Experimental Studies on Potentiation of the Antitumor Activity of 5-Fluorouracil with 3'-Azido-3'-deoxythymidine for the Gastric Cancer Cell Line MKN28 in vivo

Chikao Yasuda, Michio Kato, Daisuke Kuroda and Harumasa Ohyanagi

Department of Surgery II, Kinki University School of Medicine, 377-2 Ohno Higashi, Osaka-Sayama, Osaka 589

A new method of biochemical modulation of 5-fluorouracil (5-FU) with 3'-azido-3'-deoxythymidine (AZT) was studied experimentally. Nude mice transplanted with cells of the human gastric cancer cell line MKN28 were divided into 4 groups, i.e., control, 5-FU, AZT, and 5-FU plus AZT, and the antitumor activities were compared. Based on the assessment of tumor volume, significant suppression of tumor growth was observed in the 5-FU and 5-FU plus AZT groups ($P < 0.05$, $P < 0.01$, versus control, respectively). The thymidylate synthase (TS) inhibition rate, an index of inhibition of the *de novo* pathway, was significantly higher in the 5-FU and 5-FU plus AZT groups than in the control group ($P < 0.01$), but it did not differ from the control in the AZT group. TS-bound FdUMP tended to be higher in the 5-FU plus AZT group than in the 5-FU group. The activity of thymidine kinase (TK) and the uptake ratio of 5-bromo-2'-deoxyuridine (BrdU), indices of salvage pathway activity, were significantly lower in the AZT and 5-FU plus AZT groups than in the control group (TK, $P < 0.05$, $P < 0.01$; uptake ratio of BrdU, $P < 0.01$, $P < 0.05$, respectively). There were slight losses of body weight in the 5-FU and 5-FU plus AZT groups compared with that in the control group, but no difference between the AZT and control groups in weight loss. These findings suggest that addition of AZT plays an important role in potentiating the antitumor activity of 5-FU through both blockage of a compensatory increase of activity in the salvage pathway and also an increase in TS-bound FdUMP, and has no adverse effects. Thus, the combination of 5-FU and AZT could be useful as a new modality in gastric cancer chemotherapy.

Key words: Biochemical modulation — 3'-Azido-3'-deoxythymidine — 5-Fluorouracil — Gastric cancer — Prevention of DNA synthesis

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for solid tumors. Recently, attempts have been made to enhance the antitumor activity of 5-FU by using it in combination with other drugs.[1] The rationale for this combination chemotherapy is the enhancement of the major activity of 5-FU, i.e., inhibition of thymidylate synthase (TS), which acts in the *de novo* pathway of pyrimidine biosynthesis. Since the salvage pathway of pyrimidine biosynthesis also operates, the enhancement of TS inhibition in the *de novo* pathway by combination chemotherapy does not necessarily increase the antitumor effect.[2] Use of the combination of 5-FU and other drugs for suppression of the salvage pathway, therefore, has been attempted with thymidine and its analogues.[3] Brunetti *et al.*[4] have reported that 5-FU enhanced the cytotoxicity of the antiviral agent 3'-azido-3'-deoxythymidine (AZT) against a human colon cancer cell line, HCT8, and also that AZT potentiates 5-FU activities. However, they did not sufficiently elucidate the biochemical mechanisms involved. No examination of cell lines from human gastric cancer has yet been performed, so the present study was designed to elucidate the action mechanism of this combination chemotherapy on a gastric cancer cell line. We also assessed the ability of AZT to potentiate the antitumor activity of 5-FU, and

found evidence suggesting a synergetic effect of AZT on 5-FU by evaluation of pyrimidine biosynthesis in nude mice bearing transplanted human gastric cancer MKN28 cells. These results may open the way to the establishment of a new chemotherapeutic modality.

MATERIALS AND METHODS

Animals and tumor Six-week-old female BALB/c nude mice provided by Clea Japan, Ltd. (Tokyo), were used in this study. The human gastric cancer cell line MKN28, donated by the Japanese Cancer Research Resources Bank (Tokyo) was transplanted (10^6 cells/mouse) subcutaneously in the axillary region of nude mice. After 8 weeks of tumor growth (mean body weight of mice 22.8 g, mean tumor weight 0.75 g), mice were divided into groups of 15 mice each, and the following chemotherapy schedule was used.

Chemotherapy schedule 5-FU was provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo), and AZT was purchased from Sigma Chemical Co. (St. Louis, MO). 5-FU at 15 mg/kg/day and AZT at 200 mg/kg/day were administered by i.p.-bolus for 5 consecutive days/week for 2 weeks. In the control group, saline was injected i.p.-bolus, instead of 5-FU or AZT. In the 5-FU plus AZT

group, AZT was administered 2 h after 5-FU administration to mice. Thereafter, the animals were observed for 5 weeks.

