Jpn. J. Cancer Res. **93**, 445–452, April 2002

Cellular and Biochemical Mechanisms of the Resistance of Human Cancer Cells to a New Anticancer *Ribo*-nucleoside, TAS-106

Yuji Shimamoto,[1] Hiromi Kazuno,[1] Yuko Murakami,[1] Atsushi Azuma,[1] Katsuhisa Koizumi,[1] Akira Matsuda,[2] Takuma Sasaki[3] and Masakazu Fukushima[1]

[1]Hanno Research Center, Taiho Pharmaceutical Co., Ltd., 1-27 Misugidai, Hanno, Saitama 357-8527, [2]Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-ku, Sapporo, Hokkaido 060-0812 and [3]Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934

We have established variants of DLD-1 human colon carcinoma and HT-1080 human fibrosarcoma cells resistant to the new anticancer *ribo*-nucleosides, 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)-cytosine (ECyd, TAS-106) and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd). Both variants were shown to have decreased (3- to 24-fold decrease) uridine-cytidine kinase (UCK) activity, and exhibited cross-resistance to EUrd and TAS-106. Based on the IC_{50} values determined by chemosensitivity testing, a 41- to 1102-fold resistance to TAS-106 was observed in the resistant cells. TAS-106 concentration-dependently inhibited RNA synthesis, while its effect on DNA synthesis was negligible. The degree of resistance (14- to 3628-fold resistance) calculated from the inhibition of RNA synthesis tended to be close to the degree of chemoresistance of tested cells to TAS-106. The experiments on the intracellular metabolism of TAS-106 in the parental cells revealed a rapid phosphorylation to its nucleotides, particularly the triphosphate (ECTP), its major active metabolite. The amount of TAS-106 transported into the resistant cells was markedly reduced and the intracellular level of ECTP was decreased from 1/19 to below the limit of detection; however, the unmetabolized TAS-106 as a percentage of the total metabolite level was high as compared with the parental cells. The ratio of the intracellular level of ECTP between parental and resistant cells tended to approximate to the degree of resistance calculated from the inhibitory effect on RNA synthesis. These results indicate that the TAS-106 sensitivity of cells is correlated with the intracellular accumulation of ECTP, which may be affected by both the cellular membrane transport mechanism and UCK activity.

Key words: TAS-106 — Anticancer *ribo*-nucleoside — Resistance — Cellular membrane transport — Uridine-cytidine kinase

TAS-106 (ECyd, Fig. 1) is a new cytidine analogue showing significant cytotoxicity and antitumor activity in preclinical therapeutic models.[1–3] The results of several studies show that TAS-106 acts by interfering with RNA metabolism.[2, 4–7] Further *in vitro* studies on RNA polymerase II, an enzyme expressed in the nuclear extracts from HeLa cells, revealed that the triphosphate of TAS-106, ECTP, inhibited in a concentration-dependent manner the activity of that enzyme.[4] The action of TAS-106 on RNA polymerase-specific transcription was demonstrated to be without specificity for RNA polymerase I, II or III in K562 human leukemia cells.[5] Moreover, in preliminary experiments, RNA polymerase was inhibited competitively by ECTP with a K_i value of 21 n*M* (apparent K_m value of CTP, 8.0 μM) in isolated nuclei of FM3A mouse mammary tumor cells.[6, 7] ECTP was found to accumulate as the major intracellular metabolite following the exposure of the cells to TAS-106 for 4 h and was then very slowly eliminated from the cells.[5, 6] Therefore, intracellular accumulation and retention of the active metabolite ECTP may contribute to the potent cytotoxicity of TAS-106. This profile, considered to reflect a unique mechanism of antitumor activity and cellular metabolism, makes TAS-106 different from other antitumor nucleosides and TAS-106 is a promising candidate as a therapeutic agent for cancer patients.

The mechanisms of the antitumoral action of TAS-106 and its uracil analogue EUrd[1, 8] have been explored by Tabata *et al.*[4] using the EUrd-resistant variant, HT-1080/EUrd, from HT-1080 human fibrosarcoma cells. They had reported that the HT-1080/EUrd cells showed cross-resistance to TAS-106 because uridine-cytidine kinase (UCK, EC 2.7.1.48) activity, the enzyme also responsible for the formation of active metabolites of TAS-106 (Fig. 2), was down-regulated in HT-1080/EUrd cells when compared with the parental HT-1080 cells. Furthermore, Tsuji *et al.* had suggested that the alteration of the cellular membrane transport mechanism might partially contribute to the development of the resistance of HT-1080/EUrd cells.[9] We had expected a similar mechanism of resistance to

