Paclitaxel and SN-38 Overcome Cisplatin Resistance of Ovarian Cancer Cell Lines by Down-regulating the Influx and Efflux System of Cisplatin

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Cisplatin (DDP) is one of the key drugs used to treat patients with ovarian cancer, although resistance to DDP can occur. Paclitaxel and SN-38 (an active metabolite of irinotecan (CPT-11)) are two drugs that are effective in patients with DDP-resistant ovarian cancer. To study how these drugs may overcome the intrinsic and/or acquired resistance of cancer cells to DDP, we investigated the effect of a combination of DDP with paclitaxel and a combination of DDP with SN-38 on three ovarian cancer cell lines, RTSG (intrinsically resistant cell line), KF (DDP-sensitive cell line), and KFra (acquired resistant cell line obtained from KF). We found that these combinations showed additive to synergistic antitumor activity. A time-dependent platinum (Pt) accumulation was observed in the DDP-sensitive KF cell line, while a decrease occurred in the KFra cell line. Little accumulation was observed in RTSG. Intracellular Pt accumulation was increased in all three cell lines by exposure to paclitaxel or SN-38. Ouabain, a Na+,K+-ATPase inhibitor, decreased Pt accumulation in KF and KFra cell lines and inhibited the paclitaxel- and SN-38-induced increases in Pt accumulation in these cell lines. When we assessed the mRNA levels of the multidrug resistance-associated protein (MRP), which may be an efflux pump for DDP, the combination of paclitaxel or SN-38 with DDP down-regulated these levels, which are up-regulated by DDP alone. These results suggest that paclitaxel and SN-38 overcome DDP resistance of ovarian cell lines by controlling intracellular accumulation of DDP via both the influx and efflux systems.

Key words: Cisplatin — Paclitaxel — SN-38 — Resistance — Multidrug resistance-associated protein

Because cisplatin (DDP) is one of the main drugs used to treat patients with ovarian cancer, the control of intrinsic and/or acquired resistance to DDP is clinically significant.1) Recently, new antitumor agents, including paclitaxel and irinotecan (CPT-11) have shown promising antitumor activity in patients with DDP-resistant ovarian cancer.1–4) However, the relationship between DDP resistance and the efficacy of these new agents remains unclear. Several mechanisms of DDP resistance have been proposed, such as decreased intracellular drug accumulation, presence of a DDP detoxification system, and decreased DNA damage and/or increased repair.3–5) Some investigators have concluded that intracellular accumulation plays an important role in DDP resistance, as the intracellular platinum (Pt) accumulation is decreased in resistant cells compared with the parent cells from which they were derived.5) On the other hand, the multidrug resistance-associated protein (MRP), an efflux pump of DDP located in the cell membrane, may also function as a DDP resistance mechanism, and the role of MRP in the combined interaction between DDP and paclitaxel, and DDP and CPT-11 is not fully clarified. Since the breakdown of DDP resistance is important in clinical chemotherapy, we aimed to elucidate the mechanism of the combined interaction between DDP and paclitaxel and DDP and SN-38 (an active metabolite of CPT-11) using the DDP-resistant ovarian cell lines RTSG (intrinsically resistant) and KFra (acquired resistant), and comparing the results with those obtained using the parent cell line, KF.

MATERIALS AND METHODS

Cell lines and culture  The cell lines used were KF (poorly differentiated serous cystoadenocarcinoma), KFra (DDP-resistant KF obtained by repeated exposure to doses of DDP) and RTSG (poorly differentiated serous cystoadenocarcinoma).6) KF and KFra7) were the generous gift of Dr. Y. Kikuchi, Department of Obstetrics and Gynecology, National Defense Medical College. RTSG was established in our department and maintained in F-12 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Mitsubishi Kagaku, Tokyo), 2 mM glutamine, and 0.08 mg/ml kanamycin. KF and KFra were maintained in RPMI-1640 medium (GIBCO-BRL) supplemented with 10% FBS, 2 mM glutamine, and 0.08 mg/ml kanamycin. All cell lines were maintained
at 37°C in a humidified incubator with 95% air and 5% CO₂.

Agents DDP was purchased from Nippon Kayaku Co., Ltd., Tokyo. Paclitaxel and SN-38 were kindly supplied by Bristol-Myers Squibb Co., Ltd., Tokyo, and Daiichi Sankyo Co., Ltd., Tokyo, respectively.

