Caffeine Has a Synergistic Anticancer Effect with Cisplatin via Inhibiting Fanconi Anemia Group D2 Protein Monoubiquitination in Hepatocellular Carcinoma Cells

Yuichiro Oda, Muneaki Hidaka, and Akito Suzuki*

The Third Department of Clinical Pharmacy, Graduate School of Clinical Pharmacy, Kyushu University of Health and Welfare; 1714-1 Yoshino-machi, Nobeoka, Miyazaki 882-8508, Japan.
Received June 6, 2017; accepted August 17, 2017; advance publication released online August 31, 2017

Cisplatin is an anticancer agent and induces DNA interstrand cross-links (ICLs). ICLs activate various signaling processes and induce DNA repair pathways, including the Fanconi anemia (FA) pathway. FA complementation group D2 (FANCD2) is monoubiquitinated in response to DNA damage, leading to activation of the DNA double-strand-break repair protein, RAD51. Caffeine increases the anticancer activity of cisplatin by inhibiting DNA repair; however, details of the mechanism remain unclear. We investigated the mechanism responsible for the synergistic anticancer effect of cisplatin and caffeine in HepG2 human hepatocellular carcinoma cells, focusing on the FA pathway. Caffeine (=100 µg/mL) significantly enhanced the antiproliferative activity induced by 3.8 µg/mL cisplatin. Caffeine (200 µg/mL) promoted apoptosis and inhibited the increase in the proportion of viable cells in S phase that occurred in the presence of 3.8 µg/mL cisplatin. Both FANCD2 monoubiquitination and RAD51 expression were significantly inhibited by co-treatment with 200 µg/mL caffeine and 3.8 µg/mL cisplatin compared with cisplatin alone. In conclusion, caffeine enhances the anticancer effect of cisplatin by inhibiting FANCD2 monoubiquitination. In HepG2 cells, caffeine might inhibit the FA pathway and thereby regulate DNA damage responses such as DNA repair and apoptosis.

Key words caffeine; cisplatin; Fanconi anemia pathway; monoubiquitination

Cisplatin is a platinum complex widely used for treating solid tumors. Its cytotoxic effects are the result of DNA interstrand cross-link (ICL) formation. Cisplatin ICLs are highly toxic DNA lesions that inhibit DNA strand separation.

The homologous recombination (HR) DNA repair is induced by ICLs via the Fanconi anemia (FA) pathway. FA is a disease in which defective DNA repair leads to bone-marrow failure and the development of solid tumors, especially liver tumors. FA cells are hypersensitive to ICL-inducing agents, and FA pathway inhibition increases the anticancer effect of cisplatin. Therefore, the FA pathway plays an important role in repairing cisplatin-induced DNA damage.

The FA pathway consists of at least 16 complementation groups and their associated genes. At least 10 FA proteins form the FA core complex, which mediates the monoubiquitination of FA complementation group D2 (FANCD2) at S phase of the cell cycle. Monoubiquitinated FANCD2 (FANCD2-Ub) is a key component of the FA pathway that functionally interacts with RAD51, which is essential for strand-pairing reactions during DNA recombination.

Caffeine is a methylxanthine alkaloid that is chemically related to the adenosine and guanine bases of DNA and RNA. Caffeine increases the anticancer effect of cisplatin in hepatocellular carcinoma (HCC) cell lines via inhibiting DNA repair. In addition, a clinical trial of caffeine-potentiated chemotherapy previously reported positive results. Caffeine suppresses HR by inhibiting both RAD51 accumulation and the activity of ataxia telangiectasia and Rad-3 related (ATR) kinase, which is associated with DNA damage-induced DNA repair and apoptosis. ATR kinase-deficient cells were also found to have a reduced ability to monoubiquitinate FANCD2.

These reports suggest that caffeine increases the anticancer effects of cisplatin by interaction with the FA pathway. However, no evidence has yet been published for a relationship between caffeine and the FA pathway. Therefore, the mechanisms responsible for the synergistic anticancer effects of caffeine and cisplatin require further clarification.

This study was conducted to investigate the mechanism through which caffeine potentiates the anticancer effects of cisplatin. The combined effects of caffeine and cisplatin on cytotoxicity, the cell cycle, FANCD2 ubiquitination and RAD51 expression were investigated in a HCC cell line.

MATERIALS AND METHODS

Cell Lines and Cell Culture The human HCC cell line, HepG2 (Riken BioResource Center Cell Bank, Ibaraki, Japan), was grown in Dulbecco’s modified Eagle’s medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (Nichirei Bioscience, Tokyo, Japan) and 2 mM glutamine (PAA Laboratories) at 37°C in a humidified atmosphere containing 5% CO2.

