UCN-01 Suppresses Thymidylate Synthase Gene Expression and Enhances 5-Fluorouracil-induced Apoptosis in a Sequence-dependent Manner

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ABSTRACT

UCN-01, a protein kinase C/cyclin-dependent kinase inhibitor, suppressed thymidylate synthase (TS) protein expression in a dose-dependent manner with near complete suppression at 1 nM after a 24-h exposure in human gastric cancer cell line SK-GT5. Other protein kinase C/cyclin-dependent kinase inhibitors, including flavopiridol and safingol, had a similar effect on TS protein expression, but to a lesser degree. Moreover, UCN-01 repressed the induction of TS after 5-fluorouracil (FU) exposure by 90–95% and significantly enhanced the induction of apoptosis by FU from 4–8% with either FU or UCN-01 alone to 46 ± 1% (P < 0.005 versus either single drug, reverse sequence, or the combination) when UCN-01 was given after FU. The effect of UCN-01 on TS was associated with a dose-dependent suppression of the E2F-1 protein, a transcriptional activator of TS. Northern blot analysis revealed that TS mRNA levels decreased gradually as the concentration of UCN-01 increased, but that E2F-1 mRNA levels remained relatively unchanged. UCN-01 may provide a novel way to enhance cellular sensitivity toward FU by means of suppressing TS expression mediated mainly by down-regulation of E2F-1.

INTRODUCTION

TS is a target enzyme for FU in cancer chemotherapy. Biochemical modulation of FU activity to achieve maximal inhibition of TS has been a major area of investigation, because FU administration alone or in combination with other agents is a primary mode of treatment for many cancers including gastrointestinal malignancies (1). Using clinical biopsy samples in gastric and colorectal cancers, several studies have indicated that the increased expression of TS correlates inversely with survival and the response to FU-based therapy (2, 3). Therefore, identifying a means to decrease TS expression holds a promise to improve cancer therapy. A series of in vitro and in vivo models has demonstrated that PKC/CDK inhibitors such as UCN-01 and flavopiridol significantly enhance the induction of apoptosis by chemotherapeutic agents such as mitomycin-C, doxorubicin, and paclitaxel (4). Data also exist for the synergistic antitumor effect of PKC/CDK inhibitors in combination with FU (5, 6). Because of these results, Phase I clinical trials are under way using PKC/CDK inhibitors in combination with chemotherapy as a means of substantially increasing the chemotherapeutic effect. However, the mechanism for chemosensitivity enhancement remains essentially unknown. In this study, we have demonstrated that in the human gastric cancer cell line SK-GT5, a series of PKC/CDK inhibitors suppresses TS protein levels, with the most striking effect observed with UCN-01. Moreover, UCN-01 appears to suppress TS gene expression, mainly through the down-regulation of E2F-1 protein levels, and significantly enhances FU-induced apoptosis in a sequence-dependent manner.

MATERIALS AND METHODS

Cell Culture. Early-passage human gastric cancer cell line SK-GT5 exhibiting p53 mutation and intrinsic resistance to several chemotherapeutic agents including FU was established and characterized as described previously (7, 8). All of the cultures were maintained in standard MEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 20% heat-inactivated normal calf serum (Intergen) at 37°C in a humidified atmosphere of 5% CO₂. UCN-01 and flavopiridol were graciously supplied by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD). Safingol and FU were obtained from Eli Lilly and Hoffmann-La Roche, respectively. Aphidicolin was obtained from Sigma. The cytotoxicity of each agent for a 24-h exposure in SK-GT5 cells was determined by Alamar blue assay (9), and the concentration causing <20% growth inhibition was used in this study. Twenty-four h after passage, when cells were approximately 50–60% confluent, they were exposed to the indicated drug concentration. Cells were checked for Mycoplasma contamination at least every 3 months with a GEN-Probe Mycoplasma rapid detection kit (Fisher Scientific) and consistently tested negative.

Western Blot Analysis. Whole cell lysate containing 50 µg of protein from each sample and 2 ng of human TS protein (positive control; a generous gift from Dr. Bruce J. Dolnick, Roswell Park Cancer Institute, New York, NY) were resolved by SDS-PAGE using 8% polyacrylamide according to the method of Laemmli (10). The gels were then electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Milli-
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Panel A was probed with TS antibody first and subsequently stripped and reprobed with β-actin antibody to demonstrate equal loading of the protein. Panel B demonstrates equal loading and even transfer of the gel. The quantitation of TS protein expression by densitometric scanning is shown in the bottom panel as the fold changes from the untreated cells.
Fig. 2 Effects of UCN-01 on apoptosis induction by FU. Photomicrographs of representative fields of SK-GT5 cells stained with bisbenzimide trihydrochloride (Hoescht 33258) to evaluate nuclear chromatin condensation (i.e., apoptosis) after treatment with 50 μM FU for 24 h followed by no drug for 24 h (A), no drug for 24 h followed by 0.5 μM UCN-01 for 24 h (B), 50 μM FU for 24 h followed by 0.5 μM UCN-01 for 24 h (C), or 0.5 μM UCN-01 for 24 h followed by 50 μM FU for 24 h (D) are shown.

