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Report

Biochemical and molecular effects of UCN-01 in combination with 5-fluorodeoxyuridine in A431 human epidermoid cancer cells

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Concurrent and pre-exposure of A431 human epidermoid cancer cells to UCN-01, an investigational anticancer drug, with 5-fluoro-2′-deoxyuridine (FdUrd), which targets thymidylate synthase, produced more than additive cytotoxicty. A 24-h exposure to 10 nM FdUrd led to inhibition of TS, a 2.5-fold increase in total thymidylate synthase protein content, profound dTTP depletion and a 6.3-fold increase in the ratio of dATP to dTTP, but did not cause single-strand breaks in DNA. However, FdUrd enhanced UCN-01-associated DNA strand breaks. Concurrent thymidine exposure led to repletion of dTTP pools, and cytoprotection against FdUrd alone and with UCN-01. UCN-01 arrested cells in G_1, decreased the percentage of FdUrd-treated cells in S phase and reduced FdUrd-DNA incorporation, suggesting the latter was not important for cytotoxicity. Delayed induction of high molecular mass DNA fragmentation and poly(ADP-ribose) polymerase cleavage was observed with the combination of UCN-01 and FdUrd. These findings suggest that while FdUrd-mediated deoxynucleotide imbalance alone was insufficient to induce apoptosis in this p53-mutant cell line, it magnified UCN-01's effects, most likely by interfering with DNA repair. The clinical evaluation of UCN-01 combined with 5-fluoropyrimidines may be of interest. [© 2002 Lippincott Williams & Wilkins.]

Key words: 7-Hydroxy-staurospaurine, DNA damage, fluoropyrimidines, thymidylate synthase, UCN-01.

Introduction

UCN-01 (7-hydroxystaurospaurine) is an antibiotic isolated from *Streptomyces* species that functions as a selective inhibitor of calcium-dependent isoforms of protein kinase C (PKC).[1,2] It was selected for clinical development as an anticancer agent based on its potent anticancer activity against human cancer cell lines *in vitro*, and in several murine tumor and human xenograft models.[3] Although the concentration of UCN-01 that inhibits PKC by 50% in cell-free systems is similar to that of staurospaurine, the concentration required to inhibit protein kinase A is two orders of magnitude higher.[4] Of additional interest is the ability of UCN-01 to potentiate the cytotoxicity of various anticancer agents including γ-irradiation, mitomycin C, cisplatin, cytarabine and 5-fluorouracil (5-FUra).[5–13] Recent data suggest that UCN-01-mediated induction of apoptosis may be independent of effects on PKC.[14,15]

Information provided by Kyowa Hakko Kogyo, the pharmaceutical sponsor for UCN-01, indicated that a concurrent, 96-h exposure to 50 nM UCN-01 increased the sensitivity of A431 human epidermoid cancer cells, which are p53 mutants, to FUra by 10-fold. In the present study, we wished to investigate the mechanism of interaction in this cell line model.

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Materials and methods

Materials

UCN-01 was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). PBS (calcium- and magnesium-free phosphate buffered

saline, pH 7.4) was purchased from Biofluids (Rockville, MD), α-calf serum was from Hyclone (Logan, UT) and glutamine was from Gibco/BRL (Grand Island, NY). Isotopes ([^{3}H]FdUrd (5-fluoro-2′-deoxyuridine, 19 Ci/mmol; [5-^{3}H]dUMP, 23 Ci/mmol) and [^{14}C]thymidine, 56 Ci/mmol) were obtained from Moravek (Brea, CA). Unless otherwise stated, chemicals were obtained from Sigma (St Louis, MO).