Reagents and Antibodies The reagents used were cisplatin (Sigma-Aldrich, MO, U.S.A.), caffeine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), anti-FANCD2 and anti-RAD51 antibodies (sc-28194 and sc-8349, respectively, Santa Cruz Biotechnology, CA, U.S.A.), anti-beta-actin antibody, and horseradish peroxidase (HRP)-coupled anti-rabbit immunoglobulin G (IgG) (#4967 and #7074, respectively, Cell Signaling Technology, MA, U.S.A.). Cell Counting Kit-8 (CCK-8: Dojindo Laboratories, Kumamoto, Japan), the Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Annexin V-FITC Kit; BioVision, CA, U.S.A.; containing annexin V-FITC and propidium iodide (PI)), and PI/RNase staining buffer (Becton Dickinson Biosciences, NJ, U.S.A.) were used according to the manufacturers’ instructions.

© 2017 The Pharmaceutical Society of Japan
ological saline solution (Otsuka Pharmaceutical, Tokushima, Japan) was used for preparing caffeine and cisplatin solutions.

**Cell Proliferation Assay** Cells were seeded at $5 \times 10^3$ cells/well and incubated with cisplatin and/or caffeine for 48h. Caffeine is reported to inhibit ATR kinase with an IC$_{50}$ value of approximately 200 $\mu$g/mL. Previous studies reported IC$_{50}$ values for cisplatin in the range of approximately 2–10 $\mu$g/mL. Thus, in the present study, we used caffeine concentrations of 0–200 $\mu$g/mL and cisplatin concentrations of 0–100 $\mu$g/mL. Cell proliferation was assessed by absorption photometry using a CCK-8 assay. The IC$_{50}$ values of cisplatin in HepG2 cells were obtained by plotting the data points for the concentration–response relationships for the effect of cisplatin on cell viability in the presence and absence of caffeine.

**Apoptosis Assay** Cells were seeded at $15 \times 10^4$ cells/well and incubated with cisplatin (3.8 $\mu$g/mL, the IC$_{50}$ value obtained experimentally in the absence of caffeine in the assay described above) and/or caffeine (200 $\mu$g/mL) for 48h. Cell suspensions prepared in phosphate-buffered saline (PBS) were stained using the Annexin V-FITC Kit and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson Biosciences). Annexin V-FITC (+) PI (−) cells were considered.

### Table 1. The Difference between the IC$_{50}$ Values for Cisplatin with and without Caffeine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC$_{50}$ ($\mu$g/mL)</th>
<th><strong>p</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>3.5 ± 0.2</td>
<td><strong>&lt;0.01</strong></td>
</tr>
<tr>
<td>Cisplatin + Caffeine (100 $\mu$g/mL)</td>
<td>2.8 ± 0.1</td>
<td><strong>&lt;0.01</strong></td>
</tr>
<tr>
<td>Cisplatin + Caffeine (200 $\mu$g/mL)</td>
<td>2.8 ± 0.1</td>
<td><strong>&lt;0.01</strong></td>
</tr>
</tbody>
</table>

Caffeine co-treatment reduces the IC$_{50}$ value for cisplatin in a dose-dependent manner. Results shown are the mean±S.D. of four experiments. **p<0.01.

**Fig. 1. Antiproliferative Activity of Caffeine with or without Cisplatin in HepG2 Cells**

HepG2 cells were co-treated with caffeine (0–200 $\mu$g/mL) and cisplatin (3.8 $\mu$g/mL) and cell proliferation was measured after 48h. Cell proliferation is expressed as the percentage of viable cells. The percentage of viable cells before treatment was normalized to 100% for each sample. Results shown are the mean±S.D. of four experiments. *p<0.05, **p<0.01.

**Fig. 2. The Effect of Caffeine on Cisplatin-Dependent Apoptosis in HepG2 Cells**

HepG2 cells were treated with caffeine (200 $\mu$g/mL) and/or cisplatin (3.8 $\mu$g/mL) for 48h. Cells were then stained with an Annexin V-FITC kit and analyzed by flow cytometry. Differences in the level of apoptosis were determined using two-tailed multiple t-tests with the Tukey–Kramer test. Results shown are the mean±S.D. of three experiments. *p<0.05, **p<0.01. NS, not significant.