E-mail: simamoto@taiho.co.jp

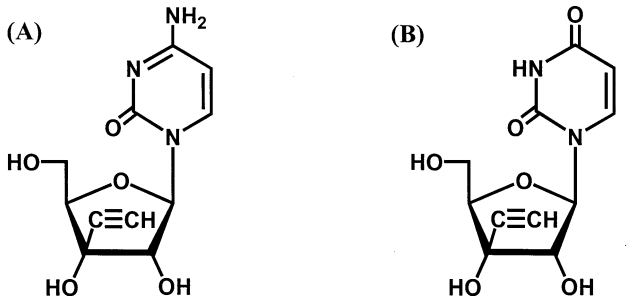

Fig. 1. Structures of TAS-106 (A) and EUrd (B).

occur in the case of TAS-106, but resistance induced by TAS-106 has not been reported yet. Therefore, to clarify the mechanism(s) of resistance and deepen knowledge on the mechanism of action of TAS-106, we made an attempt to develop TAS-106- and EUrd-resistant variants of DLD-1 human colon carcinoma and HT-1080 human fibrosarcoma cells. In the present study, we report the cellular and biochemical properties of these cancer cell lines with acquired resistance to TAS-106 and EUrd.

MATERIALS AND METHODS

Chemicals TAS-106, EUrd and 2′-deoxy-2′,2′-difluorocytidine (gemcitabine) were synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo). 5-Fluorouridine (FUR) was purchased from Sigma Chemical Co. (St. Louis, MO). 5-Fluorouracil (5-FU) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). [Cytosine-5-^{3}H]TAS-106 (5.4 Ci/mmol) was synthesized by Amersham International plc (Buckinghamshire, UK). [5-^{3}H]Cytidine (Cyd; 21.5 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). [5-^{3}H]2′-Deoxycytidine (dCyd; 26.1 Ci/mmol), [5-^{3}H]CMP, diammonium salt (26.8 Ci/mmol), [8-^{3}H]guanosine (Guo; 5 Ci/mmol) and [methyl-^{3}H]thymidine (dThd; 20 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). All other chemicals were of analytical grade from commercial sources.

Cell lines and cell culture The DLD-1 human colon carcinoma cell line, from the American Type Culture Collection (Manassas, VA), was purchased through Dainippon Pharmaceutical Co., Ltd. (Osaka). The HT-1080 human fibrosarcoma cell line and its EUrd-resistant variant HT-1080/EUrd were generous gifts from Professor Takuma Sasaki of the Cancer Research Institute, Kanazawa University (Kanazawa). A TAS-106-resistant variant of DLD-1, DLD-1/ECyd, was developed in our laboratory by continuous exposure of DLD-1 cells to TAS-106, starting with a concentration of 0.05 μM, which was stepwise increased to 1 μM. An EUrd-resistant variant of DLD-1, DLD-1/EUrd, was developed by continuous exposure to EUrd, using the same conditions as indicated for DLD-1/ECyd cells. A TAS-106-resistant variant of HT-1080, HT-1080/ECyd, was developed by continuous exposure to TAS-106, starting with a concentration of 0.05 μM, which was stepwise increased to 1 μM. DLD-1, DLD-1/ECyd, HT-1080, HT-1080/ECyd and HT-1080/EUrd cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (ICN Biomedicals, Inc., Aurora, OH) at 37°C and 5% CO_2. DLD-1/EUrd cells were maintained *in vitro* in the medium containing 1 μM EUrd, though all further experiments were performed using cells grown in a drug-free medium for 7 days.

Chemosensitivity testing The growth-inhibitory effects of test compounds on human cancer cells were determined by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma).[10] Briefly, 180 μl aliquots of an exponentially growing cell suspension ($0.5-2\times10^3$ cells/180 μl/well) were incubated in 96-well microplates for 24 h, then 20 μl of the drugs at various concentrations was added. After exposure to the drugs for 72 h, 25 μl of 2 mg/ml MTT reagent was added to each well, and the cell cultures were incubated at 37°C for 4 h. The medium was carefully removed and DMSO (150 μl) was added to each well to dissolve the formed formazan. After thorough mixing, the absorbance of each well was measured at 540 nm using a Model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA). Each experiment was performed using triplicate wells for each drug concentration and two or three independent experiments were carried out to confirm the reproducibility. The required drug concentration of IC_{50} was determined by reducing the absorbance to 50% as compared to the control.

Determination of RNA and DNA synthesis activity in whole cells Cells ($1-2\times10^4$ cells/180 μl/well) were seeded in 96-well microplates and incubated for 24 h, and then 20 μl of TAS-106 was added. After 1 h treatment with TAS-106, 10 μl of [^{3}H]Guo, a precursor of RNA synthesis, or 10 μl of [^{3}H]dThd, a precursor of DNA synthesis, was added. Cells were incubated for another 3 h, washed three times with Dulbecco's PBS(−) (Nissui Pharmaceutical Co., Ltd., Tokyo) and then subjected to trypsinization to obtain a homogeneous cell suspension. The cell suspension in each well was aspirated from the microplate and deposited on a filter material attached to a rigid plate (UniFilter plate, Packard Instrument Co., Meriden, CT) using a cell harvester (FilterMate, Packard). Then the filter material was extensively washed with distilled water to destroy the cell membrane, and once with ethanol, followed by drying with blown air. The radioactivity of the filter plates was counted using a microplate scintillation counter (Top Counter, Packard) after the addition of 25 μl of liquid scintillator Microscint 40 (Packard).

