Mechanisms of collateral sensitivity to fluorouracil of a
cis-diamminedichloroplatinum(II)-resistant human non-small lung cancer

cell line

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Summary A cisplatin(CDDP)-resistant subline of a human lung cancer cell line, PC-7/CDDP, was 4.7-fold more resistant to CDDP than the parent line in a colony-forming assay. The sensitivity of this cell line to anthracyclines, vinca-alkaloid, etoposide, mitomycin C, and bleomycin was similar to that of the parental line, PC-7. However, PC-7/CDDP exhibited 4-fold higher sensitivity to fluorouracil (FUra). Possible mechanisms associated with the collateral sensitivity to FUra were studied in PC-7/CDDP cells. The sensitivity of both cell lines to FUra did not correlate with the effect of FUra on RNA. On the other hand, FUra induced a greater reduction in dTTP pools and more single strand breaks in PC-7/CDDP than in PC-7 cells. These results suggest that the pathway for de novo deoxyribonucleotide synthesis may be a target for FUra in PC-7/CDDP cells. However, inhibition of thymidylate synthase after FUra treatment did not correlate with the DNA-directed activity of FUra. Based on the above findings, the decreased salvage synthesis of dTTP was considered a possible mechanism of the greater reduction of dTTP pools in PC-7/CDDP cells. However, the activity of dThd kinase was the same in both cell lines. In the presence of physiological concentrations of exogenous dThd in the serum, uptake of dThd was less in PC-7/CDDP cells than in PC-7 cells. Our data suggest that FUra-induced cytotoxicity in PC-7/CDDP cells is associated with the inhibition of dTTP synthesis and that the decreased uptake of dThd is a possible mechanism of the collateral sensitivity to FUra in PC-7/CDDP cells.

CDDP is one of the most effective antitumour drugs available for the clinical treatment of human cancers. However, tumours become clinically unresponsive to this drug upon continued treatment. Therefore, it is important to elucidate the mechanisms or related processes of resistance to CDDP. We previously established a CDDP-resistant subline, PC-7/CDDP, from a human lung cancer cell line, PC-7, in order to elucidate the mechanisms of resistance to CDDP and to assist in developing strategies to circumvent CDDP resistance (Hong et al., 1988; Bungo et al., 1990; Fujiiwa et al., 1990). We demonstrated that PC-7/CDDP cells do not exhibit cross-resistance or collateral sensitivity to adriamycin, daunomycin, mitomycin C, bleomycin, etoposide, or vindesine. In contrast, PC-7/CDDP cells exhibited collateral sensitivity to FUra (Ohe et al., 1990). Determination of the mechanisms of such enhanced drug sensitivity may reveal the factors that contribute to cellular sensitivity to FUra and their relation to the mechanisms of CDDP resistance. Therefore, we searched for the possible mechanisms associated with the collateral sensitivity to FUra in PC-7/CDDP cells.

The mechanism of action of FUra is complex and cytotoxicity appears to depend upon the cell type being studied. FUra is known to exert its cytotoxic effect by at least two mechanisms: incorporation of FUra into RNA and inhibition of thymidylate synthase (TS) (Chabner, 1981; Heidelberger et al., 1983). FUra is anabolised to the ribonucleotide, FUTP, FUTP is incorporated into RNA, which may alter RNA function. FUTP is also anabolised to the deoxyribononophosphate,FdUMP. In the presence of methyleneetadiphosphate cofactor, FdUMP forms a covalent ternary complex with TS, inhibiting the de novo synthesis of dTMP. Depletion of dTMP and then dTTP interferes with DNA synthesis and induces DNA strand breaks (Pagolotti et al., 1981; Lonn & Lonn, 1986; Yoshikawa et al., 1987). In addition, it has been shown that FUra can be incorporated into DNA, and this may also contribute to its cytotoxicity (Major et al., 1982; Sawyer et al., 1984). Furthermore, FUra can be catabolised to H2FUra and consequently to F-β-Ala.

We previously tried to determine the contribution of TS inhibition by FUra in PC-7/CDDP cells (Ohe et al., 1990). However, there was no correlation between the level of TS inhibition revealed by [3H]-FdUMP binding assay and sensitivity to FUra. These data suggest that the factors responsible for TS inhibition do not contribute to the collateral sensitivity of FUra. Therefore, we have examined the effects of FUra on RNA and dTTP pools to investigate further the underlying mechanisms responsible for the collateral sensitivity to FUra in PC-7/CDDP cells.