Statistical Analysis. All experiments were performed at least twice with similar results, and the results of one representative experiment are shown. The statistical significance (P) of the experimental results was determined by the two-sided Student t test.

RESULTS AND DISCUSSION

Because the effects of various PKC/CDK inhibitors on the expression of TS protein were not known, we elected to perform Western blot analysis using TS and β-actin (as an internal control) antibodies in SK-GT5 cells exposed to no drug, 300 nM flavopiridol, 50 μM safingol, and 1 μM UCN-01 for 24 h (Fig. IA). Near-complete suppression of TS protein levels was observed with UCN-01. Flavopiridol and safingol had a similar effect on TS protein expression, but to a lesser degree. Cell cycle arrest at G1-S phase was observed when SK-GT5 cells were exposed to either 1 μM UCN-01 or 5 μg/ml aphidicolin for 24 h, and the degree of TS suppression was far less in aphidicolin-treated cells than it was in UCN-01-treated cells (data not shown). Therefore, the suppression of TS protein expression by UCN-01 was not due to a nonspecific phenomenon from cellular proliferation arrest.

The TS-repressive effect by UCN-01 was further examined in SK-GT5 cells after exposure to 50 μM FU for 24 h by Western blot analysis (Fig. IB). The TS levels increased approximately 3–4-fold after a 24-h exposure to 50 μM FU (Fig. IB, Lane 2) and persisted for another 24 h in the absence of FU (Fig. IB, Lane 4). The appearance of an upper band for TS at approximately 38 kDa after FU exposure represents the ternary complex, which has been described previously by Johnston et al. (19). Exposure to 1 μM UCN-01 for 24 h significantly suppressed the induction of TS protein levels after FU exposure by 90–95% (Fig. IB, Lane 3) when compared with FU exposure followed by no drug (Fig. IB, Lane 4). In vitro and in vivo studies have demonstrated that cellular and tumor TS levels increase 24–48 h after FU exposure in colon cancer (20, 21). Whether the increase in TS after FU exposure prevents the induction of apoptosis is not known. It may very well represent a critical step that could be used to enhance the induction of apoptosis by FU and therefore increase chemosensitivity.

Given that UCN-01 significantly suppressed TS expression and the induction of TS after FU exposure, the enhancement of
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**Fig. 3** Effects of UCN-01 on TS and E2F-1 protein expression. UCN-01, TS, E2F-1, and β-actin protein levels in SK-GT5 cells exposed to 0 (no drug), 0.01, 0.1, and 1 μM UCN-01 for 24 h were examined by Western blot analysis as described in “Materials and Methods.” A duplicate blot was used for β-actin antibody probing to demonstrate equal loading of the protein.

FU-induced apoptosis by UCN-01 in SK-GT5 was evaluated by QFM (Fig. 2). Fifty μM FU and 0.5 μM UCN-01 were used. The exposure time was 24 h for each drug or no drug. Cells were treated according to the following conditions: (a) FU followed by no drug; (b) no drug followed by UCN-01; (c) FU followed by UCN-01; (d) UCN-01 followed by FU; and (e) UCN-01 in combination with FU for 24 h. Apoptosis induction was observed in 4 ± 1% of the cells treated with FU followed by no drug (Fig. 2A), 8 ± 1% of the cells treated with no drug followed by UCN-01 (Fig. 2B), 46 ± 1% (P < 0.005 versus either single drug, reverse sequence, or the combination) of the cells treated with FU followed by UCN-01 (Fig. 2C), 17 ± 1% of the cells treated with UCN-01 followed by FU (Fig. 2D), and 26 ± 1% of the cells treated with the combination of UCN-01 and FU for 24 h. With 1 μM UCN-01, similar results were observed, but with more apoptosis induction by UCN-01 alone (data not shown). These results indicate that UCN-01 significantly enhances FU-induced apoptosis in a sequence-dependent manner with maximal enhancement in FU followed by UCN-01.

As the initial approach to uncover the mechanism responsible for TS repression by UCN-01, the effects of UCN-01 on the protein expression of TS and its transcriptional activator E2F-1 were investigated by Western blot analysis in SK-GT5 cells exposed to various concentrations (0.01, 0.1, and 1 μM) of UCN-01 for 24 h. As shown in Fig. 3, both TS and E2F-1 protein levels decreased stepwise as the concentration of UCN-01 increased, with a 70–80% decrease at 0.1 μM and a >99% decrease at 1 μM for both proteins. After cellular exposure to UCN-01, we also observed that the suppression of E2F-1 protein expression preceded the suppression of TS and DHFR protein expression. These data suggest that the down-regulation of E2F-1 protein expression contributes to the suppression of the TS protein level by UCN-01.

