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UCN-01 (7-Hydroxystaurosporine) Enhances 5-Fluorouracil Cytotoxicity through Down-regulation of Thymidylate Synthetase Messenger RNA

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UCN-01 (7-hydroxystaurosporine) is a newly developed cell cycle inhibitor known to have several modes of action, including inhibition of cyclin-dependent kinase, induction of p21 and suppression of pRb phosphorylation. In order to test a combination therapy of UCN-01 and 5-fluorouracil (5-FU), growth inhibition of CRL 1420 (MIA PaCa-2; undifferentiated pancreatic carcinoma) by four different treatments was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The treatments used were UCN-01 alone, 5-FU alone, 5-FU followed by UCN-01 (5-FU/UCN-01) and UCN-01 followed by 5-FU (UCN-01/5-FU). We also assessed changes in thymidylate synthetase (TS) mRNA levels, TS activity, and 5-FU incorporation by RNA (F-RNA) for each treatment. Although treatment with UCN-01 alone, 5-FU alone, and 5-FU/UCN-01 inhibited CRL 1420 growth in a concentration-dependent manner, treatment with UCN-01/5-FU inhibited the growth of CRL 1420 synergistically at less than 1 μg/ml drug concentration. The down-regulation of TS mRNA by UCN-01 resulted in stable total TS and decreased free TS, and UCN-01/5-FU resulted in enhanced thymidylate synthetase inhibition rate (TSIR) compared to UCN-01 alone and 5-FU/UCN-01. This increased TSIR due to UCN-01 pretreatment was accompanied by elevated F-RNA concentrations in the UCN-01/5-FU treatment. The suppression of TS mRNA and TS activity by UCN-01 may lead to higher sensitivity of tumor cells to 5-FU and may explain the synergistic antitumor effect of UCN-01/5-FU. In conclusion, low concentrations of UCN-01 (from 0.01 to 1 μg/ml) may be clinically useful, affording low cytotoxicity of UCN-01, while enhancing the antitumor effect of 5-FU.

Key words: UCN-01 — Pancreatic cancer — 5-Fluorouracil — Thymidylate synthetase — mRNA

The proliferation of cancer cells is regulated by multiple signals in the cell cycle, some of which have recently been clarified.[1–5] However, few reports have been published concerning antitumor agents that regulate the cell cycle of human cancer cells. Recent studies have shown that a family of cyclin-dependent kinases (CDK) regulates human cell cycle progression. CDK activity is regulated through binding to cyclin ligands and phosphorylation by cyclin activator kinase (CAK).[6–8] Transition through G1 to S phase is regulated by activation of CDK2 by cyclin E, and the resultant cyclin/CDK complexes then phosphorylate pRb to give ppRb,[9] which then activates E2F-driven transcriptional activation.[10] E2F promotes expression of the thymidine kinase, thymidylate synthetase (TS), dihydrofolate reductase, cyclin E, and DNA polymerase-α genes, among others.

7-Hydroxystaurosporine (UCN-01) is a new antitumor agent that was isolated as a selective inhibitor of Ca^{2+} and phospholipid-dependent protein kinase C (PKC).[11–13] Evaluation of this drug on several human cancer cell lines revealed that UCN-01 is a potent antitumor agent through selective inhibition of cyclin-dependent kinase activity.[14] Based on preclinical data, UCN-01 is currently undergoing two Phase 1 clinical trials, in the United States[15] and in Japan, using different administration schedules. However, the precise mechanism of its antitumor activity is still not completely understood. Recent studies revealed that UCN-01 inhibited cell cycle progression through G1 to S phase in human cell lines,[16, 17] and enhanced the antitumor activity of several important antitumor agents, including mitomycin C, cisplatin, and 5-fluorouracil (5-FU) both *in vitro* and *in vivo*.[18–20] Johnston *et al.*[21] reported that TS plays an important role in 5-FU-based chemotherapy in primary colorectal and gastric cancer patients. UCN-01 may suppress mRNA levels of thymidylate synthetase, a target enzyme of 5-FU. In this study, we have assessed the antitumor effect of combined UCN-01 and 5-FU treatment using the pancreatic cancer cell line, CRL 1420 (MIA PaCa-2) with regard to TS activity, TS mRNA level, and 5-FU incorporation by RNA.

