Tranilast strongly sensitizes pancreatic cancer cells to gemcitabine via decreasing protein expression of ribonucleotide reductase 1

MAYUMI MITSUNO, YOSHIHIKO KITAJIMA, KAZUMA OHTAKA, KEITA KAI, KAZUYOSHI HASHIGUCHI, JUN NAKAMURA, MASATSUGU HIRAKI, HIROKAZU NOSHIRO and KOHJI MIYAZAKI

Department of Surgery, Saga University Faculty of Medicine, Nabeshima 5-1-1, Saga 849-8501, Japan

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Abstract. Gemcitabine (Gem) is a dFdC analogue with activity against several solid tumors. Gem is intracellularly phosphorylated by dCK, leading to the production of the metabolite dFdCDP. dFdCDP exhibits the cytotoxic effect by inactivating ribonucleotide reductase larger subunit 1 (RRM1), which is a rate limiting enzyme for *de novo* DNA synthesis. To date, RRM1 expression is believed to determine sensitivity to Gem in pancreatic and non-small cell lung cancer. In the present study, we found that an anti-allergic drug, tranilast strongly enhanced the sensitivity of pancreatic cancer cell line KP4 to Gem. In growth inhibition assay, 100 μM of tranilast plus 1 μM of Gem more strongly suppressed the growth of KP4 at 12.7-fold in IC_{50} than single Gem treatment, while this compound no longer affected the sensitivity to other drugs such as 5-fluorouracil, irinotecan or paclitaxel. FACS and TUNEL analysis demonstrated the increased apoptotic population in KP4 cells under tranilast plus Gem, compared with single Gem treatment. In Western blot analysis, tranilast treatment decreased RRM1 expression at protein level with dose-dependency in KP4 cells. Proteasome inhibitor MG132 disturbed the reduction of RRM1 expression in tranilast treated KP4 cells, indicating protein degradation by the activated proteasome. Transfection using siRNA against RRM1 increased the sensitivity of KP4 to Gem, suggesting that RRM1 suppression is an important step in increasing Gem efficacy. Finally, we demonstrated that tranilast reduced RRM1 protein and increased Gem efficacy in 4 other pancreatic cell lines. In a future, a novel chemotherapeutic strategy by Gem along with tranilast might improve Gem efficacy against pancreatic cancer.

Correspondence to: Dr Yoshihiko Kitajima, Department of Surgery, Saga University Faculty of Medicine, Nabeshima 5-1-1, Saga 849-8501, Japan
E-mail: kitajiy@cc.saga-u.ac.jp

Abbreviations: dFdC, 2',2'-difluorodeoxycytidine; Gem, gemcitabine; dCK, deoxycytidine kinase; dFdCDP, 5'-diphosphate nucleotide; dFdCTP, 5'-triphosphate nucleotide; RRM1, ribonucleotide reductase larger subunit 1

Key words: trainlast, gemcitabine, pancreatic cancer, ribonucleotide reductase larger subunit 1, chemo-sensitivity

Introduction

Pancreatic adenocarcinoma is one of the most lethal diseases and a frequent cause of cancer related death (1,2). Most patients with pancreas cancer have a poor outcome due to difficulty in its early diagnosis, its highly invasive with metastatic features. In patients with pancreatic cancer, the overall survival is less than 6 month after diagnosis (3) and a disease recurrence is up to 80% of the resected tumors within 2 years (4). The 5-year survival rate of patients who underwent surgical resection remains at only 15-20% (5,6).

Effective chemotherapy is thus important in order to prolong the survival of pancreatic cancer patients, especially in non-resectable or non-curative resection cases. To date, gemcitabine (Gem), (2',2'-difluorodeoxycytidine:dFdC) has been accepted as the standard treatment for advanced pancreatic cancer, since a randomized study by Burris *et al* in 1997 demonstrated that Gem treatment had a survival benefit, compared with fluorouracil (7). However, the median survival of patients with advanced pancreatic cancer treated by Gem is still about 6 months (7-9), indicating the pressing need for development of effective chemotherapy. Gem exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis. Gem is metabolized intracellularly by deoxycytidine kinase to 5'-diphosphate (dFdCDP) and 5'-triphosphate (dFdCTP) nucleotides. The cytotoxic effect is attributed to a combination of two actions performed by those two metabolites. First, dFdCDP inhibits ribonucleotide reductase larger subunit (RRM1), which is responsible for catalyzing the reaction that generates the deoxyribonucleotides required for DNA synthesis and repair; inhibition of ribonucleotide reductase (RNR) causes a reduction in the cellular concentration of the four DNA monomers. Second, dFdCTP competes with the natural deoxycytidine 5'-triphosphate (dCTP) for incorporation into the replicating DNA; once one molecule of dFdCTP is incorporated, an additional deoxyribonucleotide is added to the growing DNA strands, thereafter, DNA synthesis can no longer proceed (9,10). Especially, several *in vitro* and *in vivo* studies demonstrated the involvement of RRM1 overexpression in dFdC resistance of pancreatic as well as non-small cell lung cancer, indicating that RRM1 expression is a major predictor for Gem treatment (11-16). If some agent

decreases RRM1 expression, it is promising that the pancreatic cancer responds more strongly to Gem.

Tranilast, N-(3',4'-dimethoxycinnamoyl) anthranilic acid, has been used clinically for more than 20 years in Japan (17). This drug is taken orally and exerts the drug effect against allergic diseases such as bronchial asthma, allergic rhinitis, atopic dermatitis and allergic conjunctivitis (17,18). It has been reported that this compound is also effective in disease with excessive fibrosis such as keloid, because it inhibits fibroblast proliferation leading to the suppression of collagen accumulation (19-25). Recently, several reports showed that tranilast inhibits the proliferation of several cancer cell lines (26-29). However, no study has addressed the effect of tranilast on sensitivity to anticancer drugs in pancreatic cancer.

