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Research Article

SU5416 sensitizes ovarian cancer cells to cisplatin through inhibition of nucleotide excision repair

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Abstract. SU5416 is reported to be a selective inhibitor of vascular endothelial growth factor, and it has met with limited success in the clinic. In the present study, we investigated whether SU5416 could augment cisplatin-induced cytotoxicity in human ovarian cancer cells. When used as a single agent, 2-h exposures to SU5416 were not harmful to the cells up to doses of 100 μM. For 48-h exposures, the SU5416 IC_{20} and IC_{50} were 17 and 34 μM, respectively. When used with cisplatin, the effect of SU5416 was sequence dependent. SU5416 given first was subadditive, whereas cisplatin given first was supraadditive. Cisplatin was given as a 1-h exposure. Augmented cisplatin cytotoxicity was seen with 2-h exposures to SU5416 at doses of 17–34 μM. This was associated with a decrease in cisplatin-DNA adduct repair, as measured by atomic absorbance spectrometry. Treatment of the ovarian carcinoma cells with SU5416 was also associated with a reduced expression of ERCC-1 protein and c-jun mRNA, as well as a decrease in c-Jun and JNK activities. We conclude that SU5416 can be used to augment cisplatin-induced cell killing at doses that are non-toxic. This effect may occur through direct or indirect reduction of the activity of AP-1 and DNA repair.

Key words. SU5416; cisplatin; ovarian cancer; ERCC-1; DNA repair; JNK; AP-1.

Ovarian cancer is the leading cause of death from gynecological malignancies in the United States. The platinum-based drugs, such as cisplatin and carboplatin, are widely used to treat ovarian cancer and other solid tumors [1–5]. However, platinum resistance is a major obstacle in curing human ovarian cancer in the clinic [1, 6–8]. Although multiple mechanisms of resistance to cisplatin have been identified, increased DNA repair plays a pivotal role in the development of platinum resistance in ovarian carcinomas [7–10].

Most cisplatin-induced DNA damage is repaired by nucleotide excision repair (NER), a DNA repair pathway that removes from the genome a wide spectrum of lesions induced by UV irradiation and chemicals [11]. Evidence shows that enhanced NER is implicated in the cisplatin resistance phenotype. The human ERCC-1 (excision repair cross-complementation group 1) is a critical gene within the NER pathway. ERCC-1 is one of the three DNA endonucleases essential for the dual incision step of NER. Without a functional ERCC-1, cells do not perform cisplatin-DNA adduct repair [12]. There is a direct association between the level of ERCC-1 mRNA expression and clinical resistance to platinum-based therapy in human ovarian cancer tissues [13, 14]. Ferry et al. [15] reported recently that ERCC-1 was the only transcript examined that consistently showed a significant increase in all of the several cisplatin-resistant ovarian cancer cell

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lines, and it was present at steady-state levels which correlated with the level of cisplatin resistance (r = 0.99). We also demonstrated that cisplatin induces ERCC-1 up-regulation in several ovarian cancer cell lines which are resistant to cisplatin. We further demonstrated that activator protein 1 (AP-1) appears to be the transcriptional activator responsible for the elevated levels of ERCC-1 gene expression in A2780/CP70 ovarian cancer cells [16]. Our previous studies also showed that the induction of ERCC-1 gene expression by cisplatin can be influenced by several pharmacological agents, where the common feature is that each agent influences one or both components of the AP-1 transcription factor.

The signal transduction pathway of cisplatin-induced DNA damage and activation of ERCC-1 involved in NER remains incompletely understood. Research has shown that cisplatin can activate expression of Ras protein and cause a rapid increase downstream in the enzymatic activity of a family of serine/threonine kinases, known as the mitogen-activated protein (MAP) kinase family [17]. The MAP kinase family is classified into three subfamilies, including the c-Jun N-terminal protein kinases (JNK1 and JNK2), the extracellular signal-regulated kinases (ERK1 and ERK2), and the p38 kinases [18]. JNK activity is strongly induced in response to a variety of DNA-damaging treatments, such as UV irradiation [19], camptothecin [20], etoposide [21], and cisplatin [19, 22]. The role for this signaling pathway in cytoprotection against DNA-damage is far from clear. Evidence increasingly indicates that JNK signaling is important for cell survival [23], and activity of the JNK pathway following DNA damage is required for the DNA repair process [22]. The action of JNK in stresses and DNA damage responses is through AP-1, an important transcription factor regulating numerous genes implicated in cell growth, transformation, differentiation, and DNA repair [24–26]. JNK phosphorylates the c-Jun component of the AP-1 complex and the related transcription complexes on serine residues 63 and 73 in the NH_2-terminal domain, thereby greatly enhancing transcriptional transactivation by AP-1 and related c-Jun-containing complexes, such as the c-Jun/c-Jun homodimers or c-Jun/c-Fos heterodimers. Our previous investigations suggest that the activation of AP-1 by JNK, leading to the overexpression of ERCC-1 and other NER genes, may contribute to the enhanced DNA repair capacity in human ovarian carcinoma A2780/CP70 cells.

