP alone or in combination with either gefitinib or S3I-201 for another 24 h. The cells were stained with propidium iodide (Sigma) and analyzed through flow cytometry.

Chromatin Immunoprecipitation–PCR Assay

The chromatin immunoprecipitation assay was performed, as previously described (13). In brief, the experimental cells were treated with 1% formaldehyde for 15 min, and the cross-linked chromatin was prepared and sonicated to an average size of 500 bp. The DNA fragments were immunoprecipitated using either specific antibodies recognizing CEBPD or control rabbit immunoglobulin G (IgG) at 4 °C for 12–16 h. Following cross-link reversal, quantitative real-time PCR assays were performed using the precipitated DNA and primers corresponding to specific target gene regions. The primers were as follows:

CEBPD-A (A), 5′-GCCAAGTCCTGGTTTTGATT-3′; CEBPD (AS), 5′
-GCCCCCTCTCAGTTCTC-3';  
5'-GGTTTCACCATGTGGACCAG-3';  
5'-AGAATGGGCTTTGTCACTGTG-3';  
5'-CTCTGGTACTGGGATAACACTTGGTA-3';  
5'-TGATTTGCAAACCTTCTAGTCAAGACA-3';  
5'-CCTGGACCAGCAGCTTA-3';  
5'-AGCACAATTTGAAGGAGGAGG-3'. The amplified products were resolved using agarose gel electrophoresis and confirmed using sequencing.

Lentivirus Knockdown Assays

Viruses were produced in Phoenix cells by cotransfecting various small hairpin RNA (shRNA) expression vectors with pMD2.G and psPAX2. The lentiviral knockdown expression vectors were obtained from the National RNAi Core Facility (Genomic Research Center of the Institute of Molecular Biology, Academia Sinica, Taiwan). After the viral infection efficiency was determined, lentiviruses containing shβ-galactosidase (shLacZ) or shCEBPD were used for infecting NTUB1/P cells for 72 h at a multiplicity of infection. For all lentiviral experiments, media containing uninfected viruses were removed before further analysis. The shRNA sequences in the lentiviral expression vectors were as follows: shLacZ, 5'-CCGGTGTTCATTATCCGAAACCATCTCAGATGGTTGCCGATAATGCGAACATTTTTG-3'; shCEBPD, 5'-CCGGGCTCAGTTCAGGAGGTTGCCGATAATTGGCTGTATCCACCAGCTTTTG-3'; shABCB1, 5'-CCGGGCTCAGTTCAGGAGGTTGCCGATAATTGGCTGTATCCACCAGCTTTTG-3'; and shABCC2, 5'-CCGGCCTGTGGTGAAGCAACATATATCTCAGGATATTGTTGCTATCCACCAGCTTTTG-3'; shCEBPD, 5'-CCGGGCAGCCTTTCAACACTTGGTA-3'; shABCB1-A, 5'-CCGGGCCGACCTCTTCAACAGCATCTCGAGATTGCTGTTGAAGAGGTCGGCTTTTT-3'; shABCB1-B, 5' AGCACAATTTGAAGGAGGAGG-3'. The amplified products were resolved using agarose gel electrophoresis and confirmed using sequencing.
AGGTTTTTG-3’.

MDR Pump Activity Assays

The experimental cells were plated overnight in a growth medium at a concentration of 60000 cells/well/90 μL in 96-well plates. The following day, the cells were treated with CDDP and gefitinib or S3I-201 for 24 h. Furthermore, the cells were mixed with 100 μL/well of MDR dye-loading solution (Abcam) and incubated for 4 h at room temperature. Sample fluorescence intensity was then measured at 525 nm by using a fluorescence reader (Thermo).

Immunohistochemical Staining

Tissue sections (4 μm) were cut from paraffin-embedded tissue blocks, placed onto precoated slides, deparaffinized, and rehydrated. Their antigens were retrieved, and endogenous peroxidase was blocked, as previously described (23). Moreover, the slides were incubated for 1 h with primary antibodies for CEBPD (1:200; ab65081; Abcam), pEGFR (1:25; Tyrosine 1086-specific, Zymed), pSTAT3 (1:25; Tyrosine 705-specific, Santa Cruz), and ABCB1 (1:100, C-19, Santa Cruz). We then detected the primary antibodies by using the DAKO ChemMate EnVision kit (K5001; Carpinteria, CA, USA). To ensure immunostaining quality, a sample prepared without the primary antibody served as the negative control.