Determination of dosages of 5-FU and AZT In a preliminary test, 5-FU was administered to the mice at a dose of 30 mg/kg, 20 mg/kg or 15 mg/kg for 5 consecutive days per week for 2 weeks (data are not shown). Since marked body weight loss and treatment-related death were observed at doses of 30 mg/kg and 20 mg/kg during the test, we chose a dose of 15 mg/kg that caused a slight body weight loss of less than 10% in this study. We divided the administration of 5-FU for 5 consecutive days per week for 2 weeks in order to diminish the adverse effects and secure a good combination effect with AZT, which is active for a short period.[5] The dose of 200 mg/kg of AZT was the same as that employed in the experiment by Brunetti *et al.*[4]

Evaluation of antitumor effect The antitumor effect was assessed by measuring the change in % tumor volume, which was calculated using the following formulas:

Tumor volume=long diameter (a) ×short diameter (b)²/2

% tumor volume=posttreatment tumor volume/pretreatment tumor volume×100.

Measurement of enzyme activity and 5-FU content in tumor Tumors were removed from mice (enzyme activity, $n=5$/group; 5-FU content in tumor, $n=4$/group) at 4 h after the last administration of 5-FU in the first course in the 5-FU group and at 2 h after the last administration of AZT in the first course in both the AZT group and the 5-FU plus AZT group. They were stored frozen at $-80°C$ until measurement of biochemical parameters.

TS activity (total TS, free TS, TS inhibition rate(TSIR)) Cytosolic 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) and [³H]-FdUMP binding sites were measured by the method of Spears *et al.*[6] with some modifications. A tissue sample (500 mg) was minced with scissors, homogenized, and sonicated at 4°C in 2 ml of 50 mM KH₂PO₄ buffer, pH 7.4, containing 20 mM 2-mercaptoethanol, 100 mM NaF, and 15 mM cytidylate. The cytosol was then prepared by centrifugation. Total TS was assayed after dissociating FdUMP-bound TS (bound TS) to non-FdUMP bound TS (free TS) by 3 h preincubation at 25°C, using a 1 : 1 dilution of the cytosol with 300 mM NH₄CO₃ buffer, pH 8.1, containing 100 mM 2-mercaptoethanol, 100 mM NaF, and 15 mM cytidylate. Total TS was then determined in the presence of 6 pmol of [³H]FdUMP, 140 mM FH₄, 2.3 mM sodium ascorbate, 1.3 mM CH₂O, 0.29% bovine serum albumin, 37.1 mM 2-mercaptoethanol, 10.7 mM cytidylate, and 71.4 mM NaF for 20 min at 25°C by isolation of protein-bound radioactivity after the addition of one volume of

3.3% dextran-coated charcoal (in 0.1 N HCl at 4°C). Using the apparent free cytosol diluted with the NH₄CO₃ buffer which was not subjected to the 3 h preincubation, true free TS was then calculated by assuming that 13% of any TS bound becomes labeled with [³H]FdUMP during a 20 min assay period due to dissociation of endogenous unlabeled FdUMP. Percentage TSIR was obtained by use of the formula (1−free TS/total TS)× 100.

Bound TS, which was calculated as total TS minus free TS, was assessed for these groups, as an index of intracellular FdUMP.

Thymidine kinase (TK) activity Measurement of TK activity was performed using Taylor's method.[7] The tumor was placed in an enzyme extraction solution (1 : 5, w/v) (5 mM Tris, 0.1 mM EDTA, 1 mM 2-mercaptoethanol and 250 mM sucrose, adjusted to pH 7.5) and homogenized in ice. After ultracentrifugation at 105,000g at 4°C for 60 min, the supernatant was stored frozen at $-80°C$. A 198 μl portion of enzyme reaction solution (5 mM MgCl₂, 10 mM APT, 0.1 M Tris HCl buffer and 0.05 M Tris HCl, pH 8) and 2 μl of [³H]-thymidine were mixed, incubated at 37°C for 30 min, and preincubated with 100 μl of the enzyme extraction solution at 37°C for 15 min. The reaction was terminated by placing the reaction solution in a hot water bath at 100°C for 3 min. The reaction solution was separated by ultracentrifugation at 14,000g at room temperature for 15 min. TK was extracted on DEAE cellulose filter paper and counted in a scintillation counter.