Cytotoxicity

A431 cells (ATCC, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% α-calf serum and 2 mM glutamine. Cell counts were determined after trypsinization using a Multisizer II (Coulter, Miami, FL). After plating, the cells had a 48-h lag phase until they entered exponential growth, following which the doubling time was 21 h. To assess the effects of UCN-01, FUra, FdUrd alone or in combination, 1000 cells were plated in triplicate in 96-well plates. Forty-eight hours later, the drugs were added. After 24 h, the medium was gently aspirated, the cells were washed with PBS, and fresh medium and the desired drugs were added. After an additional 24 h, the cells were washed with PBS and incubated in fresh, drug-free medium. Cell proliferation was determined using the CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega, Madison, WI), in which MTS tetrazolium compound (Owen's reagent) is bio-reduced by cells to form a colored formazan product that is soluble in tissue culture medium. MTS was added at 72 h and after a 3-h incubation the absorbance at 490 nm was determined. The control absorbance at the end of the incubation period averaged 0.56 ± 0.04 (mean ± SEM, n=5 separate experiments). To assess any interaction between the two drugs, various combinations of UCN-01 and FUra or FdUrd were evaluated. The fraction affected values for each drug alone and the combination were calculated and the data were analyzed using CalcuSyn version 1.1 software for Windows (Biosoft, Ferguson, MO) assuming a mutually non-exclusive model.

To measure the ability of cells to recover following drug exposure, 1000 exponentially growing cells were dispensed into six-well plates in triplicate. Drugs were added 48 h later. After the desired drug exposure, the cells were washed twice with PBS and then incubated in drug-free medium for an additional 5 days. The cells were stained with 0.25% methylene blue in methanol and colonies consisting of 50 or more cells were enumerated.

FdUrd metabolism and DNA incorporation

Exponentially growing cells were exposed to [^{3}H]FdUrd (0.5 or 1 μCi/160 nmol). At the desired time point, the medium was aspirated and the cells were washed with cold PBS. For the nucleotide metabolism studies, the cells were extracted with 0.5 N perchloric acid; the acid-soluble fraction was isolated, neutralized and lyophilized. The radioactivity in an aliquot of the reconstituted sample was determined and the distribution of [^{3}H]FdUrd metabolites was measured by a reversed-phase ion-pairing HPLC method with on-line scintillation detection (Packard Instruments, Mount Prospect, IL) as previously described.[16] DNA was extracted using a PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN) as recommended by the manufacturer; trichloroacetic acid-precipitable counts retained on 0.45 μm HA filters were determined.[17]

Measurement of deoxynucleotide pools

Exponentially growing cells were exposed to no drug, 10 nM FdUrd, 0.1. or 0.2 μM UCN-01, or the combination. The cells were extracted with 0.5 N perchloric acid, neutralized, lyophilized and the samples were stored at −70°C until the time of analysis. Deoxyribonucleotide triphosphate pools were determined by a DNA polymerase assay using *Escherichia coli* Klenow fragment and synthetic oligonucleotides as template/primers.[18] The assay mixture was incubated for 15 min at 37°C, and the calibration curve for each dRTP was linear between 1 and 40 pmol of substrate.

Protein assays

A431 cells were exposed to no drug, UCN-01, FdUrd or the combination and cellular lysates were prepared from control or drug-treated cells in the presence of a protease inhibitor cocktail. Protein was quantified by the BioRad (Richmond, CA) protein assay kit using a standard curve constructed with bovine serum albumin, fraction V. Thymidylate synthase (TS) catalytic activity in cellular lysates was determined by tritium release from [5-^{3}H]dUMP as previously described.[19]

For Western analysis, equal amounts of protein were resolved by SDS–PAGE using either a 10% gel (100 μg each: TS, p53 and bcl-2) or a 7.5% minigel [40 μg poly(ADP-ribose) polymerase (PARP)]. The primary antibodies were TS106 monoclonal antibody,[20] and murine monoclonal antibodies α-tubulin

(Ab-1), PARP (Ab-2), TS, p53 (Ab-3) and bcl-2 (Ab-3) (obtained from Oncogene Research Products, Calbiochem, Cambridge, MA). Horseradish peroxidase goat anti-mouse IgG was used as the secondary antibody. The antigen–antibody complexes were visualized using the Enhanced Chemiluminescence kit (Amersham, Evanston, IL). The blots were stripped and re-probed with antibody to α-tubulin to permit correction for possible differences in protein loading. The relative quantities of proteins on scanned images were analyzed using Sigmagel for Windows version 1.0 (Jandel Scientifics, San Rafael, CA).