Each experiment was performed using 6 wells for each drug concentration and three independent experiments were carried out to confirm the reproducibility.

Measurement of intracellular TAS-106 and its nucleotides by HPLC Cells (3×10^7 cells) were preincubated for 24 h at 37°C under 5% CO_2 atmosphere. [^{3}H]TAS-106 or unlabeled TAS-106 was then added to the cells at a final drug concentration of 1 μM, and the cells were incubated for another 4, 6 or 8 h. Cells exposed to unlabeled TAS-106 were harvested in the same manner and suspended in Dulbecco's PBS(−) for cell counting. At the end of the incubation periods, the acid-soluble fraction containing cellular nucleotides was extracted with $HClO_4$ from the cells exposed to [^{3}H]TAS-106.[11] The acid-soluble fraction was neutralized with KOH and stored at −20°C until analysis by high-performance liquid chromatography (HPLC). The neutralized acid-soluble extracts were applied to a Partisil 10 SAX anion-exchange column (250×4.6 mm, Whatman, Inc., Clifton, NJ), and a linear gradient from 100% buffer A (0.005 *M* $NH_4H_2PO_4$, pH 2.8) to 100% buffer B (0.5 *M* $NH_4H_2PO_4$, pH 3.6) was run over 60 min at a rate of 2 ml/min. The elution profiles of TAS-106 and its nucleotides under these chromatographic conditions were determined by using external standards. The radioactivity associated with the respective nucleotides was measured with an on-line radioactive flow detector (FLO-ONE 500, Packard). The eluent was mixed automatically with scintillation fluid (Flo-Scint IV, Packard) at a ratio of 1:3. The amount of TAS-106 and its nucleotides in an extract was calculated based on the specific activity of [^{3}H]TAS-106. The experiments were repeated three times to confirm the reproducibility.

Enzyme assay For determination of the intracellular activities of metabolic enzymes, test human cancer cells were collected during the logarithmic growth, and cell pellets were stored frozen at −135°C. Immediately before use, the pellets were thawed, supplemented with two volumes of 10 m*M* potassium phosphate buffer (pH 7.5) corresponding to their wet weights and then sonicated. The homogenates were centrifuged at 105 000*g* for 1 h at 4°C, and the supernatants (cytosol fractions) were collected. The cytosol fractions were divided into small portions and stored frozen at −80°C until use for enzyme assays. Enzyme assays were carried out according to the method of Ikenaka *et al.*[12] with a slight modification. All the experiments were done in duplicate. The reaction rates were linear with respect to time and enzyme concentration under the conditions employed. The experiments were repeated three times and the average of the three measurements is reported as the final value. The protein concentration in human cancer cell cytosols was measured by the Bradford method[13] using a Protein Assay Dye solution (Bio-Rad), with bovine serum albumin (BSA) serving as the reference protein.

Assay of Cyd and dCyd kinase The reaction mixture, to a total volume of 125 μl, consisted of 50 m*M* Tris-HCl buffer (pH 8.0), 10 m*M* ATP (pH 7.5), 5 m*M* $MgCl_2$, 10 m*M* NaF, 0.6 m*M* (0.25 μCi/tube) [^{3}H]Cyd or 0.2 m*M* (0.25 μCi/tube) [^{3}H]dCyd, and 50 μl of enzyme solution (cell cytosol). The reaction mixture was incubated at 37°C. The reaction was stopped by heating on a heating block at 105°C for 3 min. The sample was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant, 10 μl, was spotted onto a polyethyleneimine (PEI)-cellulose F thin layer chromatography (TLC) plate (2.5×10 cm, Merck KGaA, Darmstadt, Germany) and developed with water. The phosphorylated Cyd or dCyd, remaining at the point of origin, was harvested into a vial and combined with 0.5 ml of 1 *M* HCl. The mixture was then combined with 10 ml of liquid scintillator ACS-II (Amersham) for radioactivity measurement using a Wallac 1414 WinSpectral liquid scintillation counter (Wallac Berthold Japan Co., Ltd., Tokyo).