5-FU content assay Tissue 5-FU content[8] was measured by high-performance liquid chromatography (HPLC). A 200 mg portion of tissue was homogenized in 3 ml of chilled CH₃CN with 100 mg of SiO₂. The emulsion plus 2 ml of CH₃CN was centrifuged at 3,000 rpm for 10 min. The supernatant was evaporated *in vacuo*. The residue was dissolved in 200 ml of EtOH and charged on a silica gel column. The column was rinsed with 0.5 ml of acetone, and the washes were discarded. 5-FU was eluted from the column with 2 ml of acetone. The eluent was evaporated *in vacuo*, and the residue was redissolved in 2 ml of mobile phase, n-C₆H₁₄ (AcOEt : n-C₂H₁₄ : 88% HCO₂H : H₂O=60 : 40 : 0.5 : 0.2) (3 : 2).

Uptake ratio of 5-bromo-2'-deoxyuridine (BrdU) Competitive inhibition of the salvage pathway of pyrimidine by AZT was assessed by administration of BrdU[9, 10] to mice. In the 5-FU group, BrdU at 30 mg/kg (i.p.-bolus) was administered 3 h after 5-FU administration, and in the AZT group and 5-FU plus AZT group, BrdU at 30 mg/kg (i.p.-bolus) was administered 1 h after AZT administration. Tumors were removed from mice ($n=4$/group) at 1 h after BrdU administration. The specimens were then fixed with 4% formaldehyde for 7 days, embedded in paraffin and mounted on slides for immuno-

histochemical and routine pathological examinations. The endogenous peroxidase and nonspecific binding were blocked with methanol containing 0.3% hydrogen peroxide in phosphate-buffered saline (PBS). The tissue sections were denatured for 1 h in 2 N hydrochloric acid and then rinsed extensively with PBS, pH 7.4. The sections were incubated with a 1 : 50 dilution of purified anti-BrdU monoclonal antibody (mAb) (Becton Dickinson Co., CA) in PBS containing 1% BSA and 0.1% NaN₃, and were then left at room temperature for 1 h. The slides were washed, then treated with a 1 : 20 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO, Denmark) and left at room temperature for 30 min. Control slides were stained with a mouse immunoglobulin, G2 (DAKO), instead of the anti-BrdU mAb. Finally, the slides were lightly counterstained with 10% hematoxylin. Over 1,000 cells were counted 3 times, in each section, and the uptake ratio (%) of BrdU was calculated.

Assessment of adverse drug effect As an index of adverse drug reaction (toxicity), % body weight was calculated using the following formula: % body weight = posttreatment body weight/pretreatment body weight × 100. Each group ($n = 6$) was observed for 5 weeks.

Statistical analysis The statistical significance of intergroup differences in antitumor effect, body weight loss, enzyme activity and enzyme inhibitory rate was assessed by use of the χ^2 test and t test. P values of less than 5% were considered statistically significant.

RESULTS

Inhibition of tumor growth Tumor growth was inhibited in both the 5-FU group and the 5-FU plus AZT group. In the 5-FU plus AZT group, the % tumor volume decreased below that measured before treatment. On Day 21, when the reduction in % tumor volume was maximum in the 5-FU plus AZT group, %tumor volume was 203±22% in the control group, 153±45% in the 5-FU group, 180±40% in the AZT group, and 82±9% in the

5-FU plus AZT group. Thus, tumor growth was significantly inhibited in both the 5-FU and 5-FU plus AZT groups ($P < 0.05$, $P < 0.01$, versus control, respectively). The inhibition of tumor growth in the 5-FU plus AZT group was significantly greater than that in the group given 5-FU alone ($P < 0.05$) (Fig. 1).

Effect on pyrimidine biosynthesis TSIR (%) was significantly higher at 47.3±3.7 in the 5-FU group and at 48.3 ±6.3 in the 5-FU plus AZT group than in the other groups ($P < 0.01$, versus control), but TSIR was unchanged in the AZT group. Total TS (pmol/g protein) was 10.3±2.7 in the control group, 13.7±1.8 in the 5-FU group, 9.4±3.3 in the AZT group, and 16.1±3.2 in the 5-FU plus AZT group. Compared with that in the control group, total TS was significantly higher in the 5-FU group and the 5-FU plus AZT group ($P < 0.05$), but it did not differ from the control in the AZT group (Table I). Bound TS (pmol/g protein) was 0.2±0.1 in the control group, 6.5±0.9 in the 5-FU group, 0.1±0.1 in
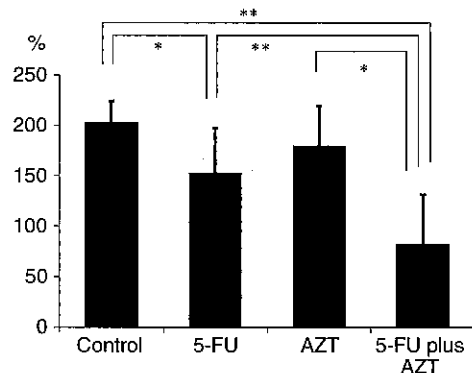