Materials and methods

Drugs and chemicals

RPMI 1640 medium and PBS were purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Other agents were obtained from the following sources: CDDP from Bristol Myers Squibb Co., Tokyo, Japan; FUra from Kyowa Hakko Kogyo Co., Ltd, Tokyo; FUDr from Mitsui Pharmaceutical Inc., Tokyo; [6-3H]-FUra (specific activity, 19.3 Ci mmol−1) and [methyl-3H]-dThd (specific activity, 60 mCi mmol−1) from Amersham Japan Ltd, Tokyo; [6-14C]-FUra (specific activity, 56 mCi mmol−1) from New England Nuclear Corp., Boston, MA. All other drugs and chemicals were purchased from Sigma Chemical Co., St Louis, MO if not otherwise reported.

Cell lines and culture

Line PC-7 derived from an adenocarcinoma of the lung with no prior exposure to chemotherapeutic agents was kindly donated by Professor Y. Hayata of the Tokyo Medical College. The CDDP-resistant cell line, PC-7/CDDP, was established in our laboratory by exposing parental cells to stepwise increased concentrations of CDDP and cloned by the

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limiting-dilution technique (Hong et al., 1988; Ohe et al., 1990). Resistance to CDDP was stable for at least 6 months in CDDP-free medium. In order for experiments, the cell lines were propagated by culturing them in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Immunobiochemical Laboratories, Fujisaka, Japan), penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹). PC-7 and PC-7/CDDP cells were grown as suspension cultures. Cell cultures were initiated by seeding the medium with approximately 5 × 10⁵ cells ml⁻¹ and grew exponentially to at least 8 × 10⁶ cells ml⁻¹. All experiments were performed with cells in the exponential phase of growth. The PC-7/CDDP were cultured for more than 2 weeks before analysis in CDDP-free medium. These cells were found to be free of mycoplasmal contamination by examination with a Hoescht stain kit for detection of Mycoplasma in cell culture (Flow Laboratories, Inc., McLean, VA).

Colonial-forming assay

Colonial-forming efficiency was assessed in a double-layer soft agar system as previously described by Ohe et al., 1989, 1990, except for the use of the same culture medium in both top and bottom layers. PC-7 and PC-7/CDDP were plated at a concentration of 3 × 10³ cells/well with various concentrations of FUrA. The culture plates were incubated at 37°C in humidified air with 5% CO₂. After continuous exposure to FUrA for 15–20 days, colonies larger than 50 μm in diameter were counted with an automatic particle counter (CP-2000, Shiraïmatsu Co., Ltd, Tokyo). All assays were performed in triplicate. The plating efficiency of cell lines without the drug was 8–9%. The effect of leucovorin (5-formyl-tetrahydrofolate) on the cytotoxicity of FUrA was determined by the same procedure as described above, except for the addition of 1-leucovorin [Lederle (Japan), Ltd, Tokyo]. In the experiments, the effect of FUrA cytotoxicity, each cell line was cultured for 3 days in RPMI 1640 supplemented with dialysed FBS (Cell Culture Laboratories, Cleveland, OH) in 6-well culture plates. Cells were then treated with FUrA at various concentrations for 3 h in a CO₂ incubator. FUrA-treated cells were washed with RPMI 1640 medium and cultured in soft agar with or without various concentrations of dTTP. The experiment was conducted for 5 days on plates grown in a CO₂ incubator. Six hours after the addition of FUrA, 25 ml of cell suspension was transferred to a 50 ml tube, 5 ml of ice-cold PBS was added and then suspension was centrifuged at 250 g for 5 min. The cell pellet was washed with 30 ml of ice-cold PBS by centrifugation as above. The volume of the cell suspension was adjusted to 200 μl by the addition of PBS to the cell pellet on ice. One hundred and fifty microlitres of the cell suspension was transferred to a 1.5 ml tube and the cell suspension remaining in the 50 ml tube was used for protein assay (BCA protein assay kit, Pierce Chemical Co., Rockford, IL) with bovine serum albumin as standard. Then 7.5 μl of ice-cold 100% TCA was added to the 150 μl cell suspension. The sample was then vortexed, placed on ice for 10 min and were centrifuged at 17,000 g for 20 s. The acid-soluble extract was neutralised by extraction with two volumes of 0.5 M tri-n-octylamine trichloro trifluoroethane. For deoxyribonucleoside triphosphate determination, ribonucleotides were destroyed by the action of periodate and metaldehyde as follows: After addition of 20 μl or 20 μl deoxyguanosine to 80 μl of neutralised cell extract, 20 μl of 0.2 M PCA was added, followed by 30 μl of 4 M methanol phosphate, pH 7.5. After incubation for 30 min at 37°C, 2 μl of 1 M rhamnose was added to destroy the remaining periodate. A Partisil-10 SAX amino-methyl cellulose column (Whatman Inc., Clifton, NJ) was used for HPLC and 0.4 M ammonium dihydrogenphosphate:acetonitrile, 10:1 (v/v), pH 3.30 was used as eluant with a flow rate of 1.5 ml min⁻¹. A 100 μl aliquot of the periodate-treated sample was injected into the column at ambient temperature, and the column eluate was monitored simultaneously at 254 nm. Compounds