In the present study, we first evaluated whether or not tranilast alters the sensitivity of a pancreatic cancer cell line KP4 to the anticancer agents Gem, 5-fluorouracil (5-FU), cisplatin (CDDP), irinotecan (CPT-11) and micro-tubule inhibitor, paclitaxel (PTX). The result revealed a novel finding that tranilast strongly increased Gem efficacy in this cell line. We thus attempted to clarify the mechanism of the sensitizing effect of tranilast on Gem efficacy. We herein present an *in vitro* model of a novel strategy using tranilast plus Gem for the treatment of pancreatic cancer.

Materials and methods

Cell line and culture. KP4, PK-8, PK-9, PK-1 and PK-59 are human pancreas cancer cell lines. KP4 and PK-59 were purchased from the RIKEN Cell Bank (Ibaragi, Japan). PK-8, PK-9 and PK-1 were purchased from the institute of development, Aging and Cancer, Tohoku University (Sendai, Japan). All cell lines were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA) and 100 μg/ml kanamycin (Meiji, Tokyo, Japan). These cell lines were grown at 37˚C in a humidified atmosphere containing 20% O_2 and 5% CO_2 in air.

Reagents. Tranilast, N-(3,4-dimethoxycinnamoyl)-anthranilic acid (MW 327.24) was a generous gift of Kissei Pharmaceutical Co., Ltd. (Nagano, Japan). Powder of tranilast was dissolved in N-,N-dimethylformamide and diluted with medium to final concentration before use. Gem was kindly supplied by Eli Lilly Pharmaceuticals (IN, USA). 5-Fluorouracil (5-FU) was purchased from Kyowa Hakko Pharmaceutical Co., Ltd. (Tokyo, Japan), cisplatin (CDDP) from Nippon Kayaku Co., Ltd. (Tokyo, Japan) and paclitaxel (PTX) from Bristol-Myers K.K. (NY, USA). Irinotecan (CPT-11) was supplied from Yakult Co., Ltd. (Tokyo, Japan) and 10 mM of stock solution was prepared in DMSO and diluted in medium at the approximate dose immediately before use. MG132, a specific proteasome inhibitor, was purchased from Sigma and prepared as a 1-mM stock in DMSO, and stored at -20˚C until use.

Analysis of drug sensitivity. Sensitivity to various drugs was analyzed by the MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay using a Cell Titer96 Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA).

KP4 was seeded in 96-well culture plates each $1x10^4$ cell/well and incubated at 37˚C in a humidified atmosphere overnight. Then the cells were exposed to tranilast for 48 h, at a concentration ranging from 0 to 400 μM. After 48 h, 20 μl of MTT was added to each well, after 4 h the reaction was stopped by addition of a solubilization/stop solution. Absorbance at 590 nm was measured using multiwell plate reader (Immuno-Mini, NJ-2300, Tokyo, Japan). All experiments were repeated three times. Every experiment was done in triplicate and data are presented as a mean ± SD value of the 3 individual experiments.

The sensitivity of KP4 to various anticancer drugs under the tranilast treatment was analyzed by MTT assay. KP4 was seeded in 96-well culture plates each $1x10^4$ cell/well. After 24 h, the cells were exposed to tranilast (concentration ranging from 0-100 μM) and each drugs for 48 h, at concentrations 0-100 μM for Gem, 0-100 μM for CDDP, 0-1 mM for 5-FU, 0-100 μM for CPT-11 and 0-1 μM for PTX.

Analysis of BrdU uptake. Uptake of bromodeoxyuridine (BrdU) in KP4 cells was performed according to the protocol of CycLex® Cellular BrdU ELISA kit (Cyclex Co., Ltd., Nagoya, Japan). Briefly, KP4 was seeded in 96-well culture plates $8x10^3$ cells/well. After 24 h, the cells were exposed to tranilast at 0, 100, 200, 300, 400 and 500 μM concentration for 48 h. BrdU was then added at final concentration of 10 μM and further incubated for 2 h. The cultured cells were treated by fixing/denaturating solution. The cells were treated by anti-BrdU antibody for 1 h, followed by HRP-conjugated anti-mouse IgG. Finally, color development by substrate reagent was measured on the micro plate reader at 450 nm (Immuno-Mini NJ-2300, Nalge Nunc International Corp., USA).

Reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from the cell lines using an Isogen® RNA extraction kit (Nippongene, Toyama, Japan). RT-PCR was carried out using the RNA LA PCR kit (AMV) Version 1.1 (Takara Biochemicals, Shiga, Japan) as previously described (30). The PCR condition following RT reaction were as follows: initial denaturation at 94˚C for 2 min followed by 30 cycles of 94˚C for 30 sec, 60˚C ribonucleotide reductase subunit 1 (RRM1) or 55˚C (ß-actin) for 30 sec and 72˚C for 60 sec. PCR for ß-actin served as an internal standard. The following sense and antisense primers were used: RRM1 primers (5'-GGAGGAATTGGTGTTGCTGT-3' and 5'-GCT GCTCTTCCTTTCCTGTG-3'), ß-actin primers (5'-TTAAGG AGAAGCTGTGCTACG-3' and 5'-TAGGTGCTTTGATGG AAGTTG-3', respectively). The proposed sizes of PCR products were 235 bp for RRM1 and 208 bp for ß-actin.