MATERIALS AND METHODS

Agents UCN-01 was supplied by Kyowa Hakko Kogyo Co., Ltd. (Tokyo). 5-FU was purchased from Kyowa

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Hakko Kogyo Co., Ltd. [6-^{3}H]5-Fluoro-2′-deoxyuridine monophosphate (FdUMP) (16.9 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA). All other chemicals used were of the highest standard grade commercially available.

Cell culture CRL 1420 (MIA PaCa-2) was obtained from the American Type Culture Collection (Bethesda, MD) and is described as an undifferentiated human pancreatic carcinoma established in continuous culture.[22] The cell line was grown in Dulbecco's modified media (DMEM), containing 10% fetal bovine serum (Filtron, Brooklyn, Australia), 100 IU penicillin, 100 μg/ml streptomycin (GIBCO, Gaithersberg, MD) and 0.25 μg/ml amphotericin B at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Evaluation of cytotoxicity We evaluated the *in vitro* chemosensitivity of tumor cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St. Louis, MO) assay reported by Mosmann[23] with some modifications as reported previously.[24–26] Cell suspensions were centrifuged, and tumor cells were suspended in DMEM supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS), diluted to 2×10^5 cells/ml, and plated into 96-well microplates (GIBCO) in a volume of 100 μl, resulting in 10^4 cells/well. Drug solutions were dissolved in DMEM and 100 μl was added to each well. Control wells contained 100 μl of cell suspension and 100 μl of DMEM containing 10% FBS. UCN-01 and/or 5-FU at concentrations from 0.01 to 1000 μg/ml was then added and plates were incubated for 72 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After incubation, a mixture of 0.4% MTT and 0.1 *M* sodium succinate dissolved in 10 μl of phosphate-buffered saline (PBS) and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA), was added and the plates were incubated for a further 3 h at 37°C. After the final incubation, dimethyl sulfoxide (Nacalai Tesque, Kyoto) was added to each well at a volume of 150 μl/well to dissolve the MTT-formazan product. Plates were then mechanically shaken for 10 min on a mixer (Model 250, Sonifier, Branson, MO) to dissolve the formazan salt. Optical density of the solutions was determined on a model EAR 340 easy reader (SLT-Labinstruments, Salzburg, Austria) at 540–630 nm. Inhibition rates were calculated using the formula: $(1-A/B)\times100\%$, where A and B represent the mean absorbance of drug-treated and control wells, respectively.

Combination cytotoxicity of UCN-01 and 5-FU To assess the combination cytotoxicity of UCN-01 and 5-FU, four treatment schedules were used for the cytotoxicity assay using CRL 1420 (Fig. 1). In treatment schedules 1 and 2, CRL 1420 was treated with UCN-01 or 5-FU, respectively, for 72 h. For schedule 3, cells were treated with 5-FU at concentrations from 0.01 to 1000 μg/ml for 36 h, followed by the same concentration of UCN-01 for 36 h. The reverse treatment order (UCN-01 followed by 5-FU) was analyzed as schedule 4. Before the second drug was added, cells were washed with PBS to remove the influence of the first drug. Inhibition rates were calculated at the end of the incubation periods as described above. Assays were performed in triplicate for each schedule, and expressed as mean average values.