Assay of CMP kinase The reaction mixture, to a total volume of 125 μl, consisted of 50 m*M* Tris-HCl buffer (pH 8.0), 10 m*M* ATP (pH 7.5), 5 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 1 m*M* (0.25 μCi/tube) [^{3}H]CMP, 1 mg/ml BSA and 50 μl of enzyme solution. The reaction mixture was incubated at 37°C. The reaction was stopped by heating on a heating block at 105°C for 3 min. The sample was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant, 10 μl, was spotted onto a PEI-cellulose F TLC plate (3×10 cm, Merck) and developed with a mixture of 1 *M* acetic acid and 1 *M* lithium chloride (1:1, v/v). Authentic samples of CMP, CDP and CTP were applied to the plate before the test sample and were visualized under a UV lamp. The spots corresponding to CDP and CTP were harvested into vials and taken up in 0.5 ml of 1 *M* HCl, then the radioactivity was measured as described above.

RESULTS

Chemosensitivity and metabolic enzyme activities of human cancer cells Tables I and II show the results of chemosensitivity testing and metabolic enzyme assays, respectively, in 6 cancer cell lines. When cells were exposed to TAS-106 for 72 h, the IC_{50} values were 0.058 μM for DLD-1, 6.088 μM for DLD-1/ECyd and 2.387 μM for DLD-1/EUrd cells. Therefore, the degree of resistance to TAS-106 for these two resistant cells was calculated to be 105 and 41, respectively. Similarly, the IC_{50} values at 72 h exposure were 0.027 μM for HT-1080, 8.299 μM for HT-1080/ECyd and 29.76 μM for HT-1080/EUrd cells. Therefore, the degree of resistance to TAS-106 for these two resistant cells was calculated to be 307 and 1102, respectively. Cross-resistance was observed to EUrd in TAS-106-resistant cells, and to TAS-106 in EUrd-resistant

Table I. IC_{50}s of Several Anticancer Agents as Determined in the DLD-1 and HT-1080 Human Cancer Cell Lines and Their TAS-106- and EUrd-resistant Variants

Cell line	TAS-106		EUrd		FUR		5-FU		Gemcitabine	
	μM	Fold[a)]	*μM*	Fold	*μM*	Fold	*μM*	Fold	*μM*	Fold
DLD-1	0.058±0.005	1	0.138	1	0.082	1	9.198	1	0.030	1
DLD-1/ECyd	6.088±0.499	105	56.00	406	9.100	111	32.55	4	0.074	2
DLD-1/EUrd	2.387±0.814	41	24.54	178	2.798	34	27.75	3	0.087	3
HT-1080	0.027±0.005	1	0.364	1	0.053	1	8.474	1	0.017	1
HT-1080/ECyd	8.299±1.293	307	226.8	623	4.222	80	19.97	2	0.070	4
HT-1080/EUrd	29.76±14.18	1102	612.7	1683	8.974	169	13.15	2	0.004	0.2

IC_{50} values for TAS-106 are mean±SD of three individual experiments. IC_{50} values for the other anticancer agents are from single representative experiments among two or three individual experiments.
a) The ratio of IC_{50}s for resistant variants to that for the respective parental cell lines.

cells developed from both DLD-1 and HT-1080 cells. Since the UCK activity measured using Cyd as a substrate was down-regulated from 1/3 to 1/24 in these TAS-106- and EUrd-resistant cells as compared with the respective parental cells, cross-resistance was also observed for FUR, as Tabata *et al.* reported.[4)] Unexpectedly, a slight increase in sensitivity to gemcitabine, a dCyd analogue phosphorylated to an active form by dCyd kinase (Fig. 2),[14–16)] was observed in HT-1080/EUrd cells, probably due to a minimal up-regulation of dCyd kinase activity as compared with the parental HT-1080 cells. In the case of HT-1080/ECyd cells, however, a slight cross-resistance to gemcitabine was observed although the dCyd kinase activity was unchanged. The reason for that remains unknown.

Table II. Metabolic Enzyme Activities of the DLD-1 and HT-1080 Human Cancer Cell Lines and Their TAS-106- and EUrd-resistant Variants

Cell line	Enzyme activity (nmol/min/mg protein)		
	Cyd kinase (Fold decrease)[a)]	CMP kinase	dCyd kinase
DLD-1	8.810±0.889 (1)	175.22±13.64	0.100±0.007
DLD-1/ECyd	1.145±0.096 (8)	138.70±15.91	0.044±0.003
DLD-1/EUrd	1.842±0.181 (5)	141.76±14.75	0.059±0.005
HT-1080	9.483±0.218 (1)	256.18±15.56	0.172±0.008
HT-1080/ECyd	3.512±0.201 (3)	227.67±25.60	0.169±0.019
HT-1080/EUrd	0.396±0.026 (24)	233.46±44.01	0.406±0.058

Enzyme activities are mean±SD of three individual experiments.
a) The decrease (fold) in enzyme activity of resistant variants as compared to the respective parental cell lines.