Western blot analysis. Pancreas cancer cell lines were seeded in 6-well culture plates at $2x10^5$ cells/well. After 24 h, the cells were exposed to tranilast (concentration range: 0-200 μM). Forty-eight hours later, cells were lysed in lysis buffer [150 mM NaNl, 50 mM Tris-HCl (pH 7.6)], 0.5% Triton X-100 and a protease inhibitor cocktail mix (Roche Diagnostic GmbH, Mannheim, Germany). Twenty micro-

grams of protein lysate was subjected to Western blot analysis as previously described (30). The primary antibodies used were mouse anti-rebonucleotide reductase monoclonal antibody (MAB3033, 1:500 dilution; Chemicon) and monoclonal anti-ß-actin (Clone AC-15, 1:10000 dilution; Sigma). After incubation with the corresponding secondary antibodies, the signals were developed using an ECL™ Plus Western blotting detection system (Amersham). For quantitative analysis, band intensities were assessed densitometrically using the LAS 3000 plus system (Fujifilm, Tokyo, Japan).

Cell cycle analysis. KP4 was seeded in 6-well culture plates $2x10^5$ cells/well. After 24 h, the cells were exposed to tranilast at 0, 100, 250 and 500 μM concentration, with or without 1 μM Gem for 48 h. For cell-cycle analysis, KP4 cells were harvested using 0.05% trypsin - 0.02% EDTA, washed with PBS and fixed in 70% ice-cold ethanol, stored at -20˚C. Later, the ethanol was removed, and the cells were stained with propidium iodide (PI) solution (50 μg/ml in PBS with 100 U/ml RNase A) (Qiagen GmbH, Hilden) for 30 min at room temperature in the dark. PI-positive nuclei were analyzed using FACS/Calibur Flow Cytometer (counting 10000 cells per sample) and Cell Quest software Version 2.0 (Becton-Dickinson Co., NJ, USA). Cell population at subG1, G1, S, G2/M phase in each experiment was estimated.

TUNEL assay. KP4 cells were seeded onto Lab-Tec II Chamber Slide™ System (Nalge Nunc International Corp., USA) $2x10^4$ cells/well and cultured for 24 h, The cells were then exposed to tranilast at 0, 100 and 250 μM with or without 1 μM Gem for 48 h. Apoptotic cells were detected using an Apoptosis detection TACS™ TdT kit (R&D, Minneapolis, MN, USA). Cells were stained for detecting apoptotic cells according to manufacturer's instructions. Cells developing brown staining in the nuclei were assessed as TUNEL positive cells. The positive cells were counted under the light microscope at x200 magnification in 3 fields. Apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/total number of cells) x 100% and the mean AI with standard deviation (SD) in 3 fields were determined.

Transient transfection of RRM1 siRNA (ds RNA). For Western blot analysis, $2x10^5$ cells of KP4 were seeded onto 6-well plates and incubated overnight. Twenty-one base pairs (bps) of double-strand (ds) RNA for RRM1, which were constructed by iGENE, Co., Ltd. (Tsukuba, Japan), was transiently transfected at 1 and 10 nM concentrations into KP4 cell for 4 h using a Oligofectamine (Life Technology, Inc., Japan). Randomly constructed dsRNA with a 21-bp length was used as a control (scramble) transfection. At 48 h after transfection, the protein was extracted from the cell using a lysis buffer [1% Triton X-100, 50 mM Tris, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The lysate was collected by centrifugation at 15000 rpm for 20 min and subjected to Western blot analysis.

Effect of RRM1 siRNA on sensitivity to Gem or CDDP was analyzed as follows: $1x10^4$ of KP4 cells were seeded onto 6-well plates. dsRNA for RRM1 was transfected in duplicate at a final concentration of 1 or 10 nM. Control (scramble) transfection was also done at 10 nM. At 24 h after transfection, the cells were exposed under Gem or CDDP at 5 μM concentration for 48 h. The cells were trypsinized and counted under trypan blue staining. Growth inhibition under 5 μM Gem or CDDP treatment was estimated in each transfection and the inhibition rate was compared between control dsRNA transfection and RRM1 siRNA.

RRM1 ds RNA design: sense 5'-GAGGAAGAAGAGA AGGAGAGGAACAAG-3' and antisense: UACUCCUUC UUCUCUUCCUCUCCUUGU-5' (iGENE, Co., Ltd.). These experiments were repeated at lease 3 times.

Inhibition of proteasome action by MG132 treatment. KP4 was seeded in 6-well culture plates at $2x10^5$ cells/well. After 24 h, 2 μM MG132 was added to the culture medium 1 h prior to tranilast (0, 100, 200 μM) treatment for 48 h. RRM1 protein levels were determined by Western blot analysis using the total cell lysate.

Statistical analysis. Data are shown as the mean ± standard deviation. The computer software Stat view for Macintosh (Abracus Concepts, Berkely, CA) was used for all statistical analysis. Comparison of means was carried out using the independent t-tests. A p-value of <0.05 was considered statistically significant. Bonferoni's correction for multiple testing was performed to determine the p-value with the number of tests.

Results

The effect of tranilast treatment on cell growth of KP4 cells. To evaluate whether or not tranilast inhibits cell growth of KP4, the cells were cultured for 48 h with tranilast at various concentrations (range, 0-400 μM). As the result, cell proliferation of KP4 was significantly reduced beginning at 200 μM tranilast, while the cell growth was not affected <100 μM (Fig. 1A).