UCN-01 has been shown to cause cell cycle arrest at G1-S phase, presumably through the dephosphorylation of pRb and the inhibition of CDK2 activity (22). The increase in hypophosphorylated pRb prohibits the release of free E2F-1 from the pRb-E2F-1 complex, thus preventing cell cycle progression into the S phase (23). The transcription factor E2F-1 has been implicated in regulating the transcription of several genes required for DNA synthesis such as TS, DHFR, and thymidine kinase (24). It has been shown that the loss of functional pRb contributes to antimetabolite resistance, because cells lacking pRb may have increased levels of TS and DHFR mediated by increased levels of free E2F-1 (25). It is conceivable that regardless of pRb status, the means of suppressing E2F-1 levels could potentially increase antimetabolite chemosensitivity by down-regulating TS and DHFR gene expression. We postulate that UCN-01 may also enhance the induction of apoptosis by

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**Fig. 4** Effects of UCN-01 on TS and E2F-1 mRNA expression. TS and E2F-1 mRNA levels in SK-GT5 cells exposed to 0 (no drug), 0.1, 0.4, and 0.8 μM UCN-01 for 24 h were examined by Northern blot analysis as described in “Materials and Methods.” The photograph of the nylon filter exhibiting the ethidium bromide fluorescence of 28 S and 18 S rRNAs is displayed in the bottom panel, demonstrating equal loading and even transfer of the gel.
methotrexate, an inhibitor of DHFR, in a fashion similar to the enhancement of FU-induced apoptosis. This hypothesis is currently under investigation in this laboratory.

To further analyze the mechanism for the suppression of TS and E2F-1 protein expression by UCN-01, Northern blot analysis using 32P-labeled TS and E2F-1 cDNA probes was performed (Fig. 4). TS mRNA levels decreased gradually as the concentration of UCN-01 increased, with ~50% decrease at 0.1 and 0.4 μM, and a 70~80% decrease at 0.8 and 1 μM (data not shown). However, the E2F-1 mRNA levels remained relatively unchanged as the concentration of UCN-01 increased, with ~30% decrease at 0.8 and 1 μM (data not shown).

IFN-γ has been shown to suppress the induction of TS protein levels after FU exposure in human colon cancer cells but is without any TS-repressive effect when used alone (21). IFN-α has been demonstrated to suppress E2F-1 mRNA expression after an 8-h exposure in Burkitt’s lymphoma cell line (26). Although the effects of UCN-01 on TS and E2F-1 expression are not the same as those of IFNs, they may share some similarities in terms of chemosensitivity enhancement. Chu et al. (21, 27) reported that the induction of TS after FU exposure was mediated by an increase in TS mRNA translational efficiency, and evidence also exists for TS translational autoregulation involving the TS protein and ternary complex. Given that there was a 20~30% discrepancy between the degrees of suppression in the TS protein and the TS mRNA levels by UCN-01, we speculate that UCN-01 may also affect TS translational autoregulation.

The regulatory mechanism for E2F-1 gene expression is not yet fully understood. Several lines of evidence have suggested that E2F-1 is controlled through precise temporal control mechanisms involving both transcriptional and post-transcriptional pathways (28, 29). Recently, it has been demonstrated that the regulation of E2F-1 protein levels could be mediated through ubiquitin-proteasome-dependent degradation, and complex formation with PBR blocks E2F-1 degradation (30, 31). Our data suggest that the down-regulation of E2F-1 protein levels by UCN-01 is mediated at the translational or posttranslational level. Additional investigations are warranted to determine whether any interactions exist between UCN-01 and the ubiquitin-proteasome system, and the effects of UCN-01 on DP-1 protein, which has been implicated directly or indirectly in the maintenance of E2F-1 function and stability. Preliminary data from our laboratory have supported the notion that UCN-01 suppresses E2F-1 at the posttranslational level through the activation of the ubiquitin-proteasome system.

We have shown UCN-01 suppressed TS gene expression mediated mainly by down-regulation of its transcriptional activator, E2F-1, in SK-GT5 cells. Furthermore, UCN-01 suppressed the induction of TS protein levels after FU exposure and significantly enhanced FU-induced apoptosis, especially when UCN-01 was given after FU exposure. The suppression of TS gene expression and the enhancement of FU-induced apoptosis by UCN-01 were similarly observed in colon cancer HCT-116 cells and breast cancer MDA-MB-468 cells. Therefore, these findings provide a novel way to modulate TS gene expression with a potential to improve FU-based chemotherapy, which has been widely used in the treatment of solid-tumor malignancies.

REFERENCES
UCN-01 suppresses thymidylate synthase gene expression and enhances 5-fluorouracil-induced apoptosis in a sequence-dependent manner.

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