Colonial-forming efficiency was assessed in a double-layer soft agar system as previously described by Ohe et al., 1989, 1990, except for the use of the same culture medium in both top and bottom layers. PC-7 and PC-7/CDDP were plated at a concentration of 3 × 10³ cells/well with various concentrations of FUrA. The culture plates were incubated at 37°C in humidified air with 5% CO₂. After continuous exposure to FUrA for 15–20 days, colonies larger than 50 μm in diameter were counted with an automatic particle counter (CP-2000, Shiraïmatsu Co., Ltd, Tokyo). All assays were performed in triplicate. The plating efficiency of cell lines without the drug was 8–9%. The effect of leucovorin (5-formyl-tetrahydrofolate) on the cytotoxicity of FUrA was determined by the same procedure as described above, except for the addition of 1-leucovorin [Lederle (Japan), Ltd, Tokyo]. In the experiments, the effect of FUrA cytotoxicity, each cell line was cultured for 3 days in RPMI 1640 supplemented with dialysed FBS (Cell Culture Laboratories, Cleveland, OH) in 6-well culture plates. Cells were then treated with FUrA at various concentrations for 3 h in a CO₂ incubator. FUrA-treated cells were washed with RPMI 1640 medium and cultured in soft agar with or without various concentrations of dTTP. The experiment was conducted for 5 days on plates grown in a CO₂ incubator. Six hours after the addition of FUrA, 25 ml of cell suspension was transferred to a 50 ml tube, 5 ml of ice-cold PBS was added and then suspension was centrifuged at 250 g for 5 min. The cell pellet was washed with 30 ml of ice-cold PBS by centrifugation as above. The volume of the cell suspension was adjusted to 200 μl by the addition of PBS to the cell pellet on ice. One hundred and fifty microlitres of the cell suspension was transferred to a 1.5 ml tube and the cell suspension remaining in the 50 ml tube was used for protein assay (BCA protein assay kit, Pierce Chemical Co., Rockford, IL) with bovine serum albumin as standard. Then 7.5 μl of ice-cold 100% TCA was added to the 150 μl cell suspension. The sample was then vortexed, placed on ice for 10 min and were centrifuged at 17,000 g for 20 s. The acid-soluble extract was neutralised by extraction with two volumes of 0.5 M tri-n-octylamine trichloro trifluoroethane. For deoxyribonucleoside triphosphate determination, ribonucleotides were destroyed by the action of periodate and metaldehyde as follows: After addition of 20 μl or 20 μl deoxyguanosine to 80 μl of neutralised cell extract, 20 μl of 0.2 M PCA was added, followed by 30 μl of 4 M methanol phosphate, pH 7.5. After incubation for 30 min at 37°C, 2 μl of 1 M rhamnose was added to destroy the remaining periodate. A Partisil-10 SAX amino-methyl cellulose column (Whatman Inc., Clifton, NJ) was used for HPLC and 0.4 M ammonium dihydrogenphosphate:acetonitrile, 10:1 (v/v), pH 3.30 was used as eluant with a flow rate of 1.5 ml min⁻¹. A 100 μl aliquot of the periodate-treated sample was injected into the column at ambient temperature, and the column eluate was monitored simultaneously at 254 nm. Compounds
were identified by their retention times and the concentrations of the compounds were determined by comparison of peak heights with those of accurately prepared standard solutions.