SU5416 is a selective inhibitor of the tyrosine kinase activity of the vascular endothelia growth factor (VEGF) receptor Flk-1/KDR [27, 28]. SU5416 has been shown to inhibit VEGF-dependent mitogenesis of human endothelial cells without inhibiting directly the growth of a variety of tumor cell types in vitro, and the inhibitory effect of SU5416 on the growth of a variety of tumor xenograft models is through the inhibition of tumor angiogenesis. The effect of SU5416 on cisplatin sensitivity in human tumors, however, has not been explored. Therefore, we undertook this study to determine the effect of SU5416 in sensitizing cisplatin activity in human ovarian cancer cells in vitro. In this work, we show that SU5416 blocks DNA repair activity and markedly increases the sensitivity to cisplatin in NIH:OVCAR-3 human ovarian carcinoma cells, and that the effect of SU5416 on ovarian tumor cells may be mediated through a JNK/AP1-dependent mechanism.

Materials and methods

Chemicals and immunoreagents

SU5416 was supplied to the National Cancer Institute (NCI) by Sugen (South San Francisco, Calif.). Cisplatin (CDDP) was obtained from the Drug Development Branch, NCI, National Institutes of Health (Bethesda, Md.). Dimethylsulfoxide (DMSO) and other chemicals were purchased from Sigma (St. Louis, Mo.). Phospho-JNK (Thr183/Tyr185) antibody was purchased from Cellular Signaling (Beverly, Mass.). Phospho-c-Jun antibody (Ser 63/73) and β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). ERCC-1 antisera were generated by Bio-Synthesis (Lewisville, Tex.).

Cell line and cell culture conditions

The cisplatin-resistant human ovarian carcinoma cell line NIH:OVCAR-3 (HTB161; American Type Culture Collection, Manassas, Va.) that has been described previously was used in all the experiments. Cells were cultured in monolayers using a RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.2 units/ml human insulin, 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies, Gaithersburg, Md.). Cells were grown in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were routinely tested for mycoplasma infection using a commercial assay system (MytoTect; Life Technologies); new cultures were established monthly from frozen stocks. All media and reagents contained < 0.1 ng/ml endotoxin as determined by *Limulus polyphemus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md.). Cell viability was determined in triplicate by trypan blue dye exclusion. Before starting the experiments, cells were grown to 70–90% confluence after subculturing. Cisplatin was initially dissolved in phosphate-buffered saline without Ca^{2+} or Mg^{2+} at 1.0 mg/ ml (3.33 µM cisplatin), and SU5416 dissolved in DMSO was serially diluted in culture medium.

Drug treatment and cytotoxicity assay

NIH:OVCAR-3 cells were exposed to cisplatin for 1 h and the cells were harvested ('zero time or no recovery')

or washed twice with fresh medium and reincubated in the absence or presence of SU5416 for 2 h. Fresh medium was replaced again and all the cells were harvested at a total of 6 h following 1 h cisplatin treatment ('recovery'). The cells exposed to cisplatin for 1 h and harvested at 6 h recovery were used as controls for measurement of overall cellular NER DNA repair activity. In separate experiments, cells were exposed to cisplatin alone, SU5416 alone, or the combination of both agents simultaneously for varying times. At the termination of incubations, cells were washed twice in PBS, released from plates by trypsinization, pelleted by centrifugation, and rapidly frozen at −70 °C for measurement of cisplatin-DNA adducts or gene expression. In some experiments, intact nuclei were isolated after breaking cells with a Dounce homogenizer. Protein concentrations were measured by the bicinchoninic acid microplate method, using bovine serum albumin as the standard.

The cytotoxicity of SU5416 or CDDP alone and the combination of both agents was measured by the microculture tetrazolium (MTT) assay. Briefly, cells were harvested by trypsinization from cultures in the exponential growth phase and suspended in fresh medium at 2000 cells/well, in 96-well plates (Costar, Cambridge, Mass.). After 24 h incubation, SU5416 (5–100 μM) and/or CDDP (5 to 100 μM) were added and the cells were further incubated for 96 h. In some experiments, cells were preincubated with cisplatin for 1 h at 20 μM (IC_{50}) and then treated with 17 μM SU5416 (IC_{20}) for 2, 48, or 72 h. The medium was then removed. The cells were washed twice using PBS, and the drug-free fresh medium was added. All cell cultures were incubated in a total 96-h incubation time. MTT (2 mg/ml in PBS) was added (50 μl/ well), and cells were incubated for an additional 3 h. Cells were centrifuged for 5 min at 2000 rpm, and the medium was removed. DMSO was then added to each well, the plates were shaken for 30 min, and the absorbance at 570 nm was read with a kinetic microplate reader (Molecular Devices, Menlo Park, Calif.). Data were collected as replicates of six wells.

Measurement of cisplatin-DNA adduct formation and repair

Tumor cell pellets frozen in tubes at −70 °C were thawed and lysed in Triton X-100 containing Tris-HCl buffer, and then cellular DNA was isolated by cesium chloride density gradient centrifugation, as previously described. The extracted DNA was dialyzed extensively against distilled water, and then lyophilized after measuring its absorbance at 260 nm. Covalent cisplatin-DNA damage was measured by flameless atomic absorption spectrophotometry, as described in detail previously. Cisplatin-DNA adducts were calculated from a platinum chloride standard curve that was included with each assay, and cisplatin-DNA damage is expressed as femtomoles of platinum per microgram of isolated DNA. The removal of cisplatin adducts from DNA from the control cells (as described above) was used as a measurement of overall cellular NER DNA repair activity.