In vitro cytotoxicity Tumor cells (1×10⁶) were seeded in a 96-well microtiter plate, the drugs were added 24 h after seeding, and the plate was placed in an incubator for a further 6 days. The cytotoxicity of the agents was evaluated by crystal violet staining, as previously described. Briefly, an equal volume of 10% formalin in phosphate-buffered saline containing 0.2% crystal violet was added to each well and the plate was incubated at room temperature for 15 min. The absorbance at 570 nm of stained cells in each well was measured using an automatic microtiter-plate reader (Multiscan MCC/340, Titertek, Flow Laboratories, Inc., Huntsville, AL). The average absorbance of cells incubated in the absence of drug was regarded as 100%, and the percentage of cell growth in each test well was calculated. Fifty-percent inhibitory concentrations (IC₅₀) of drugs were obtained from graphical plots as the concentration of cells incubated with medium alone, DDP alone (0.0001 µg/ml), paclitaxel alone (0.0008 µg/ml), SN-38 alone (0.0004 µg/ml), DDP+paclitaxel (0.0001 µg/ml+0.0008 µg/ml), or DDP+SN-38 (0.0001 µg/ml+0.0004 µg/ml) for 72 h. In the combination experiments, the drugs were exposed simultaneously, and from 1/3 to 1/4 concentrations of paclitaxel and SN-38 were used for the experiments based on our preliminary experiments. After removal of the medium, the cells were trypsinized, washed with PBS, and centrifuged. RT-PCR was carried out as described previously by Noonan and colleagues. Briefly, total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method, and 1.0 µg was used for synthesis of cDNA. Multidrug resistance-gene 1 (MDR-1) was examined by PCR amplification of the synthesized cDNA using a primer set for a 214-bp fragment. The PCR was performed for 20, 25, 30, and 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR products were separated by electrophoresis on a 2.5% agarose gel, and amplification was confirmed by photography under UV light. Detection of PCR products after 20 cycles was evaluated as +, while after 25 cycles it was +, after 30 cycles it was 2+, and after 35 cycles it was 1+. The MDR-1 and MRP3 primer sets were as follows: MDR-1: 5'-TCTGGGACTGGAATGTACGAGC-3' (as a sense primer) and 5'-TAGCAAGCGCCTGGTGATGC-3' (as a reverse primer) MRP3: 5'-ACATGACCGAGGCTACTCTTCG-3' (as a sense primer) and 5'-TAGCAAGCAGCTGGTGATGC-3' (as a reverse primer) MRP2: 5'-AGCCATAGCCTGAGCCCTTGT-3' (as a
sense primer) and 5′-AGAGTCTGAAATTGGAAAGT-GCC-3′ (as a reverse primer)
MRP3: 5′-CACAGGAACCAGAAGATAATGAG-3′ (as a sense primer) and 5′-CATGGTCGTTGGCTACATCACC-3′ (as a reverse primer)
β-Actin mRNA was used as a house-keeping gene, with the primers described previously.13)

**Statistical analysis**  Linear regression analysis was performed using Instat for Macintosh, version 2.01. The statistical significance of differences was determined by using Student’s t test. A two-tailed P value less than 0.05 was considered statistically significant.

**RESULTS**

Experiments were repeated three or four times in order to validate the assay system. Each point represents the mean value.

**Sensitivity to each agent**  Concentration-dependent cytotoxicity of each agent for each cell line was confirmed in triplicate by concentration/cytotoxicity curves. The KF cell line was sensitive to DDP with an IC50 of 0.142 μg/ml, while its DDP-resistant subline KFra was 14.7-fold more resistant to DDP, with an IC50 of 2.09 μg/ml. RTSG was relatively resistant to DDP, with an IC50 of 1.00 μg/ml. The sensitivities of these cell lines to paclitaxel and SN-38 were almost equivalent to each other; the IC50 values ranged from 0.008 to 0.012 μg/ml for paclitaxel and from 0.0016 to 0.0038 μg/ml for SN-38 (Table I).

**Isobologram analysis**  When the combination effects in each cell line were analyzed by isobologram,9–11) the combination of DDP and paclitaxel had an additive effect on RTSG (Fig. 1a), while the combination of DDP and SN-38 had a synergistic effect (Fig. 1b). On the other hand, DDP and paclitaxel, and DDP and SN-38 showed synergistic antitumor activity on KF and KFra cell lines.

| Cell line | DDP  | Paclitaxel | SN-38 |
|-----------|------|------------|-------|
| RTSG      | 1.00 | 0.0082     | 0.0031 |
| KF        | 0.142| 0.01063    | 0.001607 |
| KFra      | 2.090| 0.0122     | 0.00388 |

The cytotoxicity of the agents was evaluated by crystal violet staining, where each cell line was exposed to the agents for 6 days. Fifty-percent inhibitory concentrations (IC50) of drugs were obtained from graphical plots showing inhibition of the growth of cells by 50% relative to the control. The data are shown as IC50 in μg/ml. KF cell line was sensitive to DDP, while KFra was 14.7-fold more resistant to DDP. RTSG was relatively resistant to DDP. The sensitivities of these cell lines to paclitaxel and SN-38 were almost equivalent to each other.