Analysis of BrdU uptake was further examined to clarify the effect of tranilast on DNA synthesis in KP4 cell (Fig. 1B). As a result, uptake of BrdU was inhibited by tranilast dose-dependently. BrdU uptake was significantly decreased at >100 μM of tranilast, compared with no treatment (Fig. 1B).

The effect of tranilast on sensitivity to various anticancer drugs in KP4 cells. KP4 was exposed to various concentrations of anticancer drugs in the presence or absence of tranilast at 0-100 μM concentration. The tranilast drug sensitivity to Gem, CDDP, 5-FU, CPT-11 and PTX was assessed by MTT assay. As shown in Fig. 2A, the responsiveness of KP4 to Gem was increased with the tranilast treatment in a dose-dependent manner. Sensitivity to CDDP was also increased by tranilast less dose-dependently, while that to 5-FU, CPT-11 or PTX was no longer affected by tranilast (Fig. 2A). As shown in Fig. 2B, cell viability of KP4 was significantly decreased at 50 or 100 μM tranilast plus 1 μM Gem treatment, compared with the single Gem treatment (upper panel in Fig. 2B). The cell viability was also decreased with statistical significance in tranilast plus 10 μM CDDP, in comparison to single CDDP treatment (lower panel in Fig. 2B).

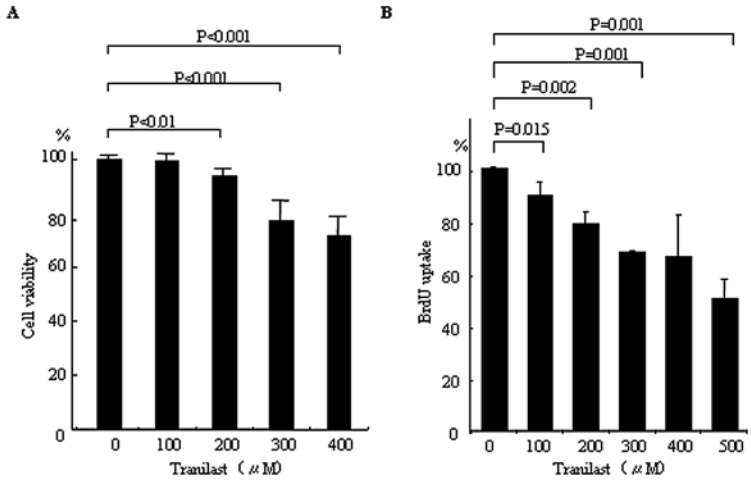

Figure 1. (A) Cell proliferation of KP4 with tranilast at various concentrations. Absorbance at 590 nm with no tranilast treatment was estimated as 100% viable in KP4 cell. Cell proliferation was inhibited by tranilast in a dose-dependent manner. (B) Uptake of BrdU in KP4 cells with treatment by tranilast at various concentrations. BrdU uptake was significantly reduced at >100 μM, compared with no treatment.

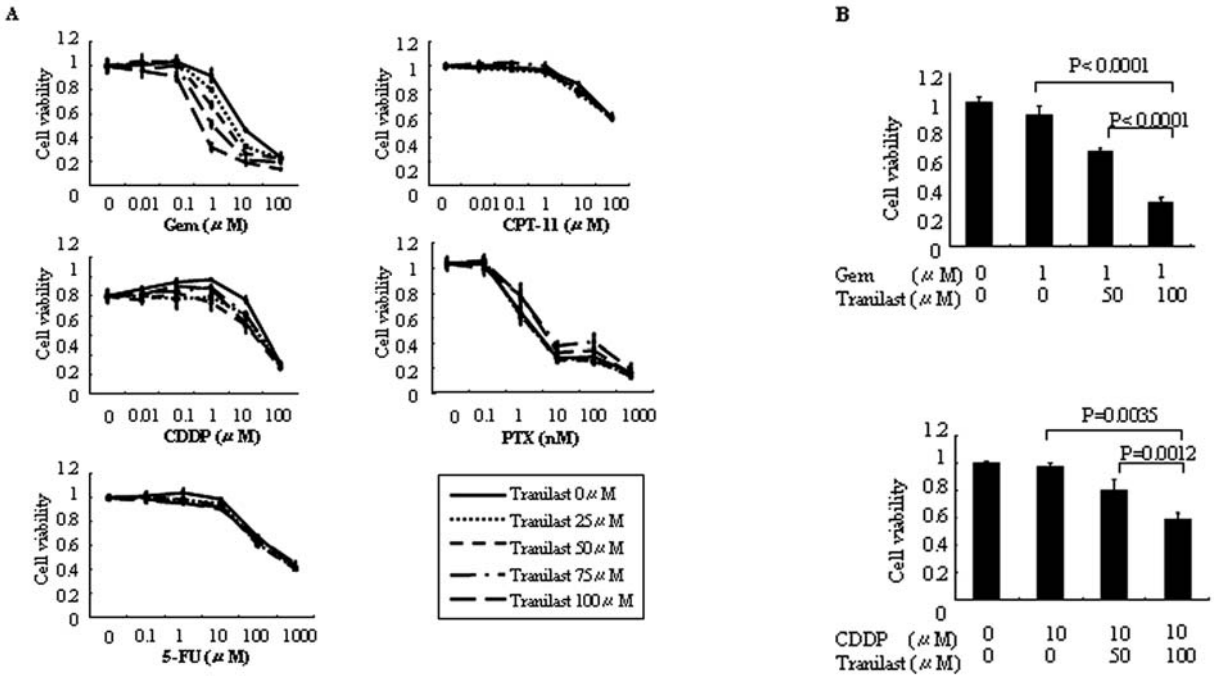

Figure 2. (A) Effects of tranilast on the sensitivity of KP4 to the anticancer drugs gemcitabine (Gem), cisplatin (CDDP), 5-fluorouracil (5-FU), irinotecan (CPT-11) and paclitaxel (PTX) were assessed in MTT assay. (B) In Gem and CDDP treatment, cell viability on the log phase of the graph (A) is represented in the histogram; the drug concentrations of Gem and CDDP were determined at 1 and 10 μM, respectively. Tranilast concentration was chosen at 0, 50 and 100 μM. Statistical analysis by Boneferoni's correction for multiple testing at p<0.0083 was statistically significant. NS, not significant.