**Alkaline elution assay**

For the determination of the FUra-induced DNA single-strand breaks, the alkaline elution technique described by Kohn *et al.* (1974, 1976) and Bungo *et al.* (1990) was used. Before the alkaline elution analysis, cells were radiolabelled by incubation with [methyl-3H]-dThd for 24 h. Then they were centrifuged, washed free of radioactivity in the medium, and incubated with 100 μM FUra for 48 h at 37°C. For the positive control of DNA single-strand breaks, cells not treated with FUra were irradiated with 5 Gy by 60Co γ-irradiation at a dose rate of 0.36 Gy min⁻¹. They were then diluted in cold PBS and gently deposited onto a 2.0 μm pore-size, 25 mm diameter polycarbonate filter (Nuclepore Corp., Pleasanton, CA). The cells were lysed on the filter by treatment for 1 h with 5 ml of a lysis solution containing 2% SDS, 25 mM Na₂ EDTA, 50 mM Tris, 50 mM glycine, and 0.5 mg of proteinase K per ml, pH 10.0. This lysis solution was allowed to flow through the filter by gravity, and then the filter was rinsed three times with 3 ml of 20 mM Na₂ EDTA, pH 10.0, to remove most of the cell protein, membrane, and RNA. The remaining DNA (more than 97% of that applied to the filter) was analyzed by elution with tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, NY)-H₂EDTA, pH 12.1, at a constant flow rate of 0.025 to 0.030 ml min⁻¹. Then fractions of the eluate were collected directly into scintillation vials on the fraction collector at 45 min intervals for 7.5 h. Aquasol II scintillation cocktail was then added to the vials and radioactivity was determined with a liquid scintillation counter.

**Cellular uptake of thymidine in short-time exposure**

PC-7 and PC-7/CDDP cells were cultured in RPMI 1640 containing 10% dialysed FBS for 1 week prior to use. Cell samples (10⁶ cells ml⁻¹) in the above fresh medium were incubated at variable temperatures (10°C, 15°C, 25°C) for 5 min before the uptake study was started. dThd and FUra uptake was then determined by a modification of the oil-stop method of Plagemann and Woffendin (1989). Briefly, a reaction mixture (50 μl) containing [methyl-3H]-dThd or [3H]-FUra (0.5–1.0 μCi ml⁻¹) was layered over oil with 20 μl of 23% sucrose layered under the oil in a microcentrifuge tube (0.25 ml). The tube was placed in the centrifuge and uptake was initiated by rapid addition of the cell suspension (150 μl) to the reaction mixture. In experiment with an inhibitor, dipyrindamole (DP) was added to the cell suspension 5 min before the addition of the suspension to the reaction mixture. The uptake was terminated by turning the centrifuge on and pelleting the cells through the oil mixture to the sucrose layer as described in the procedure for determination of the metabolism of FUra, except for using 1% SDS for cell lysis. After the addition of Aquasol II, radioactivity in the cell pellet was measured by a liquid scintillation counter. The volume of medium trapped in the cell pellet was determined by using 14C-inulin (Amersham). The values for PC-7 and PC-7/CDDP cells were 0.48 ± 0.08 μl and 0.42 ± 0.06 μl, respectively. Drug uptake data were corrected for the radioactivity of the trapped medium.

**Results**

**Cellular sensitivity to CDDP and FUra**

The PC-7 and PC-7/CDDP cells lines had almost the same amounts of DNA, RNA, and protein and about the same cellular volume (PC-7, 1.4 ± 0.7 pl; PC-7/CDDP, 1.2 ± 0.3 pl) as determined with a Coulter chanelizer model ZBI (Coulter Electronics Inc., Hialeah, FL). The cytotoxicity of CDDP and FUra in these cell lines is shown in Table 1. PC-7/CDDP cells were about 4.7 times as resistant to CDDP than PC-7 cells in a colony-forming assay. On the other hand, PC-7/CDDP cells were more sensitive to FUra than PC-7 cells. The IC₅₀ of FUra in PC-7/CDDP cells (3.6 μM) was about four times lower than that in PC-7 cells (13.1 μM) when both cell lines were treated by continuous drug exposure.

**Effect of Urd, dThd and leucovorin on the cytotoxicity of FUra**

To find out whether FUra acts mainly by inhibiting DNA synthesis or by alternating RNA function, we studied the effect of dThd and Urd on FUra cytotoxicity. dThd is thought to protect against the reduction in dTPP pools and Urd is thought to prevent the incorporation of FUTP into RNA. Urd prevented FUra cytotoxicity in PC-7 cells, but only a little prevention was observed in PC-7/CDDP cells (Table II). On the other hand, dThd prevented FUra cytotoxicity in both cell lines. The reversal effect of dThd in PC-7 cells was observed with low concentrations of dThd and reached a plateau at 1 μM. However, a higher concentration of dThd was necessary for the prevention of FUra-induced cytotoxicity in PC-7/CDDP cells. We also studied the effect of leucovorin on the cytotoxicity of FUra by a colony-forming assay (Figure 1). This reduced folate is known to enhance the inhibition of TS byFdUMP (Keyomarsi & Moran, 1986; Park *et al.*, 1988). In our study leucovorin enhanced the cytotoxicity of FUra in PC-7/CDDP cells but not in PC-7 cells. These data suggest that FUra acts mainly deoxyribonucleotide synthesis in PC-7/CDDP cells.