Fig. 1. Combined antitumor activity of DDP with paclitaxel or SN-38 on three cell lines. a), b) Combined antitumor activity of DDP with paclitaxel or SN-38 on RTSG cell line. The isobolograms indicate additive (a; DDP + paclitaxel) and synergistic (b; DDP + SN-38) effects on the RTSG cell line. c), d) The isobolograms indicate synergistic effects of DDP+paclitaxel (c) and DDP+SN-38 (d) on the KF cell line. e), f) The isobolograms indicate synergistic effects of DDP+paclitaxel (e) and DDP+SN-38 (f) on the KFra cell line.
Pt accumulation  Fig. 2 shows the time course of the accumulation of Pt in each cell line. In the KF cell line, Pt accumulated time-dependently until 6 h after drug exposure, while the accumulated Pt in the KFra cell line reached a plateau at 2 h after drug exposure and decreased at 6 h after drug exposure, resulting in almost half the area under the curve compared with its parent KF cell line. In contrast, Pt barely accumulated in cells of the intrinsically DDP-resistant RTSG cell line. When cells of the RTSG cell line were pre-incubated with paclitaxel or SN-38, the intracellular Pt accumulation increased relative to the control ($P<0.01$). There were no significant differences in the accumulation of Pt between paclitaxel and SN-38. When the intracellular accumulation of Pt in the KF and KFra cell lines was compared with or without preincubation of SN-38 and paclitaxel, both cell lines showed a significant increase in intracellular Pt ($P<0.01$), as did the RTSG cell line (Fig. 3).

Effects of ouabain on Pt accumulation  In order to clarify the mechanism of Pt accumulation by paclitaxel or SN-38, we used ouabain, an inhibitor of the Na$^+$,K$^+$-ATPase, which works as a DDP influx pump. The increased Pt accumulation induced by paclitaxel was decreased significantly ($P<0.05$) by ouabain, as shown in Fig. 4, but this phenomenon was not observed in the RTSG cell line. Similarly, ouabain decreased the intracellular Pt accumulation induced by SN-38, and this inhibition was not seen in the RTSG cell line (data not shown).

Expression of MRP and MDR-1  MRP-1 and 3 mRNAs were expressed in three cell lines (Figs. 5–8), while MRP-2 mRNA was not expressed in the wild-type KF cell line (Fig. 6). DDP alone strongly up-regulated MRP-1, 2, and 3 mRNAs in three cell lines. Paclitaxel up-regulated MRP
mRNAs in the wild-type KF cell line (Fig. 6), while this up-regulation was not remarkable in the DDP-resistant KFra subline as well as in the intrinsic resistant cell line, RTSG (Figs. 5 and 7). SN-38 also up-regulated MRP mRNAs, in particular, in the RTSG cell line (Fig. 5), and this up-regulation was moderate in the KF and KFra cell lines (Figs. 6 and 7). The combination of paclitaxel and DDP down-regulated MRP mRNAs induced by DDP alone, and the SN-38 and DDP combination completely suppressed MRP.

DISCUSSION

Several mechanisms of DDP resistance have been proposed, such as decreased intracellular accumulation of the drug, presence of a DDP detoxification system, and decreased DNA damage and/or increased repair. Some investigators have concluded that decreased intracellular accumulation has an important role in DDP-resistance, and it has been observed in most DDP-resistant cell lines. This suggests that decreased intracellular accumulation and/or increased efflux of DDP may be the rate-limiting factor for DDP resistance. Although the mechanism by which DDP passes through the cell membrane is not completely defined, some reports suggest an energy-dependent uptake mechanism in addition to passive diffusion. Andrews and colleagues reported that DDP accumulation was inhibited by ouabain and suggested that the Na⁺,K⁺-ATPase in DDP-resistant ovarian cancer cells was altered. On the other hand, the efflux system of DDP is well recognized and exists in a wide variety of cells, although its mechanism is not fully understood. MRP and/or the ATP-dependent glutathione-S-conjugate export pump are considered to control DDP efflux. MRP was reported to be one of the main efflux pumps of DDP, and it may be associated with the GS-X pump, a...
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major DDP efflux system.\textsuperscript{17–21} Recently, Kuo and coworkers reported that the increase in MRP mRNA preceded the increase of $\gamma$-glutamylcysteine synthetase mRNA, which was thought to be the rate-limiting factor for the glutathione/glutathione S-transferase (GSH/GST) detoxification system.\textsuperscript{25}