Expression of RRM1 mRNA and protein with tranilast treatment. In order to evaluate whether or not tranilast affects RRM1 mRNA or protein level, RT-PCR and Western blot analysis were performed. The expression of RRM1 mRNA was not influenced by tranilast treatment up to 200 μM concentration (Fig. 3A). In contrast, RRM1 protein level was dramatically diminished by tranilast treatment for 48 h (Fig. 3B). In the quantitative assessment, RRM1 protein level was decreased 56% at 50 μM, 22% at 100 μM and 20% at 200 μM of tranilast, compared to 100% with no treatment (Fig. 3C).

Flow cytometric analysis of cells treated with tranilast and gemcitabine. Flow cytometric analysis was examined to analyze the effect of tranilast or Gem plus tranilast on the cell cycle of KP4 cells. Proportion of cell population at subG1, G1, S, G2/M phase in cell cycle is summarized in Table I. Tranilast treatment at 100 μM did not affect the cell

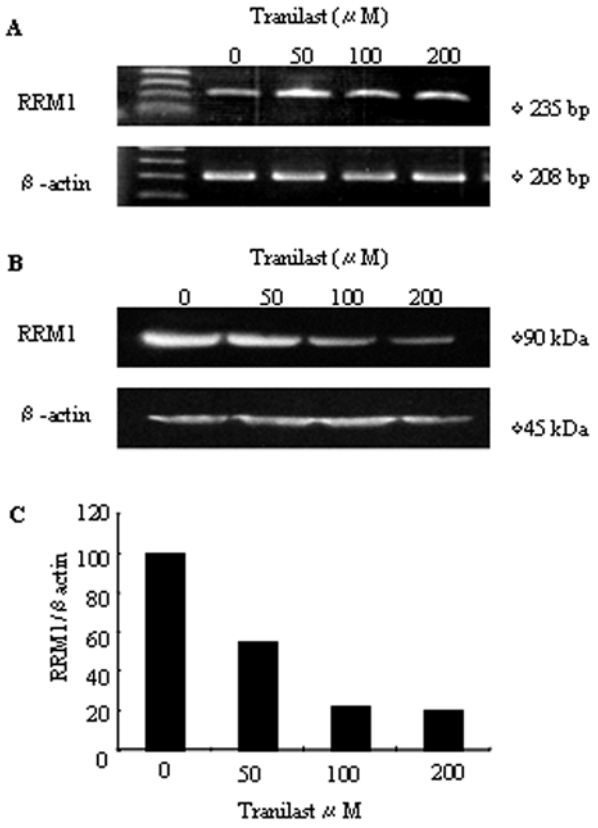

Figure 3. RRM1 expression in KP4 pancreatic cancer cell line with tranilast treatment. (A) RRM1 mRNA expression by RT-PCR analysis. The RRM1 mRNA expression level was not influenced by tranilast at 50, 100 and 200 μM concentrations. (B) RRM1 protein and control ß-actin expressions by Western blot analysis. (C) Relative expression of RRM1 protein was analyzed by densitometry. Relative intensity of RRM1 expression was calculated by the normalization of ß-actin expression and shown in the histogram. No treatment of tranilast was considered as 100%.

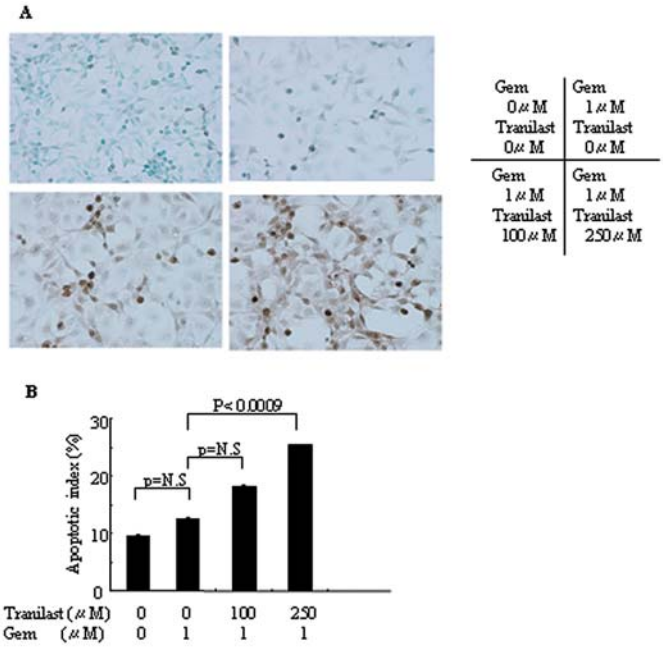

Figure 4. (A) KP4 cells treated by 0, 100 and 250 μM of tranilast with or without 1 μM Gem are shown. Apoptotic cells with nucleus stained brown were increased at treatment with 100 or 250 μM of tranilast and 1 μM of Gem. (B) Histogram of mean AI in the combination treatment of tranilast and dFdC. Mean AI is shown above each treatment. There was statistically significant difference in apoptotic index between the treatment of 1 μM Gem alone and treatment of 1 μM Gem plus tranilast at 250 μM concentration.