Immunohistochemistry Interpretation and Scoring

An expert pathologist (C.F.L), blinded to clinical and follow-up data, evaluated the immunohistochemistry results. Immunoreactivity was evaluated using both the percentage and intensity of positively stained tumors, illustrating representative
staining patterns (pEGFR, membranous and cytoplasmic; pSTAT3, nuclear; CEBPD, nuclear; ABCB1, cytoplasmic) to calculate the H-score according to the following equation: $H\text{-score} = \sum Pi(i + 1)$, where $i$ is the intensity of the stained tumor cells (0–3), and $Pi$ is the percentage of stained tumor cells for each intensity (0%–100%). This formula generates a score between 100 and 400; 100 and 400 indicate that 100% of the tumor cells were negative (0) and strongly stained (3+), respectively (23).

Animal Studies

Male 6–8-weeks-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from the Laboratory Animal Center of the National Cheng Kung University. NTUB1/P cells ($1 \times 10^7$) in 100 μL of PBS were subcutaneously inoculated into the right flanks of the mice. Once macroscopic tumors (100–125 mm$^3$) formed, the animals ($n = 4$ per group) were randomly separated into 4 groups. The groups were then administered intraperitoneal treatments as follows: the control group received the vehicle (PBS), the CDDP group received CDDP (5 mg/kg/2 d) for 3 weeks, the CDDP plus gefitinib group received CDDP (5 mg/kg/2 d) and gefitinib (10 mg/kg/2 d) for 3 weeks, and the CDDP plus S3I-201 group received CDDP (5 mg/kg/2 d) and S3I-201 (10 mg/kg/2 d) for 3 weeks. Animal weights and tumor dimensions were measured every 3 d by using calipers, and tumor volumes were estimated from the tumor length and width by using the formula $[L \times W^2]/2$, where $L$ and $W$ are the length and width, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS V.14.0 software (SPSS Inc. Chicago, IL, USA). The median immunohistochemistry H-scores for CEBPD, pEGFR, pSTAT3, and ABCB1 were used as cutoffs for separating the cases into high and low
expression groups. Furthermore, a chi-square test was used to assess the associations among CEBPD, pEGFR, pSTAT3, and ABCB1 expression. The endpoint analyzed was metastasis-free survival (MeFS), which was calculated from the dates of curative surgery and development of distal spreading. Patients lost to follow-up were censored on the final follow-up date. We plotted survival curves by using the Kaplan–Meier method and evaluated predictive differences between the groups by using the log-rank test. For all analyses, 2-sided tests of significance were conducted, with $P < 0.05$ considered significant. The statistical significance of the variations between the mean values was estimated using the SigmaPlot software package and independent Student $t$ test for unequal variances. The data were expressed as the mean ± standard error of the mean, and $P < 0.05$ was considered statistically significant.
Results
Significance of CEBPD immunoreactivity in UCUB

UCUB cells exhibited nuclear CEBPD. After dichotomizing the tumors into low and high CEBPD expression groups, we found that increased CEBPD expression was significantly associated with nodal involvement ($P = 0.013$; Supplementary Table 1). Notably, UCUB patients with high CEBPD levels had significantly worse MeFS ($P = 0.0077$; Supplementary Fig. 1A), suggesting a potential role of CEBPD in UCUB progression following postoperative adjuvant chemotherapy. Of note, the aforementioned findings remained significant in our independent cohort (Supplementary Fig. 1B)

CEBPD expression is increased in CDDP-resistant UCUB cell lines and contributes to CDDP resistance and CDDP-induced PTX cross-resistance

To investigate the involvement of CEBPD in the development of CDDP resistance, we evaluated the association of CEBPD expression and CDDP sensitivity in UCUB cell lines, including J82, TSGH8301, TCCSUP, and NTUB1. CEBPD abundance was correlated with UCUB cell resistance to CDDP-induced cell death (Fig. 1A). Our previous studies suggested that CEBPD is responsive to anticancer drugs (26, 27). We further assessed whether CDDP affected CEBPD expression in UCUB cells. In addition to the increased basal CEBPD levels being observed in CDDP-resistant NTUB1/P cells, further CEBPD induction was observed after CDDP treatment in NTUB1/P cells as well as other UCUB cells (Fig. 1B). These results suggested not only that CEBPD expression is associated with CDDP resistance in UCUB cells but also that CDDP-induced CEBPD expression leads to drug resistance of UCUB cells.