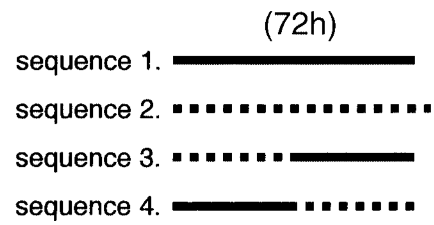

Fig. 1. Combination therapy schedules of UCN-01 and 5-FU. In treatment schedules 1 and 2, CRL 1420 cells were treated with UCN-01 or 5-FU, respectively, for 72 h. For schedule 3, cells were treated with 5-FU at concentrations from 0.01 to 1000 μg/ml for 36 h, followed by the same concentration of UCN-01 for 36 h. The reverse treatment order (UCN-01 followed by 5-FU) was analyzed as schedule 4. Assays were performed in triplicate for each treatment. ▬ UCN-01 0.01–1000 μg/ml, ▪▪▪ 5-FU 0.01–1000 μg/ml.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) To evaluate TS mRNA levels, 10^7 CRL 1420 cells were harvested after 24 h treatment with UCN-01 (0.01, 0.1, 1, 10 and 100 μg/ml) or 5-FU (1, 10, 30 and 100 μg/ml). Harvested cells were washed with PBS and homogenized. Semiquantitative RT-PCR was performed using the method of Takechi *et al*.[27] Total RNA for each homogenate was isolated using the RNeasy mini kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Total RNA yields and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. RNA integrity was checked by visualizing rRNA bands by agarose gel electrophoresis in the presence of formaldehyde.

Reverse transcription was carried out with 10 μg of total RNA in a total volume of 100 μl containing 250 pmol of oligo$(dT)_{18}$, 80 U of rRNasin RNase inhibitor (Promega, Madison, WI), and 500 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) in 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol (DTT), and 0.5 m*M* dNTPs. Initially, RNA and oligo$(dT)_{18}$ were heated at 70°C for 10 min and immediately chilled on ice, and then the other reagents were added and the mixture was incubated for 15 min at 30°C and 60 min at 42°C.

For PCR, cDNA aliquots were diluted in sterile water according to transcript abundance. Three cDNA concentrations for the TS/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer combination were used. For accurate quantification using this method, measurements must be taken in the linear phase of the reaction, so that the cDNA concentration is directly proportional to signal intensity. A range of cDNA concentrations was used to determine the linear region of the PCR. PCR primers were designed according to the human TS[28] and GAPDH[29] cDNA sequences. Primers used were as follows: TS, 5′-GAATCACATCGAGCCACTGAAA-3′ and 5′-GTG-TTACTCAGCTCCCTCAGA-3′ (product 579 bp); and GAPDH, 5′-CAACAGCCTCAAGATCATCAGC-3′ and 5′-TTCTAGACGGCAGGTCAGGTC-3′ (product 328 bp). PCR was carried out in a final volume of 50 μl containing cDNA template, TS and GADPH primers, 1.25 U of Ex *Taq* (TaKaRa, Shiga) in 10× Ex *Taq* buffer (TaKaRa), and 0.2 m*M* dNTPs, using a thermal cycler (PC-800; Astec, Tokyo). Ten picomoles of each TS primer and 2 pmol of each GAPDH primer were used in each PCR. The PCR profile consisted of an initial 3-min denaturation at 94°C, cycles of 1-min annealing at 60°C and 2-min polymerization at 72°C, and a final 10-min extension at 72°C. PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, visualized on a UV transilluminator and photographed on Type 667 films (Polaroid, Cambridge, MA). Images were scanned with an image scanner (JX-330; Sharp, Mahwah, NJ) and analyzed with Image Master ID (Pharmacia Biotech, Tokyo). Relative amounts of TS mRNA were expressed as ratios of TS to GAPDH RT-PCR products.

FdUMP binding assay Aliquots of 10^7 cells were harvested after treatment with UCN-01 and/or 5-FU to evaluate TS activity. For UCN-01 alone, CRL 1420 was treated with UCN-01 at concentrations of 0.01, 0.1, and 1 μg/ml for 24 h, and for 5-FU alone, CRL 1420 was treated with 5-FU at concentrations of 10, 30, and 100 μg/ml for 24 h. Treatment schedule 3 consisted of UCN-01 treatment (0.01, 0.1, and 1 μg/ml) for 12 h followed by 5-FU treatment (10 μg/ml) for 12 h, and treatment schedule 4 used the reverse treatment order (5-FU 10 μg/ml followed by UCN-01 at 0.01, 0.1, and 1 μg/ml). Harvested cells were washed with PBS and homogenized with three volumes of 200 m*M* Tris-HCl (pH 8.0) containing 20 m*M* β-mercaptoethanol (β-ME), 100 m*M* NaF and 15 m*M* cytidine-5-monophosphate (CMP), and centrifuged at 105 000*g* for 60 min. Resultant supernatants were used for TS activity determination according to the method of Spears *et al.*[30] using [6-^{3}H]FdUMP as the substrate. Samples for total TS determination were prepared by causing the ternary complex present in the cytosol to become fully dissociated to afford unbound TS at pH 8.0 during the preincubation period. For the samples for the determination of free TS, the dissociation step was omitted. Total TS and free TS samples were incubated with [6-^{3}H]FdUMP in the presence of 5,10-methylenetetrahydrofolate for 20 min at 30°C and radioactivity in the acid-insoluble fractions was measured using a liquid scintillation counter. The thymidylate synthetase inhibition rate (TSIR in %) was calculated as: [1 − TS free (pmol/mg protein) / TS total (pmol/mg protein)] ×100.