Effect of TAS-106 on RNA and DNA synthesis To investigate the effects of TAS-106 on RNA and DNA synthesis in DLD-1 and HT-1080 cells, [^{3}H]Guo and [^{3}H]dThd were used as precursors for macromolecular synthesis, respectively (Fig. 3). The incorporation of Guo into cellular RNA was inhibited by TAS-106 in a concentration-dependent manner with IC_{50} values of 2.3 and 1.8 *μM* for DLD-1 and HT-1080 cells, respectively. On the other hand the incorporation of dThd into cellular DNA remained unaffected by TAS-106 treatment in both cell

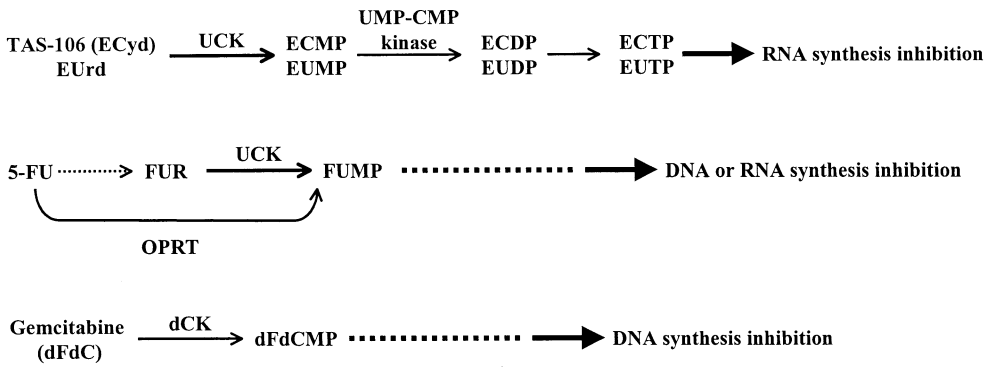

Fig. 2. Metabolic activation pathways of TAS-106 and other related anticancer agents in human cancer cells. UCK, uridine-cytidine kinase; OPRT, orotate phosphoribosyltransferase; dCK, deoxycytidine kinase.

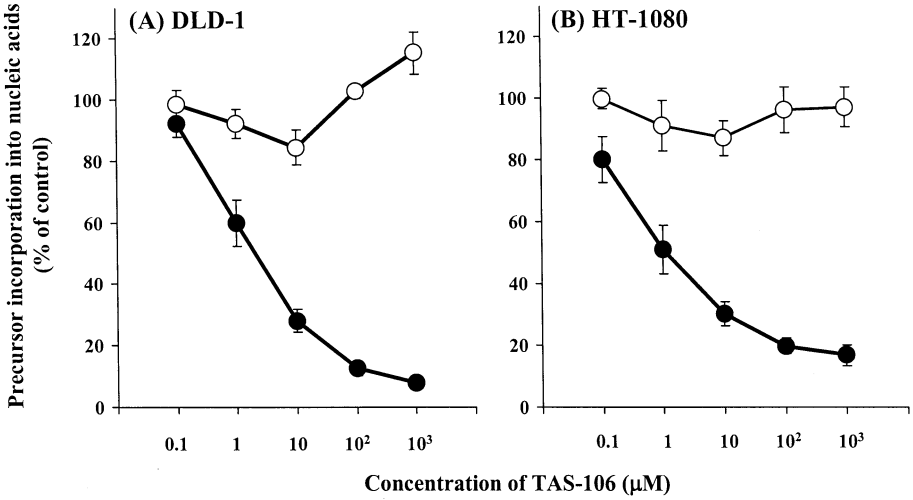

Fig. 3. Effects of TAS-106 on RNA and DNA synthesis in DLD-1 (A) and HT-1080 (B) cells. Cells were incubated with the indicated concentrations of TAS-106 for 1 h and then [^{3}H]Guo as a precursor of RNA synthesis (●) or [^{3}H]dThd as a precursor of DNA synthesis (○) was added and incubation was continued for another 3 h. The radioactivity incorporated into nucleic acids was determined as described in "Materials and Methods." Points indicate the mean of three individual experiments; bars indicate the SD.

lines. Assuming that the RNA synthesis inhibition is mainly involved in the mechanism of action of TAS-106, we have evaluated the effect of TAS-106 on RNA synthesis in TAS-106- and EUrd-resistant cells in comparison with the respective parental cells. As shown in Table III, the IC_{50} values of TAS-106 for the inhibition of Guo incorporation into cellular RNA were 158, 32.3, 645 and 6530 *μM* for DLD-1/ECyd, DLD-1/EUrd, HT-1080/ECyd and HT-1080/EUrd cells, respectively. The degrees of resistance as compared to the parental cells were thus calculated to be 69, 14, 358 and 3628 for DLD-1/ECyd, DLD-1/EUrd, HT-1080/ECyd and HT-1080/EUrd cells, respectively.