Fig. 1. Percent tumor volume after the 21st day in each group ($n = 6$) (mean±SD). The greatest reduction in percent tumor volume was observed in the 5-FU plus AZT group. 5-FU, 5-fluorouracil; AZT, 3'-azido-3'-deoxythymidine. * $P <$ 0.05 between the indicated groups. ** $P < 0.01$ between the indicated groups.

Table I. Total TS, Bound TS, TSIR and 5-FU Content in Tumor

	Total TS (pmol/g protein)	Bound TS (pmol/g protein)	TSIR (%)	5-FU content in tumor (ng/g tissue)
Control	10.3±2.7	0.2±0.1	1.4±0.9	0±0
5-FU	13.7±1.8	6.5±0.9	47.3±3.7	9±2
AZT	9.4±3.3	0.1±0.1	1.4±0.9	0±0
5-FU plus AZT	16.1±3.2	7.7±1.2	48.3±6.3	15±7
n	5	5	5	4

TS, thymidylate synthase; TSIR, thymidylate synthase inhibition rate; 5-FU, 5-fluorouracil; AZT, 3'-azido-3'-deoxythymidine.
* $P < 0.05$. ** $P < 0.01$.

Table II. TK Activity and Uptake Ratio of BrdU

	Activity of TK (fmol/mg protein/min)	BrdU uptake ratio (%)
Control	86.3±36.4	7.5±1.3
5-FU	155.6±60.8	9.7±2.1
AZT	34.0±17.4	2.2±1.0
5-FU plus AZT	30.8±21.8	4.5±1.9

TK, thymidine kinase; BrdU, 5-bromo-2′-deoxyuridine; 5-FU, 5-fluorouracil; AZT, 3′-azido-3′-deoxythymidine. * $P<0.05$. ** $P<0.01$.

the AZT group and 7.7±1.2 in the 5-FU plus AZT group; it was significantly higher than the control in the 5-FU and 5-FU plus AZT groups ($P<0.01$). Bound TS was significantly higher in the 5-FU plus AZT group than in the 5-FU alone group ($P<0.05$) (Table I). TK activity (fmol/min/mg protein) in the salvage pathway was 86.3±36.4 in the control group, 155.6±60.8 in the 5-FU group, 34.0±17.4 in the AZT group, and 30.8±21.8 in the 5-FU plus AZT group. Thus, it was significantly higher than the control in the 5-FU group ($P<0.05$) and significantly reduced in the AZT and 5-FU plus AZT groups ($P<0.05$, $P<0.01$, respectively) (Table II).

5-FU content in tumor 5-FU content (ng/g tissue) in the tumor was 0±0 in the control and AZT groups, 9±2 in the 5-FU group, and 15±7 in the 5-FU plus AZT group. Thus, the 5-FU content in tumor was significantly higher in the 5-FU plus AZT group than in the 5-FU group ($P<0.05$) (Table I).

Uptake ratio of BrdU The uptake ratio (%) of BrdU was 7.5±1.3 in the control group, 9.7±2.1 in the 5-FU group, 2.2±1.0 in the AZT group and 4.5±1.9 in the 5-FU plus AZT group; thus, it was significantly less than the control in the AZT and 5-FU plus AZT groups ($P<0.01$, $P<0.05$, respectively). A tendency toward increase was noted in the 5-FU group compared with the control (Table II).

Assessment of adverse drug effect In a comparison of toxicity among groups on Day 11, when the lowest % body weight was observed in the 5-FU plus AZT group, % body weight was 103±4% in the control group, 96±3% in the 5-FU group, 98±2% in the AZT group, and 95±4% in the 5-FU plus AZT group (mean±SD). There was significant weight loss in the 5-FU and 5-FU plus AZT groups ($P<0.05$, versus control), but the percentage losses were low, less than 10%, in both groups.