Cell cycle analysis

Following the desired drug exposure, cell nuclei were isolated, incubated in propidium iodide and DNase-free RNase for 60 min, and then gently filtered though 35-μm strainer caps into 12×75 mm polystyrene tubes. DNA histogram data were collected using a FACScan (Becton Dickinson, San Jose, CA). The list-mode files were analyzed with ModFit LT for Win32 version 2.0 software (Verity Software House, Topsham, ME).

Assessment of DNA damage

A431 cells were labeled with [^{14}C]thymidine (0.05 μCi/ml). for the final 4 h of drug exposure and induction of single-strand breaks in newly synthesized DNA was determined by alkaline elution at a fixed pH of 12.1 as previously described.[17,21] The total radioactivity was defined as the sum of the d.p.m. in each elution fraction plus that retained on the filter, less background.

To assess induction of parental DNA fragmentation, cells were exposed to either no drug, UCN-01, FdUrd or the combination for up to 72 h. The medium was removed and fresh drug was added every 24 h. At the desired times, the floating and adherent cells were collected; intact cells were embedded in agarose plugs (200 000 cells/plug), and digested *in situ* at 50°C for 48 h to remove protein and RNA. The conditions for pulsed field gel electrophoresis have previously been described.[21]

mRNA Extraction, cDNA synthesis and PCR quantitation of TS mRNA

mRNA was isolated using the QuickPrep micro-mRNA isolation kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. The mRNA was reverse transcribed using random hexamers as previously described.[22] Real-time quantitative PCR amplification was performed using specific target, doubly labeled fluorigenic probes with the ABI Prism 7700 sequence detection system (PE Applied Biosystem, Foster City, CA). The expression of the β-actin gene was used as an internal standard. The Taqman probes were labeled with 6-FAM (5′-end) and TAMRA (3′-end); the primers and probe sequences have been previously reported.[23] The Taqman mix was prepared according to the manufacturer's instructions using the $10 \times$ Taqman 1000 Reaction Gold/Buffer A kit which contains Taqman buffer A, AmpliTaq Gold 5 U/μl and 250 mM $MgCl_2$, ultrapure dNTP stock solutions (10 mM each of dCTP, dATP and dGTP, and 20 mM of dUTP). Separate primer mixes for each gene of interest were then prepared using Taqman mix, the appropriate forward and reverse primers, and the Taqman probe, and 27 μl was then placed in each 0.5-ml tube in a 96-well holder. Serial dilutions of cDNA were prepared and 3 μl of cDNA added to the Taqman primer mix. After careful mixing, 25 μl was transferred into the complementary well of a MicroAmp Optical 96-well reaction plate. After an initial hold at 95°C to permit activation of AmpliTaq Gold DNA polymerase, 40 cycles of real-time PCR were performed that consisted of 95°C for 15 s and 60°C for 1 min. The relative expression of TS to β-actin mRNA for each condition was then calculated.

Results

Cytotoxicity

A 24-h exposure to either FUra or FdUrd produced concentration-dependent inhibition of cell proliferation at 72 h as determined by bio-reduction of MTS tetrazolium by metabolically active cells to a soluble, colored formazan product: IC_{50} values were around 1.1 μM and 27 nM, respectively. The IC_{50} values of UCN-01 for 24- and 48-h exposures were 0.44 μm and 0.21 μM, respectively. To test whether UCN-01 could potentiate the cytotoxicity of either FUra or FdUrd, fixed concentrations of UCN-01 (either 0.1 μM for 24 or 48 h, or 0.2 μM for 24 h) were combined with a range of fluoropyrimidine concentrations. UCN-01 shifted the concentration–effect curve for both FUra and FdUrd to the left, consistent with enhanced cytotoxicity (FdUrd data shown in Figure 1). Both concurrent and pre-exposure to UCN-01 was associated with synergism, as reflected by a combination index < 1, and concurrent exposure to

FdUrd and 200 nM UCN-01 for 24 h produced the greatest interaction (Table 1). For each condition, the interaction of UCN-01 with FdUrd was greater than with FUra (data not shown). Therefore, additional experiments used fixed concentrations of UCN-01 and FdUrd that individually produced about 25–40% reduction in MTS staining at 72 h.