Given this information, we examined the effects of paclitaxel and SN-38 on the expression of MDR-1 and MRP mRNA in the RTSG, KF, and KFra cell lines. MRP widely exists in some normal cells, acting as an efflux pump, though its expression in ovarian normal cells is rather low. MRP mRNA has less than 6 phenotypes, and phenotypes 1 to 3 may be closely related to antitumor agents.\textsuperscript{26–28} In the present study, we examined the additive and synergistic effects of DDP with paclitaxel or DDP with SN-38, on the DDP-resistant cell lines RTSG and KFra. With these combinations, Pt accumulation was increased by pre-exposing RTSG and KFra cell lines to paclitaxel or SN-38. Simultaneously, MRP mRNA expression was up-regulated by DDP, while it was down-regulated by the combination of DDP with paclitaxel or SN-38 in the three cell lines tested. MRP-1 and MRP-3 were highly influenced by these agents, in agreement with Kool’s finding that MRP1 and MRP3 showed the highest homology among MRP phenotypes.\textsuperscript{29} DDP has many mechanisms of resistance, and these mechanisms may be related to each other. Paclitaxel is an anti-microtubule agent, and Christen and colleagues reported that paclitaxel increased Pt accumulation by altering the state of polymerization with tubulin-binding drugs.\textsuperscript{30} Yamamoto et al. concluded that paclitaxel increased Pt accumulation by down-regulating MRP,\textsuperscript{31} which was supported by other reports\textsuperscript{21, 32, 33} and our present results. On the other hand, CPT-11 is a topoisomerase I inhibitor, and Gobert and coworkers reported that the p53-mediated response to DNA damage may, at least in part, involve activation of topoisomerase I.\textsuperscript{34} There are many possible mechanisms relating to MRP and the combined effects of DDP and paclitaxel or DDP and SN-38.\textsuperscript{35}

Recently, P-glycoprotein and MRPs were re-categorized as a superfamily of ATP-binding cassette (ABC) transporter proteins, in which P-glycoprotein and MRP1 were

Fig. 6. Changes of MRP-expression in KF cell line treated with DDP, paclitaxel, SN-38 and their combination. While MRP-2 mRNA was not expressed in KF cell line, DDP alone strongly up-regulated MRP-1, 2, and 3 mRNAs. Paclitaxel and SN-38 up-regulated MRP mRNAs. The combination of paclitaxel and DDP down-regulated MRP mRNAs induced by DDP alone, and the SN-38 + DDP combination completely suppressed MRP.
Fig. 7. Changes of MRP-expression in KFra cell line treated with DDP, paclitaxel, SN-38 and their combination. In KFra cell line, MRP-1, 2 and 3 mRNAs were expressed, and were strongly up-regulated by DDP alone. Paclitaxel did not up-regulate MRP mRNAs, while SN-38 up-regulated MRP mRNAs only moderately. The combination of paclitaxel and DDP down-regulated MRP mRNAs induced by DDP alone, and the SN-38 and DDP combination completely suppressed MRP.

Fig. 8. Changes of MDR-1-expression in RTSG, KF and KFra cell lines treated with DDP, paclitaxel, SN-38 and their combination compared to MRP expression. Only the RTSG cell line expressed MDR-1 mRNA, which was up-regulated by DDP, paclitaxel, SN-38 and their combinations. KF and KFra cell lines did not express MDR-1 mRNA in control or treated groups. β-Actin probe was used as a loading control.
newly named as ABCB1 and ABCC1, respectively (http://www.med.rug.nl/mdl/humanabc.htm and http://www.gene.ucl.ac.uk/users/hesterrabc.html). In this ABC transporter superfamily, DDP is not regarded as a substrate of the MDR-1/P-glycoprotein, which is responsible for resistance to many anticancer drugs. Actually, although RTSG expressed MDR-1 mRNA, KF and KFra did not, suggesting that MDR-1 was not mainly involved in the DDP resistance in these cell lines. The intrinsically resistant cell line, RTSG, established and characterized in our laboratory showed an extremely high level of GSH (unpublished data). The resistance mechanism of RTSG was somewhat different from that of the acquired resistant cell line, KFra, i.e., undetectable intracellular Pt accumulation on exposure to DDP, expression of MDR-1 mRNA and negative ouabain-rescue. However, since paclitaxel and SN-38 increased the intracellular Pt in RTSG, and MRP mRNA of this cell line was similarly affected by the drug combination to KF and KFra cell lines, the mode of combination effect of paclitaxel + DDP and SN-38 + DDP was thought to be at least partly identical in these cell lines.

We have observed that Pt accumulation increased and MRP expression decreased after exposure of DDP-resistant ovarian cells to the combination of DDP with paclitaxel or SN-38. Although the intracellular paclitaxel and/or SN-38 might be altered by DDP, the ouabain-rescue of the increment of Pt accumulation and the down-regulation of MRP mRNA might suggest that this intracellular accumulation of Pt by paclitaxel and SN-38 at least partly accounts for the synergism of these combinations. In conclusion, these results suggest that paclitaxel and SN-38 overcome DDP resistance of ovarian cancer cell lines by down-regulating MRP mRNA. These results may suggest useful therapeutic strategies for patients with DDP-resistant ovarian carcinoma.

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