Cancer cells evade death and exhibit increased drug efflux that contributes to drug resistance. As previously mentioned, CDDP treatment could lead to PTX
cross-resistance; however, the underlying mechanisms in UCUB cells remain unclear. We assessed whether CDDP-resistant NTUB1/P cells were also PTX insensitive. NTUB1/P cells exhibited attenuated PTX sensitivity compared with NTUB1 cells (Fig. 1C). Furthermore, we examined the involvement of CEBPD in CDDP resistance and CDDP-induced PTX resistance in our system. The loss-of-function assays using shLacZ and shCEBPD were performed to verify the role of CEBPD in CDDP resistance and CDDP-induced PTX resistance. The results revealed that the attenuation of CEBPD levels by shCEBPD led to increased CDDP sensitivity and CDDP-induced PTX resistance in NTUB1/P cells (Fig. 1D). We also assessed whether CEBPD contributes to an increased drug efflux. In NTUB1/P cells, the loss of CEBPD enhanced cellular fluorescence intensity in the MDR pump activity assay (Fig. 1E), suggesting that CEBPD contributes to the increase in drug efflux.

EGFR inhibitor significantly enhances CDDP sensitivity in drug-resistant UCUB cells by reducing the CEBPD activity

A recent study reported the involvement of EGFR in drug-resistant UCUB (28); however, the mechanisms of EGFR-mediated drug resistance in UCUB remain unclear. We observed that CDDP induced EGFR expression and activity in NTUB1/P cells but not in NTUB1 cells (Fig. 2A). We then examined the EGFR loss of function by treating UCUB cells with acquired resistance with an EGFR inhibitor. This assay revealed that gefitinib treatment alone significantly induced cell death in NTUB1/P cells; however, it only marginally affected NTUB1 cells (Fig. 2B). Furthermore, we analyzed the anticancer effects of gefitinib and CDDP cotreatment. In the combined treatment, gefitinib increased NTUB1/P cell sensitivity to CDDP (Fig. 2C, compare lanes 4 and 8) and promoted NTUB1 cell death (Fig. 2C, compare lanes 3 and 7).
These results imply that EGFR plays a critical role in the CDDP resistance of NTUB1/P cells.

Abrogating STAT3 activation inhibits CEBPD expression and enhances CDDP efficiency in CDDP-resistant UCUB

CDDP induced EGFR signaling in NTUB1/P cells (Fig. 2), and activated p38 MAPK and STAT3 have been suggested to play crucial roles in CEBPD transcriptional activation (8). Therefore, we analyzed STAT3 and p38 activation in the EGF-treated NTUB1/P cells, revealing that EGF induced the activation of STAT3 (pY705) but not p38 MAPK in NTUB1/P cells (Fig. 2D). In addition to CEBPD suppression through attenuated STAT3 activation, CEBPD was suppressed in gefitinib-treated NTUB1/P cells (Supplementary Fig. 2A). To further determine whether STAT3 mediated the EGF-induced CEBPD expression in UCUB cells, we examined S3I-201 (29, 30). We first determined the effects of S3I-201 on NTUB1/P cells treated with either CDDP or EGF. Similar to our findings in gefitinib treatment in CDDP-treated NTUB1/P and J82 cells, S3I-201 inhibited CDDP- or EGF-induced STAT3 activation and CEBPD expression (Fig. 2E and Supplementary Fig. 2B). Moreover, an in vivo DNA binding assay revealed that the binding of activated STAT3 (pY705) in response to CDDP treatment was attenuated in NTUB1/P cells treated with both CDDP and S3I-201 (Fig. 2F).