Table I. Cell cycle analysis of KP4 with the treatment by tranilast and Gem.

Tranilast (μM)	0	100	250	500	0	100	250
Gem (μM)	0	0	0	0	1	1	1
SubG1 (%)	3.0	3.7	3.4	4.2	15.9	31.3	39.2
G1 (%)	74.0	73.0	79.3	89.5	70.2	55.1	47.8
S (%)	5.0	5.1	4.8	3.0	2.5	2.5	2.4
G2/M (%)	18.0	18.2	12.5	3.3	11.4	11.1	10.6

population at G1 phase (73.0%), compared with no treatment (74.0%). However, higher dose of tranilast at 250 and 500 μM increased cell proportion at G1 phase up to 79.3 and 89.5%, respectively.

On the other hand, with Gem plus tranilast, 1 μM Gem along with 100 or 250 μM of tranilast increased the percentage of sub-G1 phase up to 31.3 and 39.2% in KP4 cells, compared with 15.9% in Gem treatment alone.

TUNEL assay. Population of apoptotic cells exposed with tranilast plus Gem were estimated by TUNEL assay as shown in Fig. 4A. Number of brown color stained nuclei was counted and the apoptotic index was estimated. Mean apoptotic index (AI) was increased up to 18.3% in 1 μM Gem plus 100 μM tranilast and 25.4% in 1 μM Gem plus 250 μM tranilast, compared with 9% in no treatment and 12% in 1 μM Gem (Fig. 4B). Mean AI was significantly higher in the combination treatment of 1 μM Gem plus 250 μM tranilast, compared with 1 μM Gem treatment.

Down-regulation of RRM1 by siRNA transfection. In order to analyze the direct effect of RRM1 on Gem sensitivity, siRNA (dsRNA) against RRM1 gene was transiently transfected into KP4 cells and cell viability under Gem treatment in RRM1 siRNA transfectant was compared with that in the control siRNA transfectant. At 48 h after the transfection, RRM1 protein expression was repressed by RRM1 siRNA transfection, compared with control siRNA (Fig. 5A). Using these transfectants, viable cells with or without Gem treatment was counted. In the transfection by 10 nM control siRNA, viable cells were decreased to 52.9% at 5 μM of Gem. Cell viability with Gem treatment was further reduced 36.7 and 25.5% by RRM1 siRNA transfection at 1 and 10 nM concentration, respectively (Fig. 5B). There was statistically significant difference in cell viability between 10 nM of control siRNA and 10 nM of RRM1 siRNA transfectant with the treatment of Gem (p=0.0014). Also in the CDDP treatment, RRM1 siRNA transfection decreased more strongly the viable cells treated with 5 μM CDDP, compared with the control siRNA transfection. However, there was no significant

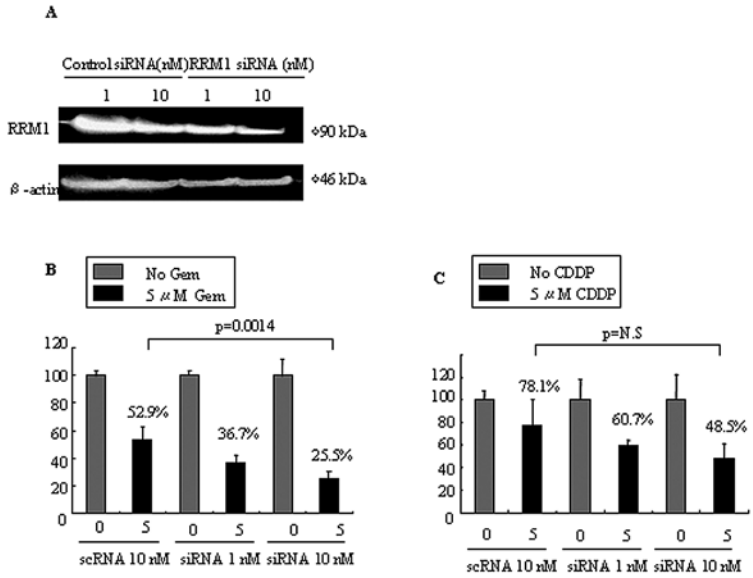

Figure 5. (A) Western blot analysis of RRM1 expression in KP4 cells transfected by siRNA against RRM1 or scRNA (control) at each 1 and 10 nM concentration. (B) Effect of RRM1 siRNA transfection on cell growth with or without Gem treatment. The percentage of cell number under 5 μM Gem treatment was estimated as the cell number with no treatment was considered 100% in each siRNA transfection. The survival percentages are given above the black bar. Cell viability of 10 nM RRM1 siRNA transfectant under Gem treatment was significantly lower than that of 10 nM scRNA transfectant. (C) Effect of RRM1 siRNA transfection on cell growth with or without CDDP treatment. Cell viability of 10 nM RRM1 siRNA transfectant with CDDP treatment was lower than that of 10 nM scRNA transfectant, although not statistically significant.

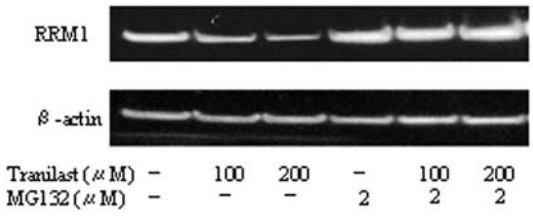

Figure 6. Effect of proteasome inhibitor MG132 on RRM1 expression with tranilast treatment in KP4 cells. Under the presence of MG132 proteasome inhibitor at 2 μM, the tranilast-induced reduction of RRM1 expression was disturbed.

difference between 10 nM of control siRNA and 10 nM of RRM1 siRNA transfectection (Fig. 5C).