Intracellular metabolism of TAS-106 The intracellular accumulation of TAS-106 and its metabolites in DLD-1, HT-1080 and their variants is shown in Figs. 4 and 5, respectively. When DLD-1 (Fig. 4A) and HT-1080 (Fig. 5A) cells were treated with 1 *μM* TAS-106 for 4 h, ECTP was detected as the major metabolite in both cells (DLD-1, 10.25 pmol/10^6 cells; HT-1080, 32.23 pmol/10^6 cells), and accounted for approximately 55% of the total amount of TAS-106 transported into the cells (DLD-1, 18.98 pmol/10^6 cells; HT-1080, 58.66 pmol/10^6 cells). The other metabolites detected in the cells following 4 h exposure to TAS-106 included the diphosphate of TAS-106 (ECDP, approximately 30–35%) and the monophosphate of TAS-106 (ECMP, approximately 10%). Unmetabolized TAS-106 accounted for only 1–2% of the total amount of TAS-106 transported into the cells. During longer treatment with TAS-106 for 6 and 8 h, the levels of all metabolites rose in both the DLD-1 and HT-1080 cells, but the proportion of each metabolite remained almost unchanged as a percentage of the whole. DLD-1/ECyd (Fig. 4B) and DLD-1/EUrd (Fig. 4C) cells, however, accumulated in general about 1/15 of the radioactivity taken up by the parental cells after 4 h exposure to 1 *μM* TAS-106 (DLD-1/ECyd, 1.19 pmol/10^6 cells; DLD-1/EUrd, 1.29 pmol/10^6 cells). The intracellular levels of ECTP were 0.50 and 0.54 pmol/10^6 cells for DLD-1/ECyd and DLD-1/EUrd cells, respectively, and those levels were 1/21 and 1/19, respectively, of the level detected in the parental DLD-1 cells. The levels of the metabolites detected in both resistant cells were as follows; ECTP (approximately 40%),

Table III. IC_{50}s of TAS-106 for Inhibition of RNA Synthesis in the DLD-1 and HT-1080 Human Cancer Cell Lines and Their TAS-106- and EUrd-resistant Variants

Cell line	RNA synthesis inhibition	
	IC_{50}, *μM*	Fold[a)]
DLD-1	2.3±1.0	1
DLD-1/ECyd	158±116	69
DLD-1/EUrd	32.3±18.7	14
HT-1080	1.8±1.2	1
HT-1080/ECyd	645±304	358
HT-1080/EUrd	6530±5993	3628

Cells were incubated with TAS-106 for 4 h and pulsed with [^{3}H]Guo during the final 3 h of the incubation. IC_{50} values are mean±SD of three individual experiments.
a) The ratio of IC_{50}s for resistant variants to that for the respective parental cell lines.

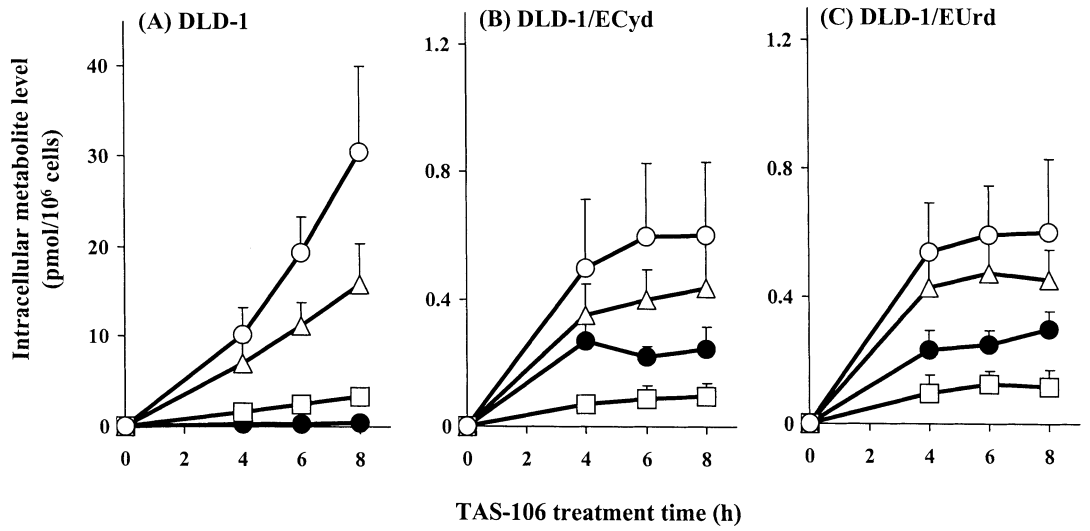

Fig. 4. Accumulation of TAS-106 and its nucleotides in DLD-1 (A), DLD-1/ECyd (B) and DLD-1/EUrd (C) cells. Cells were incubated with 1 μM TAS-106 for the indicated times. Intracellular TAS-106 (●), ECMP (□), ECDP (△) and ECTP (○) were extracted, and then analyzed by HPLC as described in "Materials and Methods." Note the difference of the scale of the vertical axis between the parental cells (A) and the resistant variants (B, C). Points indicate the mean of three individual experiments; bars indicate the SD.