**Measurement of the intracellular accumulation of FUra and its metabolism**

We measured the cellular accumulation of FUra and its incorporation into nucleic acid in each cell line by using 14C-FUra. Total accumulation of FUra in PC-7/CDDP cells was decreased to 10% of that in PC-7 cells (Figure 2a). We also measured the incorporation of FUra into the RNA fraction (Figure 2b). The incorporation of FUra into RNA was parallel with the total cellular accumulation in each cell line with approximately 50% of the total accumulation of FUra incorporated into RNA in each case. Corresponding to the inhibitory effect of Urd on FUra-induced cytotoxicity,

**Table 1** Cytotoxicity of CDDP and FUra for PC-7 and PC-7/CDDP human lung cancer cell lines

| Drug | Drug exposure | PC-7 | CIC₅₀ (μM)ᵃ | IC₅₀ ratioᵇ |
|------|---------------|------|--------------|-------------|
|      |               | PC-7/CDDP |              |             |
| CDDP | Continuous    | 1.6 ± 0.3ᵇ | 7.3 ± 3.6ᵇ | 4.7         |
| FUra | 3 h           | 75.5 ± 7.8 | 38.0 ± 12.8ᵇ | 0.50        |
|      | Continuous    | 13.1 ± 7.3 | 3.6 ± 1.8ᵇ  | 0.27        |

ᵃDrug concentration inhibiting colony formation by 50%. ᵇIC₅₀ ratio equals the IC₅₀ of the resistant cell line divided by the IC₅₀ of the parental cell line. *Each value is the mean ± s.d. of three independent experiments. ᵇP<0.01 compared to the value for PC-7 (unpaired two-tailed Student's t-test).
Table II  Suppressive effect of dThd and Urd on the cytotoxicity of FUra in PC-7 and PC-7/CDDP cells

| Compound | Concentration (µM) | IC50 of FUra (µM)* |
|----------|-------------------|---------------------|
|          | PC-7              | PC-7/CDDP           |
|          |                   |                     |
|          | 0                 | 75 (1.00)*          | 36 (1.00) |
|          | 0.1               | 73 (0.97)           | 35 (0.97) |
|          | 1                 | 74 (0.99)           | 35 (0.97) |
|          | 10                | 154 (2.05)          | 39 (1.08) |
|          | 30                | 320 (4.27)          | 43 (1.19) |
| dThd     | 0.1               | 86 (1.14)           | 40 (1.11) |
|          | 1                 | 127 (1.69)          | 42 (1.17) |
|          | 10                | 145 (1.93)          | 58 (1.61) |
|          | 30                | 125 (1.66)          | 92 (2.50) |

*Drug concentration that inhibits colony formation by 50%. Number in parentheses is the IC50 ratio which equals the IC50 of the cells treated with either Urd or dThd divided by the IC50 of the control cells.

Figure 1  Effect of leucovorin on the cytotoxicity of FUra in PC-7 and PC-7/CDDP cells. PC-7 and PC-7/CDDP cells were treated by continuous exposure to various concentrations of FUra in the presence or absence of 20 µM of leucovorin and then assayed for colony formation. Values are means ± s.d. for three determinations. ○ - PC-7; FUra; △ - PC-7; FUra + LV. • - PC7/CDDP; FUra; ▲ - PC-7/CDDP; FUra + LV.

Figure 2  Time course study of cellular accumulation of 14C-FUra and its incorporation into RNA and DNA of PC-7 and PC-7/CDDP cells. CDDP-resistant PC-7/CDDP cells and the parental PC-7 cells in the exponential phase of growth were incubated with 10 µM 14C-FUra for 6 h at 37°C. FUra accumulation and its incorporation into RNA and DNA were determined as described under Materials and methods. The data shown are the means and s.d. of triplicate assays. a, FUra accumulation in whole cells; b, FUra incorporation into RNA; c, FUra incorporation into DNA. ○, PC-7; ●, PC-7/CDDP.

Change of dTTP levels induced by FUra

Inhibition of TS activity by FdUMP results in a reduction in dTTP levels (Yoshioka et al., 1987). We did not, however, find enhanced inhibition of TS activity in PC-7/CDDP cells in our previous study; the free TS level was almost the same in both cell lines after 6 h FUra treatment (Ohe et al., 1990).
In the present study, we measured the reduction in the dTTP level (Figure 4). FUra produced greater reduction in dTTP level in CDDP-resistant cells after 6 h FUra treatment. This result is consistent with the fact that PC-7/CDDP cells showed collateral sensitivity to FUra.