Concentration of 5-FU in RNA (F-RNA) Isolation of RNA fractions and quantification of 5-FU incorporation were performed using the methods of Uchida *et al.*[31] A total of 10^7 cells was harvested after treatment with UCN-01 and/or 5-FU for evaluation of F-RNA levels. CRL 1420 cells were treated with 5-FU alone at concentrations of 10, 30, and 100 μg/ml for 24 h. In combination treatment, UCN-01 was added at concentrations of 0.01, 0.1, and 1 μg/ml for 12 h, followed by 5-FU treatment at 10 μg/ml for 12 h, or antitumor agents were added in the reverse order. Harvested cells were washed with PBS and homogenized, and RNA fractions were separated and heated to 100°C in 6 *N* HCl, then hydrated for 24 h. Finally, 5-FU was determined using gas chromatography-mass spectrometry (Model JGS-20kp, Model JMS-D 300, JEOL, Tokyo).[32]

Statistical analysis All RT-PCR, FdUMP binding assay, and F-RNA assay data are presented as the mean±SD of more than three samples. The statistical significance (P) of the experimental results was determined by Student's *t* test using Macintosh Microsoft Excel Version 2.01. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Combination effect of UCN-01 and 5-FU Fig. 2 shows the combination effect of UCN-01 and 5-FU on CRL 1420, using the different treatment orders with 72 h contact time. Interestingly, the UCN-01/5-FU sequence resulted in higher inhibition, particularly at concentrations of less than 1 μg/ml. At 0.01 μg/ml concentration, the inhibition rates of 5-FU, UCN-01, and UCN-01/5-FU were 6.5%, 16.3%, and 46%, respectively, suggesting that the antitumor activities of UCN-01 and 5-FU in UCN-01/5-FU were synergistic.

Thymidylate synthetase mRNA levels Changes in TS/GAPDH RT-PCR product ratio (TS mRNA level) in CRL 1420 after UCN-01 treatment at concentrations of 0.01, 0.1, 1, 10, and 100 μg/ml are shown in Fig. 3. The TS mRNA level in untreated CRL 1420 was 6.49. Levels reduced in response to UCN-01 in a concentration-dependent manner up to 100 μg/ml UCN-01, at which point the TS mRNA levels were almost half that of the control. In contrast, TS mRNA levels were up-regulated by 5-FU in a concentration-dependent manner (Fig. 3), reaching levels that were almost twice that of the control.

TS inhibition Table I shows the TS activity observed after the four different treatment schedules. UCN-01 did not affect TS activity, including total TS, free TS, and TSIR. 5-FU did induce total TS activity in a concentration-dependent manner, while TSIR was elevated due to a relative decrease of free TS to total TS. UCN-01/5-FU inhibited the induction of total TS and decreased free TS. As shown in Fig. 4, TSIR of untreated CRL 1420 was 13.3% which was increased 2- to 4-fold by 5-FU in a concentration-dependent manner at 10, 30, and 100 μg/ml. TSIR was markedly elevated after UCN-01/5-FU treatment in an UCN-01-concentration-dependent manner, and this increase was statistically significant compared to