RNA isolation and RT-PCR analysis

Total RNA was isolated from cells using a commercial total RNA isolation reagent kit (Life Technologies) according to the manufacturer's instructions. The amount of RNA was estimated by spectrophotometry at 260 nm. Single-stranded complementary DNAs (cDNAs) were obtained from reverse transcription of 2 μg of total RNAs using random hexanucleotides as primers (5 μM) and monkey murine leukemia virus reverse transcriptase (910 U/μl, 1 h at 37 °C). cDNAs (1 μl of RT mixture) were amplified by PCR with Taq polymerase (0.01 U/μl), dNTP (100 μM), [α-^{33}p]dATP (0.045 μCi), and specific primers (2 μM). The mixture was first heated at 94 °C for 5 min. Amplification was carried out for 34 cycles at 94 °C for 30 s, 65 °C (β-actin), and 74 °C (c-Jun) using a Perkin Elmer 9700 Thermocycler. At the end of the cycles, the reaction mixture was heated at 72 °C for 10 min. PCR products were analyzed on 8% polyacrylamide gels and visualized by autoradiography. The oligonucleotide primers for c-jun were: 5′-ATCGGAGCGCACTTCCGTGG-3′ (forward) and 5′-GAGACTCCGGTAGGGGTCGG-3′ (reverse). The oligonucleotide primers for β-actin were: 5′-ACGGCAACGTGACCTGCGAG-3′ (forward) and 5′-CCGTCAGCGTCCAGTACTTG-3′ (reverse). PCR analysis for c-jun and β-actin were carried out from the logarithmic phase of amplication. PCR-amplified products were checked by restriction enzymes. RT-PCR primers were designed inside separate exons to avoid any bias due to residual genomic contamination. Moreover, for all primers, no amplification was observed when PCR was performed on RNA preparations.

Western immunoblot analysis

The cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris pH 8.0, 1% TritonX-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithioreitol, and 2 mM pepstatin A on ice for 30 min. After centrifugation at 14,000 rpm for 20 min, the supernatant was harvested as the total cellular protein extracts and stored at −70 °C. The protein concentration was measured using Bio-Rad protein assay reagents. Forty micrograms μg of cellular protein extracts were separated by SDS-PAGE, and transferred to nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) containing 150 mM glycine and 20% (v/v) methanol. The membranes were blocked with 5% non-fat dry milk in 1× TBS containing 0.1% Tween 20 and incubated with antibodies/antisera against ERCC-1, JNK1, β-actin, phospharylated c-Jun,

and phospharylated JNK1. The protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Perkin Elmer Life Sciences), and visualized with enhanced chemiluminescence reagent (Perkin Elmer Life Sciences). The protein bands of the autoradiographs were analyzed by laser densitometry using a Molecular Dynamics densitometer.

Immunoprecipitation and in vitro kinase assay of JNK

The cells were plated in a 75-cm^2 flask overnight and then treated with various drugs at different times. Cells were washed with ice-cold PBS and scraped from the flask, and centrifuged at 4000 rpm for 10 min. The cell pellet was incubated for 15 min on ice in lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EDTA) supplemented with 1 mM EGTA, 2.5 mM sodium pyrophosphate, 2 mM aprotinin, 1 mM sodium orthovanadate, 2 mM leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 15,000 g for 15 min to clarify the supernatants. The protein content of the supernatants was determined using the Bio-Rad protein assay reagent. Forty micrograms of protein extracts were then incubated with 10 µl of antibodies against JNK for 2 h at 4 °C and 30 µl of protein A-agarose beads (Bio-Rad) were then added for an additional 1 h. The beads were then pelleted and were washed twice with incubation buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100), and twice with kinase buffer (20 mM MOPS, pH 7.6, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100, and 1 mM Na_3VO_4), then mixed with 2 µg of c-Jun Fusion Protein (Cell Signaling Tech, Beverly, Mass.), 15 µM ATP, and 10 µCi of [γ-^{32}P]ATP in 30 µl of kinase buffer. The kinase reaction was carried out at room temperature for 30 min and terminated with an equal volume of SDS sampling buffer. The samples were resolved on 12% SDS-gel electrophoresis. The JNK activities were detected by autoradiography.

Statistical analysis of data

All blots are representative of three experiments. Comparisons among treatment groups were performed with one-way analysis of variance and a post hoc Dunnett's or Student Newman-Keuls comparison as appropriate. Statistical significance was accepted if the null hypothesis was rejected with $p < 0.05$.