DNA strand breaks in cells treated with FUra

Several reports have described DNA strand breaks following a reduction in dTTP level (Yoshioka et al., 1987; Lönn & Lönn, 1986, 1988). Therefore, we examined both cell lines for formation of DNA strand breaks by the alkaline elution techniques. More DNA single-strand breaks were observed in PC-7/CDDP cells (Figure 5). These data suggest that the toxicity of FUra for PC-7/CDDP cells may be mainly due to the inhibition of synthesis of substrate for DNA replication. The degree of inhibition of TS activity is not correlated with the degree of reduction in dTTP level (Ohe et al., 1990). Moreover, PC-7/CDDP cells incorporate much less exogenous $^3$H-dThd (50 nm) into DNA than PC-7 cells (Ohe et al., 1990). Based on these data, we speculated that salvage synthesis of dTMP might be different in these cells, which could explain the greater reduction in dTTP levels in the PC-7/CDDP cells.

Short-term uptake of dThd

Our previous study did not reveal a difference in dThd kinase activity, which catalyses phosphorylation of dThd (Ohe et al., 1990). Therefore, we examined the membrane transport of dThd, the initial process of the dThd salvage pathway. Figure 6 shows short-term uptake of dThd at 25°C, demonstrated by the rapid sampling technique. Initial uptake of dThd by PC-7/CDDP cells was lower than that by PC-7 cells after treatment with 5 μM dThd (Figure 6a,b). However, the amount of dThd taken up by PC-7/CDDP cells was close to that by the parental cells at a high concentration of dThd (Figure 6b). This phenomenon was consistent with the concentration-response of dThd-suppression of FUra-induced cytotoxicity seen in the colony-forming assay. In contrast, cellular uptake of FUra was at almost the same level in both cell lines, and the amount of FUra incorporated into the cells increased depending on the exogenous FUra concentration in the same manner in both cell lines (Figure 7a,b). Under the conditions of the colony-forming assay, the concentration of dThd derived from serum is less than 1 μM (Nottebrock & Then, 1977; Schaeer et al., 1978; Sobrero & Bertino, 1986). Therefore, decreased uptake of dThd may be responsible for the decreased dTTP production in PC-7/CDDP cells. We also determined the rate of dThd transport. As the process of dThd transport was very rapid at 25°C, we decreased the temperature to determine the initial rate of dThd uptake. Although the initial rate of dThd-uptake by PC-7/CDDP cells was also lower than that by PC-7 cells at 15°C, the difference in dThd-uptake disappeared with the decrease in assay temperature (Figure 8a). An inhibitor of facilitated diffusion of nucleoside, DP inhibited the dThd uptake by both cell lines to a similar extent (Figure 8b). These data suggest that the factor associated with temperature-dependent and DP-insensitive transport of dThd may be different in PC-7/CDDP cells.
The PC-7/CDDP.

Figure 6  Short-term uptake of dThd in PC-7 and PC-7/CDDP cells 25°C. To study membrane transport of dThd, uptake of dThd was measured after short-time exposure to 14C-dThd as described in Materials and methods. a, Time course study of the uptake of 5 μM 14C-dThd. Values are means ± s.d. for three determinations. b, Concentration-dependent uptake of dThd within 5 s. The standard deviation of three determinations for each point was within 5%. a, ○ - PC-7; ○ - PC-7/CDDP. b, ○ PC-7; ● PC-7/CDDP.

Figure 7  Short-term uptake of FUra in PC-7 and PC-7/CDDP cells at 25°C. To study membrane transport of FUra, uptake of FUra was measured after short-time exposure to 3H-FUra as described in Materials and methods. a, Time course study of uptake of 5 μM 3H-FUra. Values are means ± s.d. for three determinations. b, Concentration-dependent uptake of FUra for 5 s. The standard deviation of three determinations for each point was within 5%. a, ○ - PC-7; ○ - PC-7/CDDP. b, ○ PC-7; ● PC-7/CDDP.

Figure 8  Effect of temperature and dipyridamole on the short-term uptake of dThd. Short-term uptake of 14C-dThd (1 μM) was measured at low-temperature a or in the presence of DP b as described in Materials and methods. a, Short-term uptake of dThd at 15°C and 10°C. b, Short-term uptake of dThd in the presence or absence of 10 μM DP at 15°C. The standard deviation of three determinations for each point was within 10%. a, ○ PC-7: 15°C. ● PC-7/CDDP: 15°C. △ PC-7: 10°C. ▲ PC-7/CDDP: 10°C. b, ○ PC-7. ● PC-7/CDDP. △ PC-7: DP. ▲ PC-7/CDDP: DP.