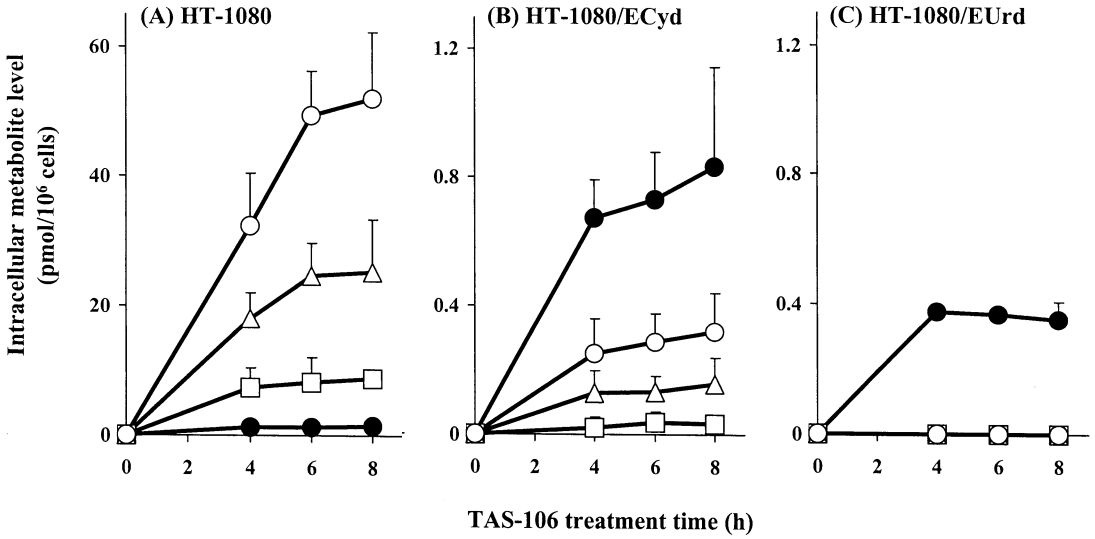

Fig. 5. Accumulation of TAS-106 and its nucleotides in HT-1080 (A), HT-1080/ECyd (B) and HT-1080/EUrd (C) cells. Cells were incubated with 1 μM TAS-106 for the indicated times. Intracellular TAS-106 (●), ECMP (□), ECDP (△) and ECTP (○) were extracted, and then analyzed by HPLC as described in "Materials and Methods." Note the difference of the scale of the vertical axis between the parental cells (A) and the resistant variants (B, C). Points indicate the mean of three individual experiments; bars indicate the SD.

ECDP (approximately 30%), unmetabolized TAS-106 (approximately 20%) and ECMP (6–7%). The proportions of the metabolites are similar to those observed in parental cells with the exception of a higher percentage of TAS-106. During longer treatment with TAS-106 for 6 and 8 h, the level of each metabolite remained almost unchanged. The intracellular metabolism of TAS-106 by HT-1080/ECyd cells is shown in Fig. 5B. The levels of total metabolites (1.07 pmol/10^6 cells) and ECTP (0.25 pmol/10^6 cells) detected in the cells after 4 h exposure to 1 μM

TAS-106 were 1/55 and 1/129, respectively, of the levels detected in the parental HT-1080 cells. The cellular level of TAS-106 (63%) was higher than that of any of its metabolites (ECTP, 23%; ECDP, 12%; ECMP, 2%). During longer treatment with TAS-106 for 6 and 8 h, the level of each metabolite remained almost unchanged. In the case of HT-1080/EUrd cells (Fig. 5C) treated with TAS-106 for 4 h, only unmetabolized TAS-106 was detected, and the levels of its metabolites were below their limits of detection. The intracellular level of TAS-106 detected in HT-1080/EUrd cells (0.37 pmol/10^6 cells) was about 1/160 of the level of total metabolites detected in the parental HT-1080 cells. Prolonged treatment with TAS-106 for 6 and 8 h did not result in further increase of its intracellular level or in the detection of other metabolites.

DISCUSSION

TAS-106 is a new and unique *ribo*-nucleoside analogue of cytidine, inhibiting RNA synthesis by blocking RNA polymerase. TAS-106 shows good antitumor activity against a panel of solid tumors of human origin *in vitro* and *in vivo*.[1–3] Thus, TAS-106 is a promising candidate as a therapeutic agent for cancer treatment. To apply TAS-106 clinically, it seems to be important to know the cellular and biochemical mechanisms of cancer cells resistance to TAS-106. Tabata *et al.*[4] have established an HT-1080/EUrd cell variant resistant to EUrd, a compound presumably activated by UCK. We expected a similar mechanism of resistance to occur in the case of TAS-106, though no such cell variant has as yet been observed. In the present study, we have therefore established several cell variants resistant to TAS-106 or EUrd to study the mechanism(s) of TAS-106 resistance. The growth rates of these resistant variants, DLD-1/ECyd, DLD-1/EUrd, HT-1080/ECyd and HT-1080/EUrd, were not significantly changed as compared to those of the respective parental cell lines, DLD-1 and HT-1080 (data not shown).