Results

SU5416 enhances cisplatin cytotoxicity and increases cisplatin sensitivity in resistant human ovarian cancer cells

To determine whether or not SU5416 has a direct antitumor action in ovarian cancer, we performed experiments to examine the effect of SU5416 on cisplatin toxicity in human NIH:OVCAR-3 ovarian cancer cells. As seen in figure 1, SU5416 was found to increase cisplatin toxicity dramatically in these cells. However, SU5416, by itself at the same concentrations, did not appear harmful to the cells (fig. 2). We also found that the effect of SU5416 is sequence dependent. Treatment of cisplatin followed by SU5416 caused a significant increase in cell killing as compared to the cells treated with cisplatin alone (figs. 1, 3). In contrast, this effect of SU5416 was not observed when cells were treated with SU5416 prior to cisplatin (fig. 3). In addition, SU5416 also increases cisplatin sensitivity in ovarian carcinoma cells. The IC_{50} was ~20 µM for cells treated with cisplatin alone. By contrast, the IC_{50} of cisplatin for cells exposed to cisplatin followed by SU5416 was reduced to ~3.8 µM, nearly a 5.5-fold difference (table 1). We also demonstrated that there was no difference when the cells were treated with SU5416 for 2, 24, or 48 h following 1 h exposure to cisplatin in our system (fig. 1). These results suggest that only a short exposure (2 h) to SU5416 is required for activity and that the drug has long-lasting effects.

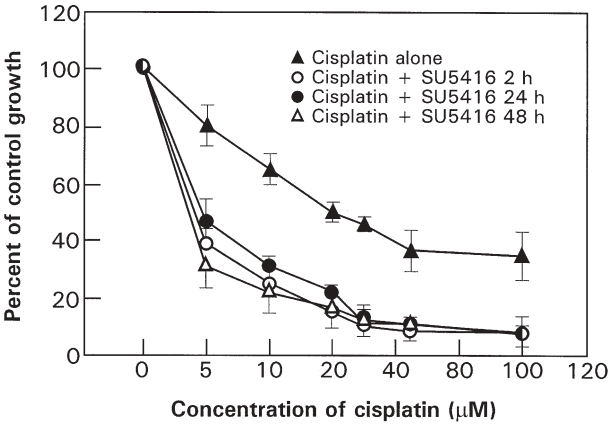

Figure 1. Effect of SU5416 on cisplatin cytotoxicity in human ovarian carcinoma cells. One thousend cells per well from NIH:OVCAR-3 cells evenly distributed in 96-well plates and exposed to cisplatin alone at the indicated doses for a 1-h pulse, or cisplatin for 1 h, followed by SU5416 at 17 µM (IC_{20} dose) for 2, 24, or 48 h. Medium was then changed and drug-free fresh medium was replenished. All cells were harvested at 96 h following 1 h cisplatin exposure. Cell toxicity was determined by MTT assays, as described in Materials and methods, and values were expressed relative to those wells where no cisplatin or SU5416 was used (100% control value). The results are expressed as the mean ± SD of three independent experiments.

SU5416 reduces repair of cisplatin-induced DNA damage in human ovarian carcinoma cells

The mechanisms underlying cisplatin resistance in human ovarian cancer and other tumors are not completely understood. Based on observations that increased DNA repair capacity contributes to the formation of drug resistance to cisplatin in human tumors, and that NER is re-

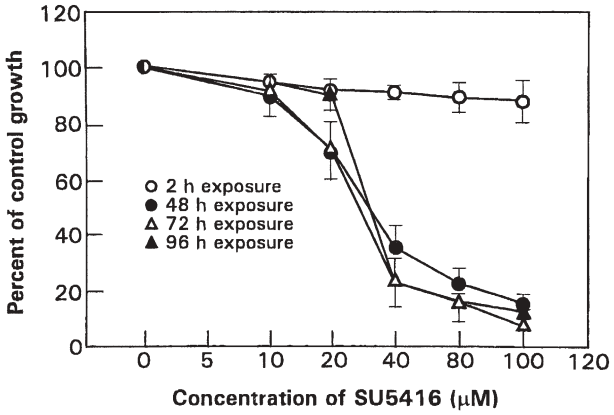

Figure 2. Effect of duration of SU5416 treatment on cytotoxicity in human ovarian carcinoma cells. One thousand cells per well from NIH:OVCAR-3 cells evenly distributed in 96-well plates and exposed to SU5416 alone at the indicated doses for 2, 48, 72, or 96 h. Medium was then changed and drug-free fresh medium was replenished. All cells were harvested at 96 h from the time when cells were exposed to SU5416. Cell toxicity was determined by MTT assays, as described in Materials and methods, and values were expressed relative to those wells where no SU5416 was used (100% control value). The results are expressed as the mean ± SD of three independent experiments.