Discussion

A CDDP-resistant cell line, PC-7/CDDP showed collateral sensitivity to FUra. The same phenomenon was also seen in a CDDP-resistant subline of a human colon carcinoma cell line, BE cells (Fram et al., 1990). There are no previous reports of mechanistic analyses of this phenomenon. Therefore, in the present study, we have investigated the possible mechanism of collateral sensitivity to FUra in PC-7/CDDP cells. Analysis of cellular FUra metabolites demonstrated that ribonucleotide synthesis from FUra (FUMP, FUDP and FUTP) in PC-7/CDDP cells was decreased to less than 18% of that in PC-7 cells. The amount of FUra incorporated into RNA of PC-7/CDDP cells was also decreased to 17% of that in PC-7 cells. Urd prevented the cytotoxicity of FUra in PC-7 cells, but not in PC-7/CDDP cells. Thus, the collateral sensitivity of FUra in PC-7/CDDP cells cannot be explained by the effect of FUra on RNA. On the other hand, dThd prevented the cytotoxicity of FUra in both cell lines although there are the possible artefacts of testing TS inhibitors with reference to dThd protection (Jackman et al., 1984). FdUMP was present at higher level in PC-7/CDDP cells than the fluoro-ribonucleotide (Figure 3). These results suggest that FUra mainly inhibits DNA synthesis in PC-7/CDDP cells. This conclusion was supported by the fact that leucovorin enhanced the cytotoxicity of FUra in PC-7/CDDP cells, but not in PC-7 cells. The decreased effect of FUra on RNA seem to contradict the enhanced sensitivity to FUra in PC-7/CDDP cells. However, the mechanism of action of FUra is very complex and it is still under question whether damage to DNA or to RNA can induce the greater cytotoxic effect.

The inhibitory effect of FUra on DNA synthesis has been hypothesised to result from inhibition of TS by FdUMP (Chabner, 1981; Heidelberger et al., 1983). However, in our preliminary study, we did not see any enhancement of TS inhibition in PC-7/CDDP cells when determined by 3H-
FdUMP binding assay (Ohe et al., 1990). In the present study, we examined PC-7 and PC-7/CDDP cells for changes occurring after inhibition of TS by FdUMP. We found that FdUra induced a greater reduction in dTTP pools and more single-strand breaks of DNA in PC-7/CDDP cells without the enhanced incorporation of FdUra into DNA. After 6 h treatment with FdUra, free TS was at almost the same level in PC-7 and PC-7/CDDP cells (Ohe et al., 1990), while FdUra induced greater reduction in the dTTP pool in PC-7/CDDP cells than in PC-7 cells. The same levels of cellular free TS could produce the same level of dTMP. Additionally, cellular dTTP levels in both cell lines were the same in the absence of FdUra (Figure 4). Accordingly, it is difficult to explain that greater reduction in the dTTP pool in PC-7/CDDP cells by FdUMP-mediated TS inhibition. It might also be possible to assume that the duration of TS inhibition was greater in PC-7/CDDP than in PC-7 cells since an in vivo study suggested the importance of the duration of TS inhibition (Houghton et al., 1986). However, the greater reduction in the dTTP pool in PC-7/CDDP cells has already been observed after 6 h FdUra treatment. Therefore, the duration of TS inhibition may not be an important factor in the mechanism of collateral sensitivity to FdUra in PC-7/CDDP cells.

Although the total TS content of PC-7/CDDP cells was 76% that of PC-7 cells, Scanlon and Kashani-Sabet (1988) reported that a CDDP-resistant ovarian carcinoma cell line, A2780 S, expressed a 3-fold increase in mRNA for TS and dihydrololate reductase and a 2.5-fold increase in the activities of both enzymes compared with the parental cells. These CDDP-resistant A2780 S cells showed cross-resistance to FdUra, FdUrd and methotrexate (Lu et al., 1988; Newman et al., 1988). Teicher et al. (1986) reported that a CDDP-resistant head and neck squamous cell carcinoma cell line (SCC-25(CP)) showed cross-resistance to methotrexate, but not to FdUra. In addition, Kikuchi et al. (1988) reported that a CDDP-resistant cell line, KFr, derived from human serous cystadenocarcinoma of the ovary, showed no cross-resistance to FdUra. Accordingly, the determinants relating to the sensitivity or resistance to FdUra in CDDP-resistant cell lines might differ with the cell line.