Since the MTS assay reflected effects on growth inhibition during drug exposure, we evaluated recovery from the cytotoxic effects of drug exposure by a colony formation assay in which cells were stained after a further 5-day incubation in drug-free cell culture medium. A 24-h exposure to 10 nM FdUrd reduced colony formation to 56% of control. A 24-h exposure to 0.2 μM or a 48-h exposure to 0.1 μM UCN-01 reduced the colony number to 69 and 82% of control, respectively, but the addition of FdUrd reduced these numbers to below 5%. These results provide supporting evidence that growth-inhibitory, but non-lethal exposures to FdUrd and UCN-01 were associated with a profound reduction in colony formation.

Table 1. Combination indices (CI) for UCN-01 plus FdUrd

UCN-01 (nM)	FdUrd (nM) 24−48 h	CI
200 h 24–48 (FI=0.42)	0.5	0.151
	1	0.113
	5	0.100
	10	0.058
	50	0.089
	100	0.044
100 h 0–48 (FI=0.22)	0.5	0.453
	1	0.450
	5	0.315
	10	0.477
	50	0.375
	100	0.180
200 h 0–48 (FI=0.53)	0.5	0.308
	1	0.290
	5	0.296
	10	0.249
	50	0.257
	100	0.195

The fractional inhibition (FI) associated with a range of concentrations was determined for each drug alone and for various combinations. The FI associated with UCN-01 is shown. CI <1 signifies synergism, whereas CI=1 or CI >1 reflects additivity or antagonism, respectively.

Effect of UCN-01 on FdUrd metabolism and DNA incorporation

Following a 4-h exposure to [^{3}H]FdUrd, FdUMP (5-fluoro-2′-deoxyuridine monophosphate) was the predominant metabolite, accounting for 96 $\pm$ 2% (mean $\pm$ SE, n=5 experiments), while FdUrd comprised the balance. Neither 5-FU nor FdUTP were seen. Both pre-exposure to 0.1 μM UCN-01 for 20 h