CEBPD mediates CDDP-induced ABCB1 expression

As previously mentioned, ABC transporters contribute to drug resistance by increasing drug efflux, thereby reducing toxicity. Therefore, we assessed which ABC transporters could respond to CEBPD induction in NTUB1/P cells. Among the ABC transporters, ABCB1 and ABCC2 demonstrated specific transcript upregulation in
response to CEBPD treatment (Supplementary Fig. 3A). High ABCB1 levels were observed in patients with UCUB (31, 32) and have been suggested to be involved in PTX resistance. We observed that ABCB1 transcripts were present at higher levels in NTUB1/P cells and were further activated in NTUB1/P cells treated with CDDP; however, this activation did not occur in NTUB1 cells (Fig. 3A). Furthermore, exogenously expressed CEBPD induced ABCB1 transcription in NTUB1/P cells (Fig. 3B), and CEBPD knockdown attenuated the CDDP-induced ABCB1 reporter activity (Fig. 3C). RT-PCR was used for determining whether exon 1 of ABCB1 could be transcribed in NTUB1/P cells (Supplementary Fig. 3B). Various 5'-flanking regions and ABCB1 locus fragments were cloned into a reporter vector for CEBPD-responsive region identification (Fig. 3D, left panel, exon and intron mapping according to ABCB1 cDNA clone NM_000927.4). Six putative CEBPD binding motifs within the aforementioned fragments were predicted using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html), and a reporter assay revealed that the −21/+386 bp region was significantly responsive to exogenous CEBPD expression (Fig. 3D). To determine whether CEBPD can directly bind the CEBPD-responsive motifs within the −21/+386 bp region, we performed a ChIP assay. The results showed CEBPD binding, which was responsive to CDDP treatment in NTUB1/P cells (Fig. 3E). These results suggested that CDDP-induced CEBPD directly binds to ABCB1 exon 1 and upregulates its transcription.

Gefitinib and S3I-201 reduce ABCB1 and ABCC2 transcript levels and enhance CDDP and PTX sensitivity in CDDP-resistant NTUB1/P and J82 cells

We next assessed ABCC2 and ABCB1 mRNA levels following the treatment with either CDDP and gefitinib or CDDP and S3I-201 in NTUB1/P and J82 cells. The
ABCC2 and ABCB1 transcript levels were responsive to CDDP treatment; however, they were suppressed in NTUB1/P (Fig. 4A) and J82 cells (Supplementary Figs. 4A and 4B), following the treatment with either gefitinib or S3I-201. In addition, an efflux assay assessing the ABCB1 transporter activity was used for examining CDDP-induced PTX resistance. The results revealed that gefitinib and S3I-201 inhibit fluorescent MDR indicator efflux in NTUB1/P and J82 cells (Fig. 4B and Supplementary Fig. 4C). These results suggested that gefitinib- and S3I-201-attenuated ABCB1 enhanced CDDP cytotoxicity in CDDP-resistant UCUB cells.

Our results suggested that the EGF/pSTAT3 signaling axis mediates CDDP-induced CEBPD transcription and that increased CEBPD contributes to ABCB1 transcription and expression in CDDP-resistant UCUB cells. We further demonstrated that gefitinib and S3I-201 increased NTUB1/P and J82 cell sensitivity to CDDP and PTX (Figs. 4C and 4D and Supplementary Fig. 4D, respectively). These results suggested that gefitinib and S3I-201 recover CDDP and PTX sensitivity in CDDP-resistant cells. In addition, CDDP and PTX are the substrate of ABCC2 and ABCB1, respectively (21). To further assess the specific involvement of ABCB1 and ABCC2 in cross-resistance, a cell viability assay was conducted in ABCB1 or ABCC2 knockdown NTUB1/P cells. The results showed that the loss of ABCC2 in NTUB1/P cells is sensitive to CDDP but minor to PTX treatment (Fig. 4E). Moreover, the loss of ABCB1 in NTUB1/P cells is specifically sensitive to PTX but not to CDDP treatment (Fig. 4F). In addition, to verify that CEBPD expression is correlated with pEGFR, pSTAT3, and ABCB1 expression in patients with UCUB, we performed immunohistochemical assays. The results revealed that CEBPD expression was significantly associated with EGFR and STAT3 activation ($P = 0.010$ and $<0.001$, respectively).
respectively). Moreover, ABCB1 expression in UCUB was significantly associated with high CEBPD and pSTAT3 levels ($P < 0.001$; Table 1 and Supplementary Table 2 and Fig. 5).