**Fig. 3. Changes in Expression of Cisplatin-Induced FANCD2-Ub and RAD51 in the Presence or Absence of Caffeine in HepG2 Cells**

HepG2 cells were co-treated with caffeine (200 $\mu$g/mL) and 3.8 $\mu$g/mL cisplatin for 24h. (a) FANCD2-Ub, FANCD2 and RAD51 protein expression were analyzed by Western blot. β-Actin served as a control. FANCD2-Ub/FANCD2 ratio (b) and RAD51/β-actin ratio (c) were calculated based on quantification of the band density. Significant differences in the FANCD2-Ub/FANCD2 ratio and RAD51 expression were determined by two-tailed multiple t-tests with the Tukey–Kramer test. Results shown are the mean±S.D. of three experiments. *p<0.05, **p<0.01. NS, not significant.
apoptotic.15)

**Western Blotting** For FANCD2 and RAD51 detection, cell lysate samples (4.5 µg) were separated by polyacrylamide gel electrophoresis followed by electrophoretic transfer to polyvinylidene difluoride membrane. After blocking with EzBlock Chemi (Atto Corporation, Tokyo, Japan), membranes were washed with PBS and incubated with primary antibody (anti-FANCD2 and anti-beta-actin, 1:1000 dilution; anti-RAD51, 1:500 dilution) at 4°C for 24 h. The membranes were then washed with PBS and incubated with HRP-coupled anti-rabbit IgG at room temperature for 1 h. After washing with PBS, proteins were visualized by chemiluminescence reagent using the Luminata Forte Western HRP Substrate (Merck Millipore Corporation, MA, U.S.A.). Protein expression levels were measured by quantitative densitometry (ImageQuant LAS 4000, General Electric Healthcare, Buckinghamshire, England).

**Cell Cycle Analysis** Cells were seeded at 15×10⁴ cells/well and incubated with cisplatin (3.8 µg/mL) and/or caffeine (200 µg/mL) for 24 h. After washing with PBS, cells were fixed in ice-cold 70% ethanol and stored at −20°C for 24 h. Fixed cells were incubated in PI/RNase staining buffer and the cell cycle distribution was analyzed by flow cytometry using FACSCalibur.7)

**Statistical Analysis** The data shown are expressed as the mean±standard deviation (S.D.), with the number of observations indicated. The Student’s t-test was used for comparison between two groups, and the two-tailed multiple t-test with the Tukey–Kramer test for more than two groups. Probabilities of less than 5% (p<0.05) were considered significant.

**RESULTS**

**Caffeine Increases the Antiproliferative Effect of Cisplatin** We first examined whether caffeine influences the antiproliferative effect of cisplatin. Table 1 shows the IC₅₀ values of cisplatin in HepG2 cells. For each experiment, a concentration of 3.8 µg/mL cisplatin (the IC₅₀ value) was used to record the control response. Concomitant application of caffeine (200 µg/mL) significantly decreased the IC₅₀ value for cisplatin. Figure 1 shows that caffeine alone has no effect on cell proliferation; however, in the presence of cisplatin, caffeine concentrations of ≥100 µg/mL cause a significant reduction in cell viability.

**Caffeine Increases the Level of Cisplatin-Dependent Apoptosis** Caffeine alone had no effect on HepG2 cell apoptosis (Fig. 2). In contrast, cisplatin significantly increased the level of apoptosis (14.5±2.9%) compared with the non-treated control group (2.6±0.1%). The level of apoptosis was further increased by the combined application of cisplatin and caffeine (24.0±0.9%).

**Caffeine Inhibits Cisplatin-Induced FANCD2-Ub and RAD51 Expression** We conducted a preliminary investigation of time-dependent changes in cisplatin-induced DNA repair activity. RAD51 expression was significantly increased after cisplatin treatment for 24 h (data not shown). Consequently, to identify the mechanism of caffeine effects on the FA pathway, FANCD2 and RAD51 expression levels were measured after a treatment period of 24 h.

Changes of FANCD2 and RAD51 expression were assessed at 24 h after the application of cisplatin (3.8 µg/mL) and/or caffeine (200 µg/mL). Cisplatin alone significantly increased both the FANCD2-Ub/FANCD2 ratio and the level of RAD51 expression (Fig. 3). However, caffeine co-treatment reduced the cisplatin-dependent increase in the FANCD2-Ub/FANCD2 ratio by roughly half. Furthermore, caffeine co-treatment completely inhibited the cisplatin-dependent increase in RAD51 protein levels.