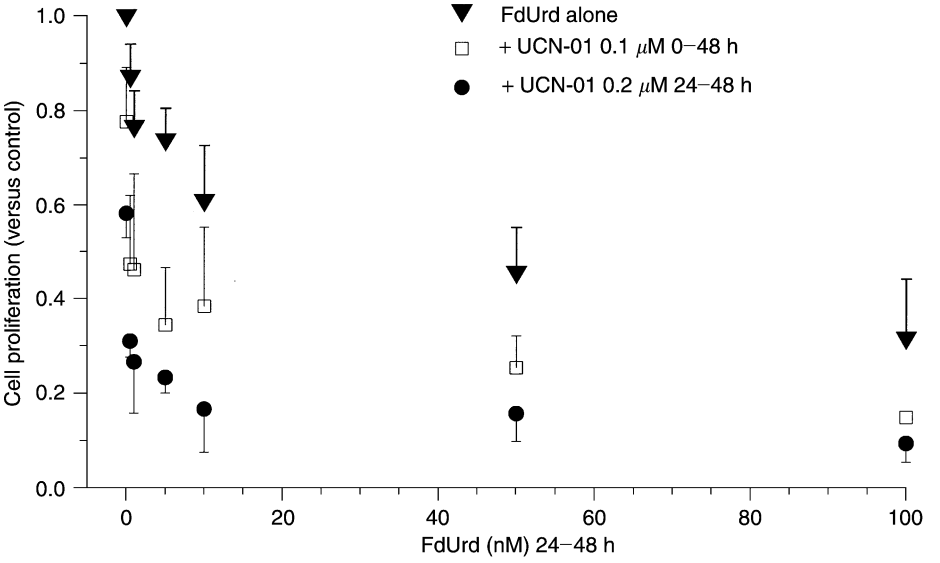

Figure 1. Cytotoxicity of FdUrd alone and in combination with UCN-01. Exponentially growing A431 cells were plated in replicates in 96-well plates (1000 cells/well). After the lag phase, the cells were exposed to the either diluent or 0.1 μM UCN-01. After 24 h, the medium was gently aspirated, the cells were washed, fresh drug-free medium was replaced, and either diluent, 0.2 μM UCN-01 and FdUrd at the indicated concentrations was added for 24 h. The cells were incubated in drug-free medium for the final 24 h, after which MTS reagent was assayed. The data are from four separate experiments done in replicate and the results are shown as the mean $\pm$ SE.

and concurrent exposure to 0.2 μM for the final 4 h decreased the amount of FdUMP formed by 1.7- and 2.9-fold, respectively (Table 2). The FdUMP detected in the acid-soluble fraction represents free metabolite that is not bound to TS, so a crucial issue is the potential impact of reduced FdUMP levels on TS inhibition (see the following section).

Incorporation of [^{3}H]FdUrd was detected in DNA, but not in RNA. Pre-exposure to 0.1 μM UCN-01 for 20 h prior to FdUrd (given for an additional 4 h) and concurrent exposure to 0.2 μM UCN-01 for 4 h significantly decreased FdUrd into DNA by 2.7- and 7.7-fold, respectively. The possible basis for this effect will be discussed later. Although 5-fluoropyrimidine incorporation into DNA contributes to cytotoxicity in some models, the observation that the combination of UCN-01 and FdUrd led to enhanced cytotoxicity despite a marked decrease in [^{3}H]FdUrd-DNA incorporation suggests this mechanism is not pertinent in the current model.

Impact of FdUrd alone or with UCN-01 on TS activity

TS protein expression in exponentially growing A431 cells is relatively low in comparison with other cancer cell lines (data not shown). TS catalytic activity in non-drug treated cells was only 3.6 ± 0.4 pmol/min/mg protein (mean $\pm$ SE, n=6 separate experiments) and the radioactivity in the supernatant (reflecting free ^{3}H released from the carbon-5 position of [5-^{3}H]dUMP) averaged only 1.7 ± 0.1-fold above background. Therefore, this assay was not sensitive enough to assess possible enzyme inhibition in the presence of FdUrd.

TS protein content was next evaluated by Western blot. Exposure to UCN-01 0.1 μM for 24 to 48 h, or to 0.2 μM for 4 or 24 h did not substantially alter the amount of TS protein compared to control (Figure 2). Exposure to FdUrd 10 nM for 4 and 24 h led to the appearance of TS bound in the ternary complex, reflected by a slower migrating protein band just above the 35-kDa-free TS band.[20] The total TS protein content (bound plus free) was 1.7- and 2.5-fold greater than controls after a 4- and 24-h exposure to FdUrd alone. The addition of UCN-01 to FdUrd did not affect the extent of ternary complex formation in this cell line.

We next determined TS mRNA expression relative to that of β-actin mRNA. The value for control cells harvested at 24 and 48 h was 9.23×10^{-3} and 7.55×10^{-3}. TS mRNA expression was 0.85- and 0.77-fold of the control level after cells were exposed to 10 nM FdUrd from 20 to 24 and 24 to 48 h, respectively (Figure 3). The FdUrd-associated increase in total TS protein content is therefore likely due to a post-transcriptional phenomenon. Exposure to UCN-01 0.1 μM for 24 and 48 h was associated with a modest decrease in TS mRNA expression, while TS mRNA expression was somewhat lower with 0.2 μM from 20 to 24 and 24 to 48 h. More pronounced effects were seen with the combination of UCN-01 and FdUrd.