The greater reduction in dTTP pools in PC-7/CDDP cells suggests the decrease in capacity of dTTP synthesis by a non-TS mediated process. dTMP can be synthesised from dThd rather than from DUMP in de novo synthesis. Accordingly, another proposed determinant of dTTP production is the availability of dThd through the salvage pathway (Notbeck & Then, 1977; Howell et al., 1978; Sobrero & Bertino, 1986). We previously showed that less exogenous 3H-dThd (50 nM) was incorporated into DNA of PC-7/CDDP cells into that of PC-7 cells, without a significant difference in dThd kinase activity (Ohe et al., 1990). Therefore, we examined the membrane transport of dThd, the initial process of the dThd salvage pathway. Short-term uptake of dThd was lower in PC-7/CDDP cells than in PC-7 cells. As shown in Figure 6a, it is clear that uptake of dThd was decreased in PC-7/CDDP cells. The uptake of dThd for 5 s does not mean the initial rate of dThd uptake because dThd uptake is saturated within 5 s at 25°C. However, it is possible that the difference in dThd uptake for 5 s was reflected in the difference in the membrane transport of dThd since the rate of dThd uptake was low in the second phase as shown in the time course experiment. A decreased initial uptake of dThd in the linear phase was also observed in PC-7/CDDP cells at 15°C. As shown in Figure 6b, the dThd uptake of dThd uptake between PC-7/CDDP and PC-7 was greater in the lower exogenous dThd concentrations at 25°C. This phenomenon is consistent with the concentration response of dThd suppression of FdUra-induced cytotoxicity seen in the colony-forming assay. Lower concentrations of dThd (0.1 and 1 μM) did not modify the FdUra-induced cytotoxicity in PC-7/CDDP cells, and higher concentrations (10 and 30 μM) were necessary for the modification of the cytotoxicity (Table II). These data clearly demonstrate that the rate of dThd uptake at a low concentration (1 μM or less) is too low to support DNA replication in the absence of endogenous dTMP synthesis. Therefore, it is possible that FdUra-induced cytotoxicity in PC-7/CDDP cells is associated with the inhibition of dTTP synthesis and that the decreased uptake of dThd is the mechanism of the collateral sensitivity to FdUra in PC-7/CDDP cells.

Two dThd-membrane transport systems have been identified in mammalian cells (Belt, 1983; Vijayalakshmi & Belt, 1988; Jarvis, 1989; Plagemann & Woffendin, 1989). One is a symmetrical, non-concentrative and facilitated diffusion system with broad substrate specificity which has low affinity for dThd. The other is a sodium- and energy-dependent and concentrative transport system which has high affinity for dThd. It may be that the exogenous dThd passes through the cell membrane mainly by the high-affinity transporter in our culture condition. In addition, further analysis of dThd-membrane transport showed that the difference in the levels of dThd uptake disappeared at low temperature and an inhibitor of facilitated diffusion, DP, inhibited dThd uptake in PC-7/CDDP and PC-7 cells to a similar extent. Another inhibitor, 6-[(4-nitrobenzyl)thio]-9-D-ribofuranosylpurine also inhibited dThd uptake by both cell line (data not shown). These data suggest that a dThd-transporter sensitive to these inhibitors exists not only in PC-7 cells but also in PC-7/CDDP cells. Therefore, we now hypothesise that the decreased uptake of dThd is related to the difference in the energy-dependent transport of dThd. However, more detailed analysis is required to elucidate the mechanisms of the decreased uptake of dThd in PC-7/CDDP cells.

Recently, there have been many studies that demonstrate a qualitative inverse relationship between drug sensitivity to CDDP and total intracellular CDDP concentration in many CDDP-resistant cell lines, exceptions to which include A2780 S cells (Richon et al., 1987; Waund, 1987; Andrews et al., 1988; Hosper et al., 1988; Kraker & Moore, 1988; Kuppen et al., 1988; Newman et al., 1988; Bungo et al., 1990; Fujiwara et al., 1990). Interestingly, the accumulation of CDDP in PC-7/CDDP cells was 30% of that in PC-7 cells (unpublished data). Although CDDP is believed to enter cells by passive diffusion, an energy-dependent CDDP accumulation mechanism has been demonstrated (Andrews et al., 1988). It has also been suggested that alteration in drug uptake might be one of the simplest and earliest events leading to CDDP resistance (Richon et al., 1987). These findings further suggest that the decreased uptake of dThd is associated with the decreased uptake of CDDP in PC-7/CDDP cells. Although the mechanisms underlying the decreased uptake are still unclear, we speculate that common changed process(es) exist in CDDP and dThd uptake. The possibility of such factors related to uptake of CDDP and dThd warrants further study in order to clarify the mechanisms of drug resistance.

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