As previously mentioned, S3I-201 can abolish CEBPD activation to promote the CDDP sensitivity of CDDP-resistant UCUB cells. To determine whether CEBPD and ABCB1 were also responsive to CDDP treatment and were suppressed by gefitinib and S3I-201, we studied a CDDP-resistant nasopharyngeal HONE1 cancer cell line, HONE1/R (Supplementary Fig. 4E). CEBPD and ABCB1 expression was higher in HONE1/R cells than in parental HONE1 cells (Supplementary Fig. 4F). As observed for NTUB1/P cells, S3I-201 also inhibited ABCB1 transporter efflux activity (Supplementary Fig. 4G) and sensitized HONE1/R cells to CDDP (Supplementary Fig. 4H).

Gefitinib and S3I-201 significantly enhance CDDP therapeutic efficacy in CDDP-resistant UCUB

Our *in vitro* results suggested that gefitinib and S3I-201 treatment significantly sensitized CDDP-resistant UCUB cells to CDDP. To further evaluate these results *in vivo*, the effects of CDDP, gefitinib, S3I-2-1, combinations of CDDP/gefitinib and CDDP/S3I-201 treatments were examined in a NTUB1/P-xenografted NOD/SCID mouse model. In accordance with previous results, gefitinib and S3I-201 have a fewer effect on NTUB1/P tumor burden (58% and 52% inhibition, respectively, at day 9). Importantly, CDDP/gefitinib and CDDP/S3I-201 treatments significantly reduced the tumor burden (82% and 81% inhibition, respectively, at day 9) and weight (Fig. 5). Furthermore, pSTAT3, CEBPD, and ABCB1 levels in NTUB1/P tumors were verified. As shown in Supplementary Figs. 6A and 6B, pSTAT3, CEBPD, and
ABCB1 levels and ABCC2 mRNA levels were reduced in the CDDP/gefitinib- and CDDP/S3I-201-treated NTUB1/P xenografted NOD/SCID mice; this result was consistent with the reduction in tumor size.
Discussion

CDDP and PTX are widely used in UCUB therapy. CDDP ultimately triggers cell death and is commonly used in the clinical therapy of many cancers, including UCUB. Resistance can be pre-existing (intrinsic) or drug induced (acquired). As previously mentioned, UCUB therapy remains associated with persistent high rates of local and distant failure resulting from the acquisition of chemoresistance (33). CEBPD expression was higher in patients with UCUB than in patients with normal urothelial tissue (34). Our findings indicated that CEBPD upregulated ABCB1 and ABCC2 expression, revealing an increased drug efflux activity in CEBPD-abundant UCUB cells. These results suggested not only that CEBPD plays a functional role in intrinsic and acquired resistance but also explained why patients with UCUB tend to develop drug resistance and become desensitized to CDDP and PTX (35, 36).

CEBPD is a CDDP-responsive gene. In this study, we demonstrated that the EGFR/STAT3 signaling axis participates in CDDP-induced CEBPD transcription in UCUB cells, contributing to CDDP and CDDP-induced cross-resistance to PTX by activating ABCC2 and ABCB1 expression (Fig. 3). This study also provided a novel insight into the mechanism underlying acquired resistance and linked EGFR signaling with ABCB1-mediated PTX cross-resistance in CDDP-resistant UCUB. Furthermore, treatment with either gefitinib or S3I-201 reduced ABCB1 expression in CDDP-resistant HONE1 cells and in CDDP-resistant UCUB cells. Thus, CEBPD plays a functional role in promoting drug resistance, and CEBPD inhibition may be an effective approach for reducing the occurrence of drug resistance.

Forced CEBPD overexpression induces growth arrest and death in cancer cells (9). Increased CEBPD levels have been observed in response to treatment with HMDB (10), dexamethasone (37), retinoic acid (37), and 5-azacytidine (38). Furthermore, CEBPD have been suggested to contribute to apoptosis. It has been recently suggested
to play dual roles in pro and antitumor processes under certain conditions (39-41). Furthermore, upregulated caspase 3 and caspase 8 activity has been reported in response to CEBPD induction in prostate cancer (41), and CEBPD was involved in antiapoptosis in astroglialoma U373MG cells by upregulating the antiapoptotic gene ZNF179 (40). These observations imply that CEBPD may serve dual roles in controlling cell survival and regulating cell fate in a cell-specific manner. Herein, we revealed that the EGFR/STAT3 pathway contributes to CEBPD activation and confers a drug-resistant activity that includes cross-resistance. In addition, a previous study showed that the overexpression of EGFR is sensitive to CDDP treatment through a ligand-independent pathway in U87 glioma and MB468 breast cancer cells (42). In our system, the basal levels of pEGFR and pSTAT3 are higher and coincident with basal CEBPD abundance in NTUB1/P cells (Fig. 1A and Supplementary Fig. 7). Therefore, treatment of CDDP could further enhance the activities of pEGFR and pSTAT3 and coordinately CEBPD abundance in NTUB1/P cells (Fig. 2A), indicating a unique but unknown protein/pathway is responsive to CDDP in the CDDP-resistant cells to strengthen the activation of pEGFR and pSTAT3. More importantly, it also provided a motivation for initiation of clinical studies with inhibition of the EGFR/STAT3 signaling pathway when combined with CDDP to prevent the development of therapeutic resistance and thus significantly impact overall survival in patients with UCUB.