Effect of proteasome inhibitor, MG132 on RRM1 expression in KP4 cells with tranilast. To examine whether or not proteasome-mediated degradation acts on the RRM1 suppression by tranilast, KP4 cells were exposed to tranilast in the presence or absence with the specific proteasome inhibitor MG132. As shown in Fig. 6, RRM1 was decreased by tranilast at 100 and 200 μM concentration. However, the reduced RRM1 expression by tranilast was completely abolished with MG132 treatment.

The effect of tranilast treatment on dFdC sensitivity in other pancreas cell lines. We investigated whether or not the synergistic effect of tranilast toward Gem sensitivity is found in the pancreatic cancer cell lines PK-8, PK-9, PK-1 and PK-59. Expression of RRM1 protein in these cells under tranilast treatment at concentration of 50, 100 and 200 μM was analyzed in Western blot and the expression level was quantitatively assessed (Fig. 7). As a result, RRM1 expression declined with tranilast treatment (ranging from 62.3 to 5.1% at 200 μM tranilast) (Fig. 7). The effect of tranilast on Gem sensitivity was then analyzed in MTT assay and the IC_{50} for Gem was calculated in all the cell lines including KP4. As summarized in Table II, 50 or 100 μM tranilast plus Gem treatment significantly decreased IC_{50} for Gem in KP4, PK-8, PK-9, PK-1 and PK-59 cells, compared with single Gem treatment.

Discussion

Ribonucleotide reductase (RNR) catalyzes the conversion of ribonucleotides into the correspondent 2'-deoxyribonucleotides in the rate-limiting step for the biosynthesis of DNA (9,10,31). This enzyme thus has long been regarded as an important target for cancer therapies. The enzymatic activity of RNR was shown to be dependent on the formation of a complex between two different dimers composed of larger subunit (RRM1) and smaller subunit (RRM2) (31). Various inhibitors targeting on RRM1 or RRM2 have been developed and the *in vitro* and *in vivo* effects examined (31). Among the RNR inhibitors, Gem inhibits RRM1 activity by which Gem metabolite, dFdCDP is misincorporated at the active site, leading to no more DNA replication (31). Recently, clinical use of Gem has been approved by the FDA for the treatment of patients with non-small cell lung cancer and adenocarcinoma of the pancreas (7,8). Current reports demonstrated that overexpression of RRM1 is linked to Gem resistance in human cancers (11-16). Using DNA array system, Davidson *et al* isolated RRM1 as the most critical gene determining Gem resistance in non-small cell lung cancer cell line (11). Thereafter several studies reported a significant

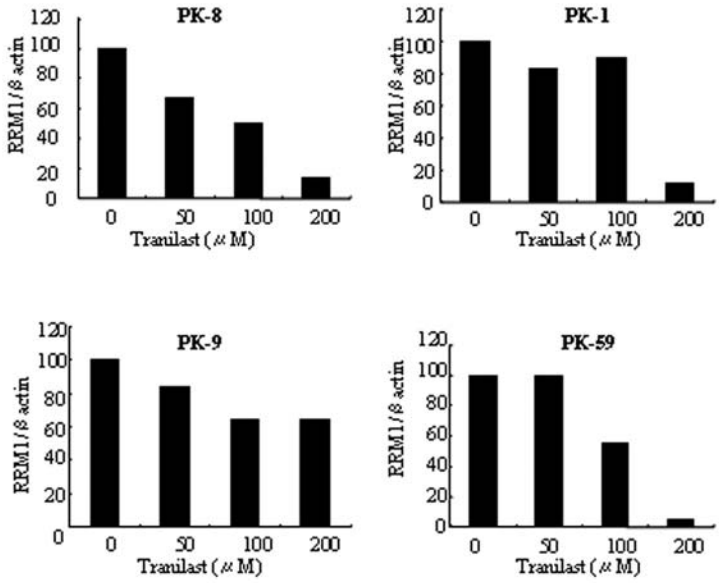

Figure 7. (A) Western blot analysis of RRM1 expression using the pancreatic cancer cells PK-8, PK-9, PK-1 and PK-59 with tranilast treatment at 50, 100 and 200 μM. Intensity of RRM1 signal in each cell was analyzed in LAS3000 and normalized by that of ß-actin. The relative intensity (%) was calculated as no treatment of tranilast and considered 100%.

Table II. IC_{50} values of Gem in pancreatic cancer cells with tranilast treatment at various concentrations.

Cell lines	Tranilast (μM)	IC_{50} (μM)	SD	P-value
KP4	0	9.29	0.12	
	50	4.83	0.29	<0.0001
	100	0.73	0.06	<0.0001
PK-8	0	78.8	75.0	
	50	45.1	15.5	0.039
	100	8.6	1.6	0.017
PK-9	0	96.1	26.5	
	50	76.7	8.85	0.0048
	100	72.2	56.9	0.011
PK-1	0	108.1	6.4	
	50	75.1	17.7	0.038
	100	62.2	8.7	0.0018
PK-59	0	84.3	1.9	
	50	41.4	7.5	0.0007
	100	11.1	3.5	<0.0001

p<0.05; statistically significant.

correlation between the RRM1 expression and Gem resistance in non-small cell lung carcinoma cell lines and tissues (12-14). In pancreatic cancer, literature demonstrates that RRM1 is a key molecule in Gem resistance through both *in vitro* and clinical models (15,16). These reports indicate that attenuation of RRM1 expression in both pancreatic and non-small cell lung cancer might contribute to overcome the resistance to Gem.