DISCUSSION

In recent years, biochemical modulation of the most commonly used chemotherapeutic agent for solid tumors, 5-FU, has been performed by using CDDP,[11, 12]

LV[13, 14] and MTX[15, 16] as modulators. This potentiation of 5-FU cytotoxicity involves the enhanced inhibition of TS activity, which converts 2-deoxyuridine-5-monophosphate to thymidylate by methylation in the *de novo* pathway. However, when the *de novo* pathway is inhibited by the combination of 5-FU and modulators, pyrimidine synthesis via the salvage pathway might increase, thus decreasing the cytotoxicity of chemotherapy. Vogel *et al.*[17] have attempted to use thymidine clinically as a modulator of 5-FU. Although the excess amount of thymidine in salvage synthesis did exert a strong antitumor effect, it increased the incidence of neutropenia due to myelosuppression, and consequently the effect of thymidine on the salvage pathway could not be fully explored. Grem and Fischer[18] reported that the combination of 5-FU and dipyridamole, which blocks nucleoside transport on the cell membrane, had an increased antitumor effect, owing to blockage of the salvage pathway. AZT was found to have anti-AIDS activity,[19] and also to inhibit TK activity in human lymphocytes, and was further found to reduce deoxythymidine triphosphate in the intracellular pool.[20] Brunetti *et al.*[4] reported that the combination of 5-FU and AZT was more cytotoxic than 5-FU or AZT alone against a human colon cancer cell line in *in vitro* and *in vivo* experiments. Experimental studies on the 5-FU and AZT combination have subsequently been reported for ovarian and pancreatic cancers.[21] However, the precise mechanism of the enhanced cytotoxicity of the combination of 5-FU and AZT has not yet been elucidated.

We therefore performed the present study with a gastric cancer cell line, since the fundamental chemotherapeutic agent, 5-FU is most commonly used against this cancer. Despite a slight inhibitory effect of 5-FU and no effect of AZT on tumor growth, the finding of definite inhibition of tumor growth with combined use of 5-FU and AZT strongly suggests that potentiation of the antitumor effect and cytotoxicity of 5-FU can be induced by this combination at doses which have little cytotoxicity, separately.

To elucidate the mechanism of enhanced cytotoxicity of this chemotherapy, we assessed the effect of chemotherapeutic agents in both the *de novo* and salvage pathways of pyrimidine biosynthesis, by measuring total TS, bound TS, TSIR and TK. The finding of no significant differences in increase of TSIR and increase of total TS activity between the 5-FU group and the 5-FU plus AZT group suggests that AZT has no direct effect on the TS inhibition by 5-FU. However, the finding that, compared with those in the 5-FU group, significantly higher values of 5-FU content in the tumor and of bound TS activity in the 5-FU plus AZT group suggests that uptake of 5-FU increased with enhanced uptake of orotic acid and that synthesis of FdUMP from 5-FU was increased via the *de*

novo pathway in compensation for the inhibition of TK activity by AZT. Besides its principal activity on salvage pyrimidine synthesis, AZT therefore induces enhanced uptake of 5-FU into tumor tissue and inhibits the *de novo* pathway by potentiating the activity of bound TS.

It is known that BrdU is incorporated into DNA via the salvage pathway,[22] because its incorporation is inhibited by AZT, a competitive inhibitor of the salvage pathway of pyrimidine, in mice. We will next discuss the pyrimidine salvage synthesis in terms of changes of TK activity and BrdU uptake ratio. The finding of a significant increase in TK activity and a tendency toward increase in BrdU uptake in the 5-FU group suggest that a compensatory increase of salvage occurs to counteract the depression of the *de novo* pathway in pyrimidine synthesis, and that this increased salvage lessens or eliminates the antitumor effect of 5-FU. In spite of the increase in the activity of TK by 5-FU, AZT markedly suppressed the TK activity. Decreased activity of TK in the 5-FU plus AZT group suggests that TK inhibition by AZT was induced through an allosteric effect[23] and canceled out the elevation of TK activity by 5-FU. The BrdU uptake ratios were correlated with TK activities in the 5-FU and 5-FU plus AZT groups, implying that combined use of AZT with 5-FU not only blocks the compensatory increase in salvage synthesis, but also suppresses the salvage pathway to the level of activity present with AZT alone. In addition, when a high dose of 5-FU is administered, inhibition of TK activity will probably occur as reported by Nord and Martin.[24] In our experiment, elevations of total TS and TK activities through protein synthesis were observed after the administration of 5-FU. Therefore, we consider that 5-FU does not affect RNA function and mainly inhibits DNA synthesis.

We conclude that AZT synergetically potentiates the cytotoxic and antitumor effects of 5-FU through enhancement of *de novo* inhibition and depression of the salvage pathway. The finding of loss of body weight of less than 10% over the course of chemotherapy suggests that combined use of 5-FU and AZT at appropriate doses of each has definite antitumor effects without undue adverse effects on the host.

ACKNOWLEDGMENTS

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