P-gp was the first factor known to modulate MDR and is the most widely studied ATP-dependent drug efflux pump. P-gp is encoded by ABCB1 and is considered to mediate MDR by reducing the intracellular accumulation of cytotoxic drugs and compounds. Many types of cancers express high ABCB1 levels and are unresponsive to chemotherapy (43, 44); ABCB1 expression has been considered a predictor of poor
response to chemotherapy and overall survival (45, 46). Our results also indicated that UCUB cells with high expression of CEBPD are strongly resistant to CDDP and PTX because of ABCC2 and ABCB1 activation (Fig. 4), particularly in CDDP-resistant UCUB cells. Thus, we speculated that CEBPD activation should, at least in part, contribute to the proapoptotic activity in response to anticancer drugs; however, its activation conferred acquired resistance via ABCB1 and ABCC2, promoting the resistance of anticancer drug-induced cytotoxicity. In addition to forming a homodimer, CEBPD can heterodimerize with other C/EBP family members, including CEBPA and CEBPB (47). Moreover, ABCB1 is upregulated by CEBPD and CEBPB in anticancer drug-resistant breast cancer cells (48). In accordance with this study, our results also suggested the importance of examining the response and involvement of CEBPB in CDDP-treated and -resistant UCUB cells in the future. The association between ABC transporters with bladder cancers was previously established. Recent studies have reported the overexpression of ABC transporters in chemotherapeutic patients with bladder cancer (32, 49). CEBPD is also responsive to stimuli involving the activation of either p38 MAPK or STAT3. Therefore, it is reasonable to speculate that anticancer treatments, particularly those promoting CEBPD overexpression and p38 MAPK and STAT3 activation, tend to lead to the development of drug-resistant cancers.

Chemoresistance in several cancer types has been linked to STAT3 activation. Recently, constitutively active STAT3 was shown to lead to cellular transformation, suggesting an oncogenic role. STAT3 has also been suggested to confer an enhanced cellular survival following genotoxic treatments (11). Moreover, STAT3 pathway inhibition has been shown to result in growth arrest, apoptosis, and chemosensitivity in several human malignancy models (50). We previously demonstrated that CEBPD
inhibition increased UCUB sensitivity to CDDP (19). In this study, we demonstrated that CEBPD attenuation in UCUB and CDDP-resistant UCUB cells sensitized them to CDDP and CDDP-induced PTX resistance (**Fig. 1**). We further revealed the novel finding that ABCB1 responds to CEBPD upon CDDP treatment by activating EGFR and STAT3 in UCUB and CDDP-resistant UCUB cells. Therefore, inactivating either EGFR or STAT3 can sensitize UCUB and CDDP-resistant UCUB cells to CDDP and even to PTX. In addition to gefitinib, we demonstrated that S3I-201 inhibited CDDP-induced CEBPD and augmented CDDP and PTX sensitivity.
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References

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Figure Legends

Figure 1. CEBPD expression is responsive to CDDP and is associated with CDDP and PTX cross-resistance

A, CDDP affects cell viability and CEBPD expression in various UCUB cells. UCUB cell lines were treated with various concentrations of CDDP for 24 h. CEBPD expression levels were evaluated using Western blotting with the indicated antibodies, and cell viability was examined using the CCK8 assay. TSGH8301 (TSG), TCCSUP (TCC), NTUB1 (NTU), and CDDP-resistant NTUB1/P (NTP) cells.