Tranilast has been found to inhibit the release of transforming growth factor (TGF)-ß, interleukin (IL)-1ß, prostaglandin (PG) E2 and IL-2 from human monocytes and macrophages (18). The result led to the clinical use of this compound for allergic diseases. Several studies have demonstrated the growth inhibition effect by tranilast in several types of cancer cells (26-29). Platten *et al* reported growth inhibition of malignant glioma cells by tranilast treatment (27). Shime *et al* showed that tranilast treatment induced growth arrest of leiomyoma cells at G0/G1 phase with p21 and p53 expression (29).

In the present study, tranilast treatment significantly inhibited cell growth of KP4 cells at 200-400 μM (Fig. 1A). Furthermore, uptake of BrdU was inhibited by tranilast treatment at more than 100 μM concentration. FACS analysis revealed the increased G1 fraction by the high dose treatment of tranilast at 250 or 500 μM (Table I). These results indicated that tranilast treatment inhibited cell growth of KP4 and rendered the cells arrested at G1 phase, at more than 250 μM concentration. Previous reports demonstrated similar findings that 300 μM of tranilast induced growth arrest in both glioma and leiomyoma cells (27,29).

On the contrary, combination experiment using anti-cancer drugs revealed that tranilast remarkably increased Gem sensitivity of KP4 cells at concentrations ranging from 25 to 100 μM (Fig. 2).

As tranilast at 100 μM did not affect cell growth of KP4 (Fig. 1A), it was indicated that tranilast synergistically enhanced Gem efficacy to KP4 cells. This compound also increased the efficacy of CDDP, although the synergistic effect was less than that to Gem (Fig. 2). However, tranilast no longer influenced the sensitivity to 5FU, CPT-11 or PTX in KP4 cells (Fig. 2A). This notable difference provided us a

hypothesis that tranilast could alter the expression of some important molecule determining Gem or CDDP sensitivity. As expected, tranilast dramatically reduced protein expression of a target molecule of Gem, RRM1 dose-dependently at 50-200 μM (Fig. 3B, C). This result indicated that the dose-dependent reduction of RRM1 protein resulted in the decreased uptake of BrdU (Fig. 1B) and G1 phase arrest at 250, 500 μM tranilast in FACS analysis (Table I). On the other hand, apoptotic effect by Gem was strongly accelerated in KP4 under the combined treatment at 100 and 250 μM tranilast (Table I, Fig. 4). It is thus speculated that tranilast decreases RRM1 expression and thereafter the Gem metabolite, dFdCDP, might easier inactivate the smaller amount of RRM1, leading to the strong induction of cell apoptosis in KP4.

To further confirm the effect of RRM1 expression in drug sensitivity to Gem or CDDP, transfection experiment using siRNA was carried out. The result demonstrated that siRNA against RRM1 significantly reduced the number of living KP4 cells exposed at 5 μM of Gem, compared with the control siRNA transfection, indicating that the sensitizing effect of tranilast on Gem mainly depends on the reduced RRM1 expression (Fig. 5B). Sensitivity to CDDP was also increased in RRM1 siRNA transfectant (Fig. 5C), although there was no statistical significance. CDDP is believed to kill cancer cells by binding to DNA and interfering with its repair mechanism. Harrington *et al* previously reported that CDDP irreversibly inhibits RNR activity via binding to RRM1 (32). Bepler *et al* showed that transfection of RRM1 siRNA increased sensitivity not only to Gem with 100-fold range, but also to CDDP with 2-fold range in non-small cell lung cancer cells (14). These reports support our result that RRM1 suppression by tranilast also contributes to the elevated CDDP sensitivity in KP4 cells.

To investigate the mechanism of RRM1 suppression, RRM1 expression was analyzed with tranilast along with MG132. As a result, RRM1 suppression by tranilast was abolished by MG132 treatment (Fig. 6). This result suggested that RRM1 degradation proceeds through the activated proteasome pathway. Proteasome inhibitor MG132 might cancel the tranilast effect on the enhancement of Gem toxicity through the inhibited RRM1 degradation. At present, the more precise mechanisms such as poly-ubiquitination of RRM1 protein under tranilast treatment, leading to RRM1 degradation by proteasome, remain to be elucidated.

To assess the feasibility of combined tranilast plus Gem therapy, the effect of tranilast on Gem sensitivity was investigated using other pancreatic cancer cell lines. In Western blot analysis, RRM1 expression was decreased in PK-8, PK-9, PK-1 and PK-59 by tranilast treatment (Fig. 7). In MTT assay, tranilast significantly decreased the IC_{50} for Gem in the cell lines as shown in KP4 (Table II). These results indicated that tranilast decreased RRM1 expression in all the pancreatic cancer cell lines tested and the reduced RRM1 expression is necessary to enhance the responsiveness to Gem.

Previous report showed that the concentration of tranilast in human plasma was estimated at 30-300 μM in case of daily oral intake of 600 mg tranilast for the treatment of allergic patients, suggesting the reasonable setting of tranilast concentration in the present *in vitro* study (22). Tranilast is usually taken orally and found to have few side effects except eosinophilic cystitis (33). In conclusion, tranilast induces protein degradation of RRM1 in pancreatic cancer and thereafter Gem might easier inactivate the smaller amount of RRM1, resulting in strong inhibition of cancer growth. Therefore, Gem plus tranilast would be a promising combination therapy against this malignancy. A clinical trial comparing the single Gem treatment with Gem plus tranilast should be performed in pancreatic cancer patients.

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