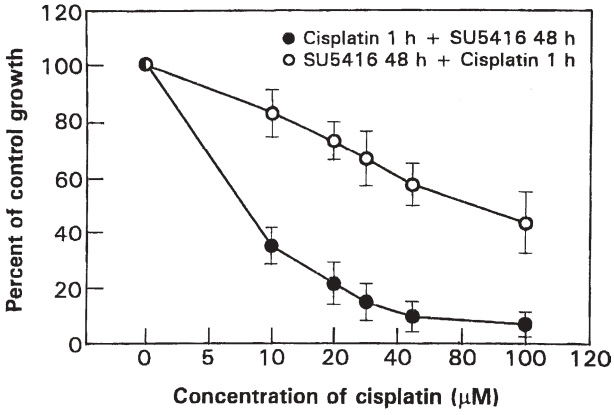

Figure 3. Effect of the sequence of SU5416 treatment on cisplatin cytotoxicity in human ovarian carcinoma cells. 1,000 cells per well from NIH:OVCAR-3 cells were evenly distributed in 96-well plates. The cells of one group were exposed to cisplatin at the indicated doses for a 1-h pulse. Medium was then changed and fresh medium containing 17 μM SU5416 (IC_{20} dose) was replenished. Cells were incubated with SU5416 for 48 h. In another group, cells were treated with SU5416 for 48 h, followed by exposure to cisplatin for 1 h. Medium was then changed and drug-free fresh medium was replenished. All cells of both groups were harvested at 96 h following 1 h cisplatin exposure. Cell toxicity was determined by MTT assays, as described in Materials and methods, and values were expressed relative to those wells where no cisplatin or SU5416 was used (100% control value). The results are expressed as the mean ± SD of three independent experiments.

Table 1. The VEGF inhibitor SU5416 enhances cisplatin sensitivity in human ovarian cancer cells.

Drug	IC_{50} (μM)
Cisplatin	20.6 ± 2.6
SU5416	34.3 ± 1.5
Cisplatin + SU5416	3.8 ± 0.8

Cytotoxicity by cisplatin alone or cisplatin followed by SU5416 (2 h) in NIH:OVCAR-3 cells was determined as described in the legend to figure 1. Cytotoxicity by SU5416 alone was assessed by the incubation of the cells with SU5416 at 0, 5, 10, 20, 40, 80, or 100 μM for 96 h. Cells were then harvested and SU5416 toxicity was determined by MTT assays, as described in Materials and methods. Values of percent of control growth were plotted against the concentrations of the drugs being used, and the IC_{50}, the dose of drug that reduces the survival rate in the cell line to 50% of the control value, for cisplatin or SU5416 was identified from the plots. The results are representative of three separate experiments.

sponsible for the repair of cisplatin-DNA adducts in human cells, we would like to know if reducing NER activity accounts for SU5416 enhancing cisplatin sensitivity in resistant human ovarian cancer cells. To examine the effect of SU5416 on DNA repair efficiency, we determined the removal of cisplatin-damaged DNA isolated from cisplatin-treated NIH:OVCAR-3 cells. As shown in figure 4, after exposing cells to 20 μM cisplatin for 1 h, we measured 100 fmol platinum/μg DNA. In cells treated with cisplatin alone, the extent of platinum-DNA adduct was decreased to around 60 fmol platinum/μg DNA during a 6-h recovery period. Figure 4 shows that there was significant DNA repair in these cells in which approximately 40% of platinum-DNA lesions were removed. In contrast, SU5416 treatment significantly diminished the repair of cisplatin-DNA adducts in this system, yielding about 80 fmol platinum/μg DNA which is a 33% higher level of DNA platination than in cells exposed to cisplatin alone at 6-h recovery time. These data indicate that down-regulation of DNA repair activity and increase in cisplatin platination by SU5416 are positively associated with enhanced cytotoxicity of cisplatin in resistant human ovarian cancer cells.

Effect of SU5416 on cisplatin-dependent induction of ERCC-1 expression

We next wanted to understand whether the effect of SU5416 on NER DNA repair gene expression contributes to the down-regulated DNA repair capacity by SU5416. We chose to study the effect of SU5416 on ERCC-1 expression in our system, because resistant ovarian cancer cells and ovarian cancer tissues have ample ERCC-1 protein that contributes to their highly efficient DNA repair capability, and that ERCC-1 is a marker gene for NER activity. We measured ERCC-1 mRNA and ERCC-1 protein levels in NIH:OVCAR-3 cells treated with cisplatin or SU5416 alone, or the combination of both agents. We

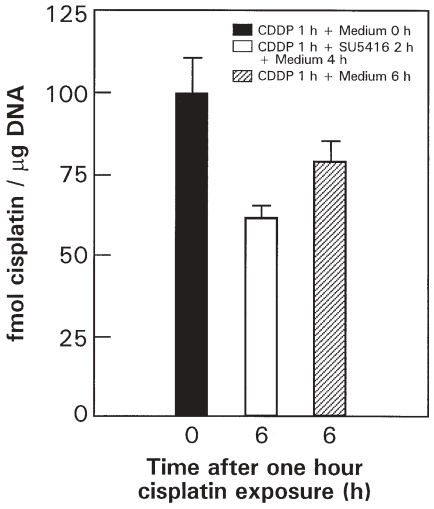

Figure 4. Effect of SU5416 on cisplatin-DNA adduct repair in human ovarian cancer cells. NIH:OVCAR-3 cells were exposed to cisplatin alone at its IC_{50} dose of 20 μM for a 1-h pulse. Cells were harvested immediately after cisplatin exposure or following a 6-h recovery period; or cells were treated with cisplatin for 1 h, followed by SU5416 at its IC_{20} dose of 17 μM for 2 h. Drug-free fresh medium was then replenished, and cells were harvested at a 4-h recovery period. Cellular DNA was isolated by cesium chloride buoyant density gradient centrifugation, and the total content of DNA-bound platinum was measured by atomic absorption spectrometry, with Zeeman background correction. The results are expressed as the mean ± SD of three separate determinations. Error bars representing the standard deviation for each column are shown. *CDDP*, cisplatin; *fmol*, femtomole.