B, CEBPD expression is responsive to CDDP treatment and sustained in all UCUB cells. A Western blot was conducted with lysates harvested from CDDP-treated all UCUB cell lines for 24 h. Western blot analysis of CDDP-treated UCUB cell lysates.

C, NTP cells are insensitive to PTX treatment. NTU and NTP cells were treated with or without PTX and assessed for cell viability by using the CCK8 assay after 24 h of incubation.

D, CEBPD attenuation in NTP cells sensitizes them to CDDP and PTX. NTP cells were pretreated with lentiviruses containing either shLacZ or shCEBPD (shCD1 and shCD2). Following 48 h of incubation, experimental cells were treated with CDDP or PTX for 24 h and then assessed for cell viability by using the CCK8 assay.

E, CEBPD affects the ABC transporter-mediated pump activity in NTP cells. NTP cells were pretreated with lentiviruses containing either shLacZ or shCEBPD (shCD2). The cells were then treated with or without CDDP. After 24 h, an MDR dye-loading solution was incubated in each well for 4 h, and the resulting fluorescence intensity was detected using an ELISA reader. In this figure, *** and ** denote a significant difference with $P < 0.001$ and <0.05, respectively. CTL: control
Figure 2. Abrogation of EGFR and STAT3 activation inhibits EGF and CDDP-induced CEBPD expression in CDDP-resistant NTUB1/P cells

A, EGFR and STAT3 expression and activity levels increased in NTUB1/P (NTP) cells. Western blot analysis was performed using lysates from NTUB1 (NTU) and NTP cells treated with CDDP for 24 h and the indicated specific pEGFR (pY1085), pSTAT3 (pY705), EGFR, STAT3, and α-tubulin antibodies.

B, gefitinib attenuated NTU and NTP cell survival. NTU and NTP cells were treated with gefitinib at the indicated doses for 24 h, and cell death was examined using propidium iodine staining.

C, gefitinib enhances NTU and NTP cell sensitization to CDDP. NTU and NTP cells were treated with either gefitinib or CDDP for 24 h, and cell death was assayed using PI staining.

D, CEBPD expression in NTP cells is associated with STAT3 activity following EGF treatment. NTP cells were treated with EGF for the indicated periods, and lysates were harvested at the indicated time points. Antibodies recognizing pSTAT3 (pY705), STAT3, pp38 (T182/Y180), p38, CEBPD, and α-tubulin were used for Western blot analysis.

E, a STAT3 inhibitor, S3I-201, inhibits EGF and CDDP-activated CEBPD expression. Western blot analysis was performed using the indicated antibodies and lysates of cells pretreated with S3I-201 for 1 h and then stimulated with EGF or CDDP for 24 h.

F, CDDP enhances binding of STAT3 to the CEBPD promoter in NTP cells. Sonicated chromatin was employed in ChIP assays by using STAT3 or control IgG antibodies and then subjected to PCR analysis after eliminating DNA-binding proteins, as described in the Materials and Methods. In this figure, ***, **, and * denote a significant difference with $P < 0.001$, $<0.01$, and $<0.05$, respectively.
Figure 3. CEBPD upregulates $ABCB1$ expression following CDDP treatment

A, $ABCB1$ transcripts are abundant in NTUB1/P (NTP) cells. qPCR assays were performed using total RNA harvested from NTUB1 (NTU) or NTP cells treated with CDDP for 24 h.

B, CEBPD induces $ABCB1$ transcription. qPCR assays were performed using the total RNA harvested from NTP cells transfected with expression vectors with and without CEBPD cDNA (CD and control [CTL], respectively).

C, Loss of CEBPD attenuates CDDP-induced $ABCB1$ reporter activity. NTP cells were pretreated with lentiviruses containing shLacZ or shCEBPD (shCD2), transfected with $ABCB1$ reporter (AB-I, diagram shown as follows), and treated with or without CDDP. Luciferase assays were performed after 24 h by using lysates harvested from the experimental cells.

D, Identification of the CEBPD-responsive region within the $ABCB1$ 5'-flanking region and intron 1. Schematic of the putative CEBPD motifs (oval) within the human $ABCB1$ 5'-flanking region and exon/intron 1 (−1000 to +917 bp) and various $ABCB1$ reporters. For reporter assays, NTP cells were transfected with the indicated $ABCB1$ reporters and pCDNA3/HA with or without CEBPD cDNA (CD and CTL, respectively).