Since dTTP depletion is a consequence of TS inhibition, we measured the effects of the drugs on dTTP pools. The endogenous dTTP pools averaged 33.7 pmol/10^6 cells (Figure 4, left). A 4-h exposure to 10 nM FdUrd led to significant depletion of dTTP to 2.0 pmol/10^6 cells. UCN-01 given alone did not appreciably affect dTTP pools compared to control and did not further enhance dTTP pool depletion when given with FdUrd. These results are consistent with the Western analysis results. FdUrd given alone or with UCN-01 was associated with a 6- to 8-fold increase in the dATP to dTTP ratio, signifying profound deoxyribonucleotide imbalance (data not shown).

Concurrent exposure to thymidine concentrations that were not toxic when given alone provided complete protection against FdUrd toxicity using the

Table 2. FdUrd metabolism and DNA Incorporation (mean $\pm$ SE)

FdUrd	UCN-01	FdUMP (pmol /10^6 cells)	FdUrd (fmol/μg DNA)
10 nM $\times$ 4 h	none	2.11 ± 0.55	–
10 nM $\times$ 4 h	0.1 μM $\times$ 24 h	1.27 ± 0.26	–
10 nM $\times$ 4 h	0.2 μ M $\times$ 4 h	0.73 ± 0.28	–
10 nM $\times$ 24 h	none	–	0.54 ± 0.14
10 nM $\times$ 24 h	0.1 μM $\times$ 24 h	–	0.20 ± 0.07[a]
10 nM $\times$ 24 h	0.2 μ M $\times$ 24 h	–	0.07 ± 0.07[b]

Exponentially growing A431 cells were exposed to [^{3}H]FdUrd (0.5 or 1 μCi) for either 4 h (h 20–24) or 24 h alone or in combination with UCN-01 at the indicated concentrations. After drug exposure, FdUrd metabolism (n=5 separate experiments) and DNA incorporation (n=3 separate experiments) were determined as described in the methods. There was trend for lower FdUMP formation in the presence of either UCN-01 condition compared to FdUrd alone (p=0.066, t-test). The amount of [^{3}H]FdUrd incorporated into DNA in the presence of UCN-01 was significantly lower: 0.1 μM, [a]$p < 0.05$; 0.2 μM, [b]$p < 0.01$ (t-test).