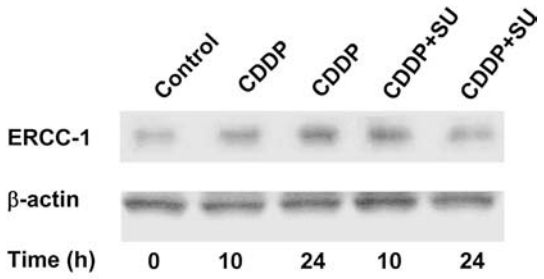

Figure 5. Western immunoblot analysis of the effect of SU5416 on ERCC-1 protein expression in NIH:OVCAR-3 cells. Cells were exposed to 20 μM of cisplatin alone for a 1-h pulse; or to cisplatin 1 h, followed by SU5416 at 17 μM for 2 h. Cellular protein was sequentially extracted at 10 or 24 h following 1 h cisplatin exposure. A total of 25 μg of cellular extract protein isolated from cisplatin-treated or cisplatin+SU5416-treated NIH:OVCAR-3 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with ERCC-1 antiserum.

confirmed our previous observations from A2780/CP70 cells that the cellular levels of ERCC-1 mRNA were increased severalfold within 24 h of exposing NIH:OVCAR-3 cells to cisplatin (data not shown). ERCC-1 protein levels were also increased significantly in these cells (fig. 5). Intriguingly, SU5416 abrogated the cisplatin-induced increases in ERCC-1 mRNA (data not shown) and ERCC-1 protein levels (fig. 5) in this model, suggesting that the inhibitory effect of SU5416 on DNA repair is due, at least in part, to the reduced ERCC-1 level and decreased NER activity.

Effect of SU5416 on AP-1 level and c-Jun activity in human ovarian tumor cells

We recently demonstrated that cisplatin induces ERCC-1 expression in A2780/CP70 ovarian cancer cells and that this induction of ERCC-1 is dependent upon AP-1 activity [16]. Therefore, we would like to know whether SU5416 has any effects on AP-1 (Jun/Fos) in ovarian carcinoma cells. We first examined the effect of SU5416 on the expression of c-Jun mRNA in NIH:OVACR-3 cells using an RT-PCR approach. The expression of *β*-actin was used as an internal control for the efficiency of each RT-PCR reaction. As seen in figure 6, cisplatin alone induced transient expression of c-jun mRNA in a time-dependent manner, which peaked at 10–24 h with a four-fold increase in this system. In contrast, SU5416 antagonized the cisplatin-dependent increases in c-jun mRNA and the effect of SU5416 in this regard was also time dependant. The difference between the c-jun mRNA levels stimulated by cisplatin alone or by cisplatin plus SU5416 is significant in these cells ($p < 0.05$). The levels of c-jun mRNA did not change in cells treated with SU5416 alone for up to 24 h, as compared to those in the control cells (data not shown). We also found that cisplatin induced increases in c-Fos protein levels in ovarian tumor cells, and that the increases in c-Fos by cisplatin were abrogated by SU5416 (data not shown).

We then determined whether SU5416 exposure alters the activity of c-Jun protein in our model. Consistent with the RT-PCR analysis of c-jun mRNA (fig. 6), the levels of c-Jun protein phosphorylation at serine residues 63 and 73 were increased at 3–6 h with an approximate six- to eightfold increase following the cisplatin exposure (fig. 7). In contrast, treatment of the cells with SU5416 resulted in a marked decrease in c-Jun phosphorylation levels, as compared to those in cells treated with cisplatin alone at each corresponding time point (fig. 7). These data indicate that a reduced level of AP-1 and activity of c-Jun by SU5416 paralleled the decreased level of ERCC-1 and the reduced activity of NER in human ovarian carcinoma cells.

Effect of SU5416 on JNK1 activity in human NIH:OVCAR-3 ovarian carcinoma cells

Finally, we tested the effect of SU5416 on JNK1 activity in ovarian tumor cells. Studies have shown that JNK1 activity is increased upon cisplatin treatment in different cell types. Accordingly, we examined JNK1 activity in NIH:OVCAR-3 cells following exposure to cisplatin or SU5416 alone, or cisplatin plus SU5416 for varying lengths of time. JNK1 activity was assayed by an in vitro kinase assay using a GST-c-Jun (1–135) fusion protein as

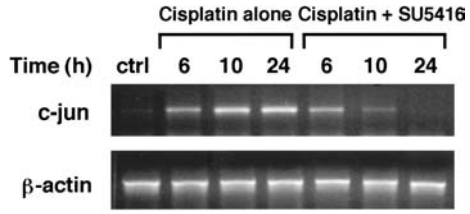

Figure 6. RT-PCR analysis of the effect of SU5416 on c-jun mRNA expression in NIH:OVCAR-3 cells. Cells were exposed to 20 μM of cisplatin alone for a 1-h pulse, or cisplatin for 1 h followed by SU5416 at 17 μM for 2 h. Total cellular RNA was sequentially isolated at 6, 10, or 24 h following 1 h cisplatin exposure. A 237-bp segment of c-jun cDNA and a 731-bp segment of β-actin cDNA were amplified by RT-PCR and the aliquots of amplified DNAs were electrophoresed through a 1.5% agarose gel. ctrl, control.