E, CDDP enhances CEBPD binding to the $ABCB1$ region containing putative CEBPD binding motifs in NTP cells. Sonicated chromatin was subjected to ChIP–PCR analysis by using CEBPD or control IgG antibodies, as described in the Materials and Methods. In this figure, *** denotes a significant difference ($P < 0.001$).
Figure 4. EGFR/STAT3 inhibition attenuates transporter efflux and promotes CDDP- or PTX-induced death in NTUB1/P cells

A, gefitinib and S3I-201 inhibit CDDP-induced $ABCC2$ and $ABCB1$ transcription. NTUB1/P (NTP) cells were treated with CDDP alone or in combination with either S3I-201 or gefitinib for 24 h. qPCR assays were used for measuring $ABCC2$ and $ABCB1$ transcript levels.

B, gefitinib and S3I-201-mediated the inhibition of ABCB1 transporter pump activity in NTP cells. NTP cells were treated with CDDP alone or in combination with either gefitinib or S3I-201 for 24 h. An MDR dye-loading solution was then added to each well and incubated for 4 h. Fluorescence intensity was then detected using an ELISA reader.

C and D, gefitinib and S3I-201 enhance NTP cells sensitivity to CDDP and PTX. NTP cells were treated with CDDP or PTX alone or combination with or without either gefitinib or S3I-201 for 24 h. Cell viability was measured using the CCK8 assay. In this figure, *** denotes a significant difference ($P < 0.001$).

E and F, attenuation of ABCC2 and ABCB1 in NTP cells sensitizes to CDDP and PTX, respectively. NTP cells were pretreated with lentiviruses containing shLacZ (LacZ) or shABCC2 or shABCB1. After 48 h of incubation, experimental cells were treated with CDDP or PTX for 24 h and then examined for cell viability by CCK8 assay. In this figure, ***, significant difference ($P < 0.001$). **, significant difference ($P < 0.05$).
Figure 5. Antitumor effects of CDDP treatment alone or in combination with either gefitinib or S3I-201 on NTUB1/P-xenografted NOD/SCID mice

Effects of the intraperitoneal administration of CDDP, gefitinib, S3I-201, CDDP/gefitinib, or CDDP/S3I-201 on NTUB1/P (NTP)-xenografted NOD/SCID mice. A total of 24 NOD/SCID mice were subcutaneously inoculated with NTP cells \((1 \times 10^7)\) and divided into 6 groups. Following solid tumor formation, the animals were intraperitoneally injected with a control vehicle or CDDP (5 mg/kg/2 d), gefitinib (10 mg/kg/2 d), S3I-201 (10 mg/kg/2 d), CDDP and gefitinib, or CDDP and S3I-201.
Fig. 1
Fig. 2
Fig. 3

A

B

C

D

E

Folds of relative ABCBI mRNA

Folds of relative ABCBI mRNA

Folds of relative luciferase activity

-1000

-1000

-1

-21

+163

+917

-21

Lnc

AB-C

Lnc

AB-I

Lnc

AB-II

Lnc

AB-III

-1000

-21

+163

+917

-950

-657

-15

+250

CDDP

Input

αCEBPD

αIgG

IP

A

B

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Fig. 4
Fig. 5
Table 1. Correlations between expression status of pEGFR, pSTAT3, CEBPD, and ABCB1 in muscle invasive UCUB

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Expression status</th>
<th>Case No.</th>
<th>pSTAT3 Exp.</th>
<th>p-value</th>
<th>CEBPD Exp.</th>
<th>p-value</th>
<th>ABCB1 Exp.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFR</td>
<td>Low Exp.</td>
<td>39</td>
<td>23</td>
<td>16</td>
<td>0.092</td>
<td>25</td>
<td>14</td>
<td>0.010*</td>
</tr>
<tr>
<td></td>
<td>High Exp.</td>
<td>40</td>
<td>16</td>
<td>24</td>
<td></td>
<td>14</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>pSTAT3</td>
<td>Low Exp.</td>
<td>39</td>
<td>-</td>
<td>-</td>
<td>29</td>
<td>10</td>
<td>28</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>High Exp.</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>30</td>
<td>11</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CEBPD</td>
<td>Low Exp.</td>
<td>39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>High Exp.</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>33</td>
</tr>
</tbody>
</table>

* Statistically significant
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