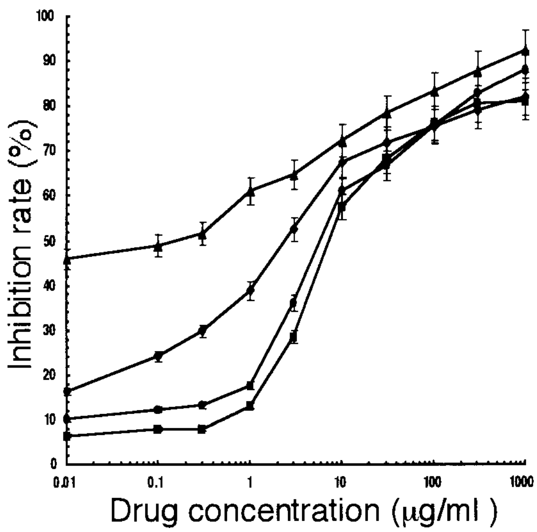

Fig. 2. The cytotoxicity of the four different treatments after 72 h. The synergistic effect of UCN-01 and 5-FU was observed for the UCN-01/5-FU sequence at concentrations less than 1 μg/ml. ◆ UCN-01, ■ 5-FU, ▲ UCN-01/5-FU, ● 5-FU/UCN-01.

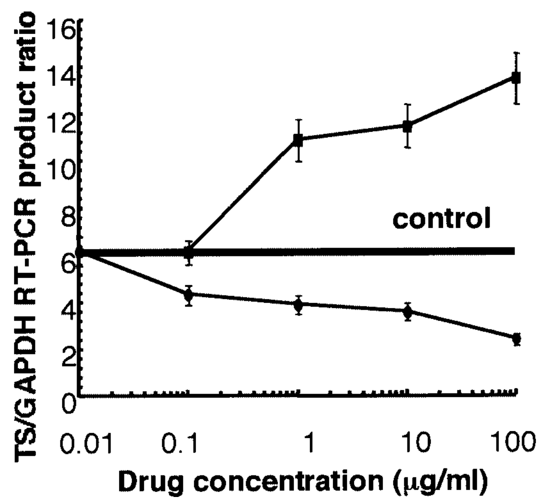

Fig. 3. Correlation between drug concentration and TS mRNA level. Plots showing the correlation between concentration of UCN-01 and TS/GAPDH RT-PCR product ratio (●) and concentration of 5-FU and TS/GAPDH RT-PCR product ratio (■). TS/GAPDH RT-PCR product levels are expressed as ratios to GAPDH internal standard.

Table I. TS Activities in CRL 1420

Drug concentration[a]	Sequence[b]	TS total[c]	TS free[d]	TSIR[e]
Control		2.54±0.56	2.20±1.03	13.3±19.5
UCN-01	UCN-UCN	2.65±0.72	2.26±0.85	14.9±9.8
0.01	UCN-FU	1.45±0.92*	0.99±0.31	31.4±17.0
	FU-UCN	1.41±0.55*	1.12±0.51*	20.9±6.9
UCN-01	UCN-UCN	2.59±1.28	2.11±0.75	18.4±6.7
0.1	UCN-FU	1.40±0.86*	0.75±0.29*	46.3±25.6*
	FU-UCN	1.82±1.12	1.39±0.77*	23.8±9.5
UCN-01	UCN-UCN	2.68±1.49	2.00±1.29	25.4±8.4
1	UCN-FU	1.25±0.39*	0.60±0.37*	51.9±33.2*
	FU-UCN	2.22±1.45	1.64±0.88*	26.1±14.2
5-FU alone	10	4.43±2.88	3.28±1.60*	26.1±3.8
	30	5.71±2.59*	3.89±1.57*	31.7±4.7
	100	6.28±2.20*	3.01±1.57*	52.1±21.1*

a) In combination treatment, UCN-01 at a concentration of 0.01, 0.1, or 1 μg/ml for 12 h and 10 μg/ml of 5-FU were used.
b) UCN-UCN: treatment with UCN-01 for 24 h, UCN-FU: treatment with UCN-01 for 12 h, followed by 10 μg/ml of 5-FU for 12 h, FU-UCN: the reverse treatment order of UCN-FU.
c) TS total, in pmol/mg protein.
d) TS free, in pmol/mg protein.
e) TS inhibition rate (%): 1−(TS free/TS total).
* $P<0.05$ relative to control.