**Caffeine Inhibits the Cisplatin-Dependent Increase in the Proportion of Viable Cells in S Phase** Caffeine-induced cell cycle arrest occurs at G1 phase and thus decreases the proportion of cells in S phase.60) Monoubiquitination of FANCD2 mainly occurs during S phase.7) Therefore, we assessed the influence of caffeine on cell viability in each cell cycle stage in HepG2 cells (Fig. 4). Treatment with cisplatin alone significantly decreased the proportion of viable cells in G1 phase, but significantly increased the proportion of viable cells in S phase, compared with the relevant controls. Notably, caffeine inhibited the cisplatin-dependent increase in the proportion of viable cells in S phase.

**Fig. 4. Differences in the Proportion of Viable Cells in Different Cell-Cycle Stages**

HepG2 cells were treated with caffeine (200 µg/mL) and cisplatin (3.8 µg/mL) for 24 h. Viable cells were counted by flow cytometry. Significant differences in cell cycle distribution were determined by two-tailed multiple t-tests with the Tukey–Kramer test. Results shown are the mean±S.D. of three experiments. *p<0.05, **p<0.01. NS, not significant.
Cisplatin ICLs activate apoptotic and DNA repair signaling pathways in many cancer cell lines, including lung cancer, HCC and ovarian cancer.\textsuperscript{2,17–19} Cisplatin-induced DNA repair is regulated by the FA pathway.\textsuperscript{23} Caffeine inhibits DNA repair and increases the anticancer effects of cisplatin.\textsuperscript{79} Mechanisms responsible for the influence of caffeine on the anticancer effects of cisplatin have been suggested to involve several proteins, such as ATR kinase, ataxia telangiectasia mutated (ATM) kinase, and p53 upregulated modulator of apoptosis (PUMA).\textsuperscript{12,20} However, effects of caffeine on FA signaling are not well studied.

In the present study, we showed that caffeine increased cisplatin-dependent antiproliferative activity and promoted cisplatin-dependent apoptosis in HepG2 cells. A preliminary investigation was performed to determine whether the xanthine derivatives, caffeine, theophylline, and theobromine, influence the antiproliferative effects of cisplatin in HepG2 cells. Only caffeine was found to increase the antiproliferative effects of cisplatin (data not shown). Caffeine was previously reported to increase the cisplatin-dependent antiproliferative effects and apoptosis in a dose-dependent manner.\textsuperscript{20–23} Our present study confirms these results in HCC cells. We found that apoptotic cells were significantly increased in the presence of cisplatin with caffeine compared with cisplatin alone. These results are different from those of previous studies.\textsuperscript{79} At the present time, we do not know the reason for this discrepancy, although it may be due to different concentrations of caffeine used in the analyses.

We demonstrated that co-application of caffeine and cisplatin significantly reduces both the FANCD2-Ub/FANCD2 ratio and RAD51 expression compared with cisplatin alone, and that caffeine significantly inhibits the cisplatin-dependent increase in the proportion of viable cells in S phase. Cisplatin was previously reported to increase the proportion of viable cells in S phase, which was reversed by caffeine co-treatment in cervical cancer cell.\textsuperscript{23} These findings suggest that the inhibition of the increase in the proportion of viable cells in S phase in the presence of both cisplatin and caffeine is associated with decreasing amounts of FANCD2-Ub. The present study suggests that caffeine co-treatment enhances the anticancer activity of cisplatin by decreasing FANCD2-Ub and RAD51 expression and thus decreasing the proportion of cells in S phase of the cell cycle. A previous study showed that caffeine increased the antiproliferative effect of other ICL-inducing agents, including mitomycin C.\textsuperscript{24} The authors suggested that the combination of these two agents evoked a synergistic effect that seemed to be relevant to the S phase of the cell cycle. However, the detailed mechanism of the synergistic action remains unknown.

We observed that caffeine potentiates cisplatin-dependent apoptosis. It was recently reported that FANCD2 down-regulation by small-interfering RNA in MG-63 osteosarcoma cells induces apoptosis.\textsuperscript{25} Therefore, it is thought that either FANCD2-Ub downregulation or FANCD2 dysfunction causes apoptosis. Therefore, the function of FANCD2 might be closely related to apoptosis. We think that caffeine-dependent inhibition of the FA pathway might regulate DNA damage responses such as DNA repair and apoptosis.

We conclude that in HepG2 cells caffeine enhances the anticancer effects of cisplatin via effects on the FA pathway.

We would like to thank Ms. A. Ohashi and Ms. S. Dohi for their help in this study.

Conflict of Interest The authors declare no conflict of interest.

ACKNOWLEDGMENTS We thank Ms. A. Ohashi and Ms. S. Dohi for their help in this study.

REFERENCES

15. Takahashi K, Tatsunami R, Tempo Y. Methylglyoxal-induced apo-