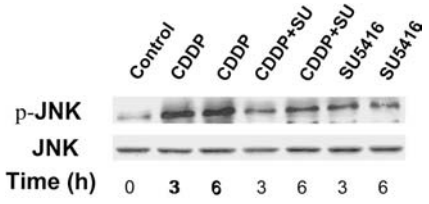

Figure 8. Western immunoblot analysis of the effect of SU5416 on the levels of JNK1 protein phosphorylation in NIH:OVCAR-3 cells. Cells were exposed to 20 μM of cisplatin alone for 1 h or 17 μM of SU5416 alone for 2 h, or cisplatin for 1 h followed by SU5416 for 2 h. Cellular protein was sequentially extracted at 3 or 6 h following 1 h cisplatin exposure. A total of 25 μg of cell extract protein isolated from cisplatin and/or SU5416-treated NIH:OVCAR-3 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with a phospho-JNK1 (Thr183 and Tyr185) antibody (Santa Cruz) or with a JNK1 antibody as controls.

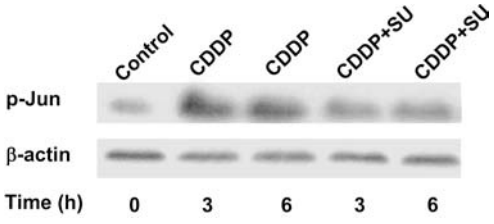

Figure 7. Effect of SU5416 on the levels of c-Jun protein phosphorylation in NIH:OVCAR-3 cells. Cells were exposed to 20 μM of cisplatin alone for a 1-h pulse; or cisplatin for 1 h followed by SU5416 at 17 μM for 2 h. Cellular protein was sequentially extracted at 3 or 6 h following 1 h cisplatin exposure. A total of 25 μg of cell extract protein isolated from cisplatin-treated or cisplatin+SU5416-treated NIH:OVCAR-3 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with a phospho-Jun antibody (Santa Cruz) or with a β-actin antibody as controls.

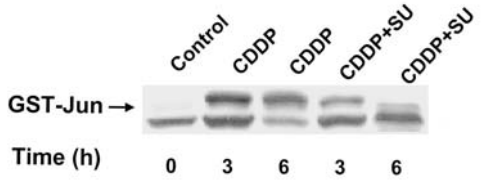

Figure 9. Effect of SU5416 on JNK kinase activity in NIH:OVCAR-3 cells. Cells were exposed to 20 μM of cisplatin alone for 1 h, or cisplatin for 1 h followed by SU5416 at 17 μM for 2 h. Cellular protein was sequentially extracted at 3 or 6 h following 1 h cisplatin exposure. A total of 40 μg of cell extract protein isolated from cisplatin and/or SU5416-treated NIH:OVCAR-3 cells was subjected to immunoprecipitation by JNK antibodies, and JNK kinase activities were determined by in vitro kinase assays with GST-Jun fusion protein as the substrate of JNK, as described in Materials and methods.

a substrate, and the JNK phosphorylation level was assessed by Western blot analysis using an anti-phospho-JNK1 polyclonal antibody. Total JNK protein level was monitored using antibodies capable of recognizing both phosphorylated and unphosphorylated forms of the protein. As seen in figure 8, the levels of phosphorylated JNK were increased by seven- to ninefold, as compared to the control following exposure of the cells to 20 μM cisplatin. The cisplatin-induced JNK activity was due to an increase in JNK kinase activity, as evaluated by an in vitro kinase assay (fig. 9), but not secondary to elevation of JNK protein levels, as determined by Western blot analysis. The latter shows that there were no significant changes in JNK1 protein levels following cisplatin stimulation (fig. 8, lower blot). By contrast, the cisplatin-induced JNK activity was significantly blocked by SU5416 treatment in our cell systems (figs. 8, 9). Interestingly enough, the changes in JNK activity were associated with alternations in the levels of c-Jun phosphorylation in cells treated with cisplatin alone or cisplatin plus SU5416 for each time point. These findings suggest that the association and interaction between JNK and c-Jun may be involved in SU5416 action in cisplatin sensitivity and DNA repair activity in human ovarian cancer cells.

Discussion

Ovarian cancer has a poor prognosis due to the frequent appearance of a drug-resistant state. An alternative therapeutic approach may lie in combinations of conventional chemotherapeutic agents with new classes of drug. A large number of studies have been conducted using combinations of drugs designed to augment the activity of cisplatin and attenuate resistance to the drug.