UCN-01 alone, 5-FU/UCN-01 and 5-FU alone at concentrations of 10 and 30 μg/ml.

Concentration of F-RNA F-RNA levels increased exponentially in a concentration-dependent manner (Fig. 5A) after treatment of 5-FU alone. When low concentrations of 5-FU (10 μg/ml) were used after UCN-01 treatment, F-RNA levels increased in a UCN-01 concentration-dependent manner, this increase being statistically significant compared to the reverse sequence (Fig. 5B). After UCN-01 pretreatment, F-RNA levels also increased compared with 5-FU alone at the same 10 μg/ml concentration; this difference was also statistically significant at $P<0.05$.

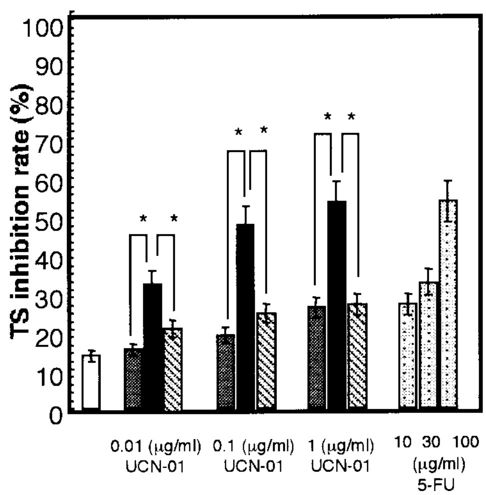

Fig. 4. TS inhibition rate (%) in untreated CRL 1420 cells and in cells after the four treatments. TSIR (%) was determined as [1 – TS free (pmol/mg protein) / TS total (pmol/mg protein)]× 100. Blank box: untreated (control), gray box: UCN-01 alone, solid (black) box: UCN-01/5-FU (10 μg/ml), shaded box: 5-FU (10 μg/ml)/UCN-01, spotted box: 5-FU alone. ★ $P<0.05$.

DISCUSSION

Previous reports have indicated that UCN-01 induces preferential G1 phase accumulation in several types of human cell lines, although the mechanism by which this occurs has not been clearly defined.[14, 16, 17] In the human cell cycle, transition through G1 to S phase is regulated by the activation of CDK2 by cyclin E and/or cyclin A. CDK4 and/or CDK6 are also key regulators in progression due to their regulation of the phosphorylation state of pRb, the retinoblastoma susceptibility tumor suppressor gene product. Phosphorylation of pRb results in a loss of affinity for the E2F family of transcription factors.[33–35] The E2F family is responsible for governing the transcription of many genes necessary for progression through the S phase, including thymidine kinase, thymidylate synthetase, dihydrofolate reductase, cyclin E, DNA polymerase-α, and others.[10]

We speculated that UCN-01 might suppress the mRNA levels of TS as a transcription inhibitor, resulting in the inhibition of TS-induction by 5-FU that would lead to increased 5-FU-cytotoxicity. In order to test a combination therapy involving UCN-01, we used 5-FU, which inhibits