As shown in Fig. 1, TAS-106 is a Cyd analogue with an acetylene group at the 3′-position in the ribose moiety, and EUrd is its uracil analogue.[1, 8] It has been suggested that TAS-106 and EUrd might have a similar mechanism of action, based on mean graph analysis, which revealed that the two compounds show similar inhibitory spectra against various human cancer cell lines *in vitro*.[1] This idea was proved to be true since cross-resistance was actually observed to EUrd in TAS-106-resistant cells, and to TAS-106 in EUrd-resistant cells. Both *ribo*-nucleoside analogues, TAS-106 and EUrd, must be phosphorylated by UCK to exert cytotoxic effects, as illustrated in Fig. 2. In the TAS-106- and EUrd-resistant cells, the UCK activity was down-regulated as compared with the respective parental cells, while the UMP-CMP kinase activity was not much changed. TAS-106 and EUrd are largely resistant to inactivation by Cyd deaminase and uridine phosphorylase, respectively (data not shown). Therefore, the decrease in UCK activity in these resistant cells was expected to be a main cause of resistance to TAS-106 and EUrd. Cross-resistance was also observed for FUR, and not for 5-FU. These results suggest that FUR and 5-FU are probably phosphorylated to their monophosphate, 5-fluorouridine 5′-monophosphate (FUMP), through different pathways in human cancer cells. As shown in Fig. 2, FUR may be phosphorylated in human cancer cells to FUMP by UCK, while 5-FU may be phosphorylated to FUMP by orotate phosphoribosyltransferase.[17]

TAS-106 concentration-dependently inhibited RNA synthesis, while its effect on DNA synthesis was negligible. The IC_{50} values for inhibitory effect of TAS-106 on RNA synthesis following 4 h drug exposure were 13 to 219 times higher than the IC_{50} values for chemosensitivity testing following 72 h exposure to TAS-106, but the degree of resistance calculated from the inhibitory effect on RNA synthesis in TAS-106- and EUrd-resistant cells tended to be close to the degree of chemoresistance to TAS-106.

When we measured the intracellular accumulation of TAS-106 metabolites, the amount of TAS-106 transported into the parental DLD-1 and HT-1080 cells increased linearly with time up to 6 or 8 h and ECTP was detected as the major metabolite in both cells. The other TAS-106 metabolites included ECDP, ECMP and unmetabolized TAS-106, in descending order, and each of the metabolites remained almost unchanged with time as a percentage of the total metabolites derived from TAS-106. These results suggest that TAS-106 is rapidly phosphorylated to ECMP, ECDP and ECTP after being taken up by DLD-1 and HT-1080 cells. On the contrary, the amount of TAS-106 taken up by TAS-106- and EUrd-resistant cells was markedly reduced. Additionally, the levels of TAS-106 and its nucleotides reached plateaus within 4 h exposure to TAS-106 in these resistant variants, indicating the presence of intracellular regulatory mechanism(s) maintaining the amount of TAS-106 transported into these cells at constant levels. Thus, the alteration of nucleoside transport mechanisms, both decrease in uptake and increase in efflux, may be one of the important factors for acquisition of resistance by cancer cells to TAS-106 and EUrd, as suggested by Tsuji *et al.*[9] Moreover, the unmetabolized TAS-106 as a percentage of the total intracellular metabolite level was much higher in these TAS-106- and EUrd-resistant cells than in their parental cells. The apparent increase in the accumulation of unmetabolized TAS-106 is caused by the down-regulation of UCK activity, which in turn prevents the further metabolic step to ECTP, an active metabolite that is unable to leave the cell due to the presence of the phosphate function. The activity of UCK is actually several-fold decreased in the resistant variants, though at this stage it is difficult to conclude whether this is due to

decreased expression at the protein level or altered affinity of the enzyme for TAS-106. Consequently, the ratio of the intracellular level of ECTP after 4 h treatment with 1 μM TAS-106 in the resistant cells to that of the respective parental cells tended to approximate to the degree of resistance calculated from the inhibitory effect of TAS-106 on RNA synthesis after 4 h exposure.

In conclusion, the sensitivity of the cells to TAS-106 was correlated with the inhibition of cellular RNA synthesis by TAS-106, and further the inhibition of cellular RNA synthesis by TAS-106 was correlated with the intracellular accumulation of ECTP. In addition, we consider that both alterations of cellular membrane transport (decrease in uptake and increase in efflux) and intracellular phosphorylation (down-regulation of UCK activity) are involved in the development of the resistance of cancer cells to TAS-106 or EUrd.

(Received October 16, 2001/Revised January 9, 2002/Accepted January 21, 2002)

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