Anti-angiogenesis represents a promising new approach in human cancer therapies, and it is currently under intense investigation. SU5416 is a selective inhibitor of VEGF receptors that is currently undergoing clinical evaluation as an anti-cancer agent for the treatment of advanced malignancies [27–29]. SU5416 has a potent mechanism-based activity (blockade of VEGF signaling

through inhibition of the Flk-1/KDR RTK activity) [27, 28] capable of targeting all solid tumors. Therefore, SU5416 may be beneficial in treating ovarian cancer and other tumors because of the anti-angiogenic property of this compound. However, the effect of SU5416 on cisplatin activity in human tumors is completely unknown. In the present in vitro study, we showed for the first time that SU5416 enhances cisplatin cytotoxicity and increases cisplatin sensitivity in resistant human ovarian cancer cells. This effect of SU5416 is sequence dependent. Indeed, the effect of SU5416 was only observed in the ovarian carcinoma cell line, when SU5416 was added after cisplatin, suggesting that the drug is effective only after cellular DNA has been damaged by cisplatin. We also observed in this study that only a short exposure (2 h) to SU5416 is required for activity and that the drug has long-lasting effects. Furthermore, we demonstrated that SU5416 reduces repair of cisplatin-induced DNA damage in human ovarian carcinoma cells and that the action of SU5416 is also in a sequence-dependent manner, which is in line with the effect of SU5416 on cisplatin cytotoxicity. These results suggest that SU5416 enhances cell killing or reduces cell survival of cisplatin-treated cells by, at least in part, reducing the repair efficiency of cisplatin-caused DNA damage.

Although a number of studies have shown that JNK is activated in response to cisplatin treatment, the signaling pathway leading to JNK activation and the role for this pathway in determining cell fates (survival or death) are unclear at this time. Several studies have provided evidence indicating that the JNK pathway contributes to cisplatin-induced apoptosis [30]. However, others have suggested that JNK signaling is important for cell survival [23]. Such differential effects observed from one study to another could reflect cell type specificity and different treatments of cisplatin, such as cisplatin dose and exposure time.

The molecular basis underlying the double roles of JNK in apoptosis and survival remains unknown. Based on our and others' observations, we propose a possible explanation. A transient increase in JNK activity may activate the DNA repair and cell survival pathways, while continuous and sustained elevation of JNK activity may lead to activation of the apoptosis pathways that cause cell death. In our recent studies using human ovarian cancer cells, we found that cisplatin treatment led to an increase in ERCC-1 DNA repair gene expression and AP-1 activation through a JNK signaling pathway. Others have also shown that cellular damage induced by DNA-damaging agents including cisplatin results in activation of the JNK pathway involving AP-1 [22] and that AP-1 may be the transcription factor responsible for expression of six NER genes [31] and maybe some other DNA repair genes. These studies suggest that the JNK pathway may mediate a physiological response to DNA damage, such as induction of some DNA repair and damage response proteins. This signal transduction pathway may protect against cisplatin-induced DNA damage, and this response may be required for DNA repair and cell survival following cisplatin treatment [22].

In the current study, SU5416 inhibition of JNK and c-Jun activities, as well as ERCC-1 expression in a resistant human ovarian carcinoma cell line, suggests a therapeutic potential for this compound in the treatment of cisplatin-resistant ovary malignancies. SU5416 reduces JNK/AP-1 activity, and DNA repair gene expression may account for its suppressive effect on DNA repair capacity and its augmenting effect on cisplatin cytotoxicity, and this action of SU5416 may be greatly beneficial in patients with cisplatin-resistant ovarian cancer and other neoplasms. Drugs like SU5416 inhibiting DNA repair will increase the accumulation of total platinum and the formation of DNA-platinum adduct, as well as the rate of DNA damage in cisplatin-treated cells, and these may result in a significant enhancement of the anti-tumor effect of cisplatin. Although we have known that SU5416, as an anti-angiogenic, is a small-molecule indolinone RTK inhibitor, which inhibits its RTK targets via binding in the conserved adenosine triphosphate-binding site within the kinase domain of the receptor, the mechanism of its action in enhancement of cisplatin sensitivity and inhibition of DNA repair capacity, the specific signaling events involving JNK, and the levels at which they are blocked by SU5416 are not understood at this point. Further investigations using subclones of ovarian tumor cell lines with knockout JNK signaling genes are necessary to define the mechanism of action of SU5416 in inhibition of JNK activation and the JNK/AP1-dependent signaling pathway leading to a decrease in proficiency of DNA repair, and these would give us further insights into the mechanisms for the effect of SU5416 on the altered cisplatin cytotoxicity and DNA repair activity in human ovarian cancer cells.

In summary, our studies indicate that the anti-angiogenic SU5416 can increase sensitivity to the chemotherapeutic agent cisplatin and inhibit repair of cisplatin-induced DNA damage. The mechanism underlying the effects of SU5416 in NIH:OVCAR-3 human ovarian cancer cells is definitely complex and may involve inhibition of JNK/AP1-dependent pathways for the activation of a cellular DNA repair pathway. However, our findings do suggest that SU5416 treatment targets the DNA repair protein ERCC-1/NER pathway as one component of the mechanism. The potent synergy of cisplatin and SU5416 in vitro against ovarian carcinoma cells suggests that combinations of SU5416 and cisplatinum may be more effective against this disease in the clinic.

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