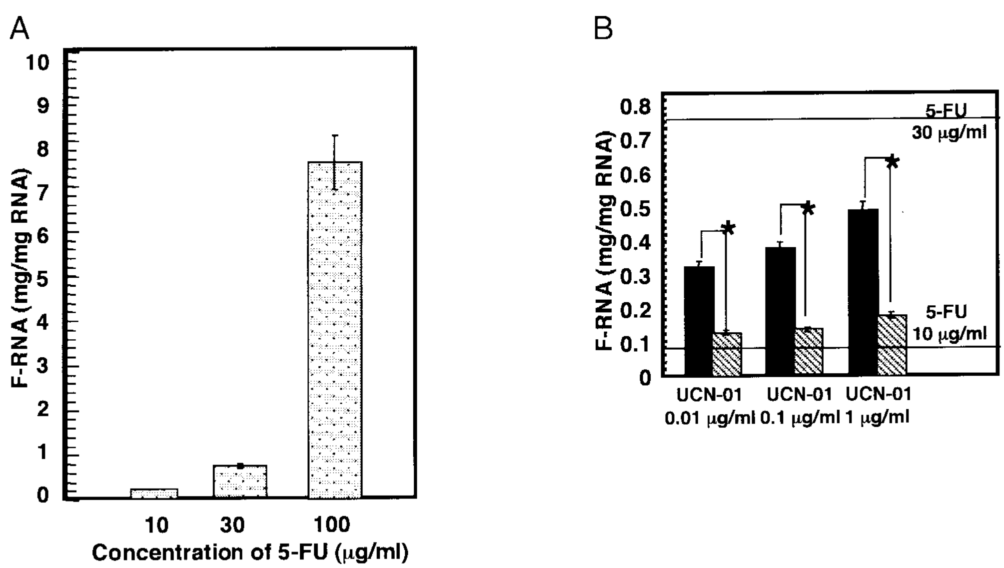

Fig. 5. Comparison of F-RNA levels among three different treatments. A (spotted box) shows the concentration of 5-FU in RNA after 5-FU treatment. B shows the F-RNA level after UCN-01/5-FU treatment (solid box), or 5-FU/UCN-01 treatment (shaded box). Concentrations of 0.01, 0.1, or 1 μg/ml of UCN-01 and 10 μg/ml of 5-FU were used. ★ $P<0.05$.

cell growth through the suppression of TS, a target enzyme of FdUMP. Treatment with UCN-01 alone, 5-FU alone, and 5-FU followed by UCN-01 inhibited the growth of CRL 1420 in a concentration-dependent manner (Fig. 2). However, treatment with UCN-01 followed by 5-FU inhibited the growth of CRL 1420 synergistically (Fig. 2). This suggested that UCN-01 might have inhibited the induction of total TS by 5-FU, resulting in the synergism in the UCN-01/5-FU sequence. Hsueh *et al.*[36] demonstrated that UCN-01 dephosphorylated ppRb and inhibited the activation of E2F, resulting in incomplete TS transcription. To examine this hypothesis, we assessed the changes in TS RT-PCR products, normalized against GAPDH RT-PCR products, using CRL1420 cells treated with UCN-01 or 5-FU. Although TS mRNA was up-regulated by 5-FU in a concentration-dependent manner, TS mRNA was inhibited by UCN-01 in a concentration-dependent manner. The suppression of TS mRNA was obvious even at the low UCN-01 concentration of 0.1 μg/ml, where a synergistic effect was observed in combination with 5-FU. The down-regulation of TS mRNA by UCN-01 resulted in significantly decreased free TS and increased TSIR in the UCN-01/5-FU sequence, compared with UCN-01 alone and the 5-FU/UCN-01 sequence.

Two main modes of action have been proposed for 5-FU, through its active metabolites, FdUMP and 5-fluoro-UTP.[37] FdUMP is thought to form a ternary complex with 5,10-methylenetetrahydrofolate and TS, which inhibits DNA synthesis, while 5-fluoro-UTP is incorporated into cellular RNA, resulting in RNA dysfunction.[32] From our results, we suggest that UCN-01 suppresses TS mRNA at the transcriptional level, as suggested by Hsueh *et al.*,[36] which results in the inhibition of TS activity, which in turn may account for the synergistic antitumor effect of UCN-01 followed by 5-FU. The incorporation of 5-FU into RNA might also account for the synergism in the UCN-01/5-FU sequence. Actually, the UCN-01/5-FU sequence had a synergistic cytotoxicity at 0.01 μg of UCN-01 per ml, where UCN-01 did not down-regulate TS mRNA but increased the incorporation of 5-FU into RNA. This phenomenon may be related to the result that the cytotoxicity of 5-FU was enhanced at higher concentrations, where F-RNA was remarkably increased, while total TS was enormously induced, free TS was relatively increased, and TSIR was elevated. However, the mechanism of the increase of F-RNA at 0.01 μg of UCN-01 per ml is unclear, and should be clarified in further experiments.

We conclude that low concentrations of UCN-01 (from 0.01 to 1 μg/ml) might be clinically useful, affording reduced cytotoxicity of UCN-01, as indicated by preclinical animal toxicology models,[38] and enhancing the cytotoxicity of 5-FU through down-regulation of TS mRNA and TS activity.

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