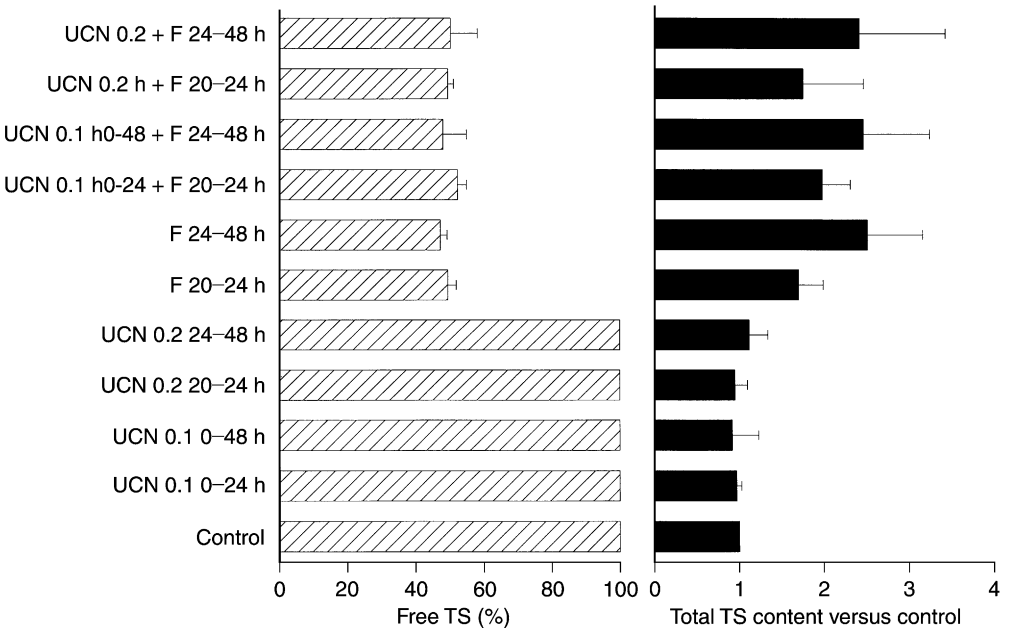

Figure 2. Effect of FdUrd and UCN-01 on TS ternary complex formation. A431 cells were exposed to diluent, UCN-01 for 24–48 h, FdUrd 10 nM from either 20 to 24 or 24 to 48 h, or the combination. Cellular lysates were prepared, and 100 μg protein was resolved on a 10% SDS–PAGE gel using a 1:100 dilution of TS106 monoclonal antibody and a 1:5000 dilution of goat anti-mouse IgG antibody. The signal was detected by enhanced chemiluminescence. The left panel shows the percent unbound TS protein, while the right panel shows total TS protein content (free plus bound). The data are presented as the mean $\pm$ SE and are from four to six separate experiments.

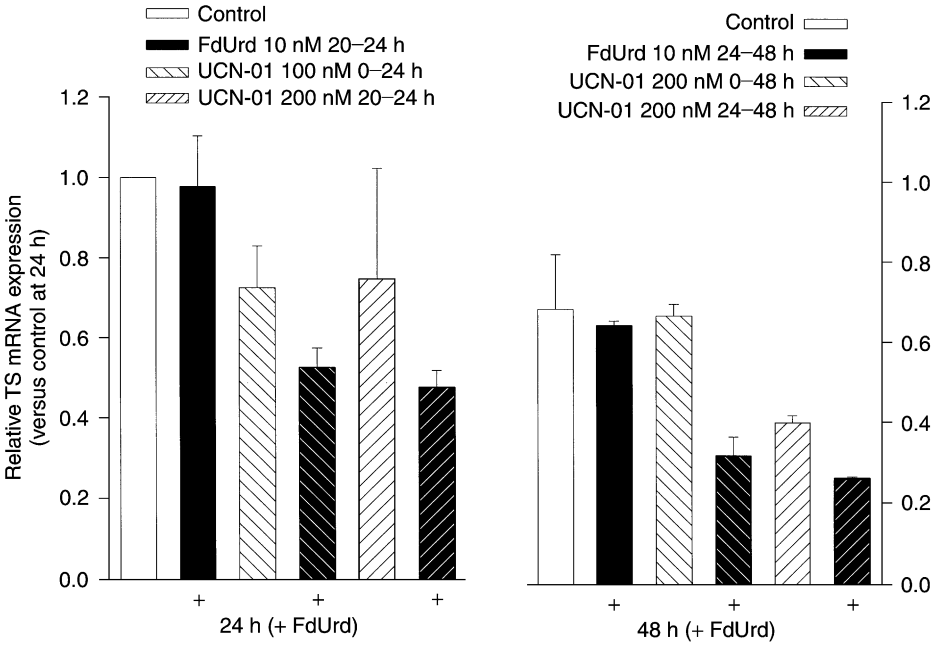

Figure 3. TS mRNA expression during UCN-01 and FdUrd exposure. A431 cells were exposed to UCN-01 and FdUrd at the indicated concentrations and durations, and then harvested and frozen at -70°C. mRNA was purified, and mRNA expression of both TS and β-actin was determined by a quantitative RT-PCR method. The left and right panels show mRNA expression of TS relative to β-actin in cells harvested at 24 and 48 h, respectively. The data for each condition are presented relative to the control TS mRNA expression at 24 h. The data, from two separate experiments, are shown as the mean $\pm$ 1/2 range.

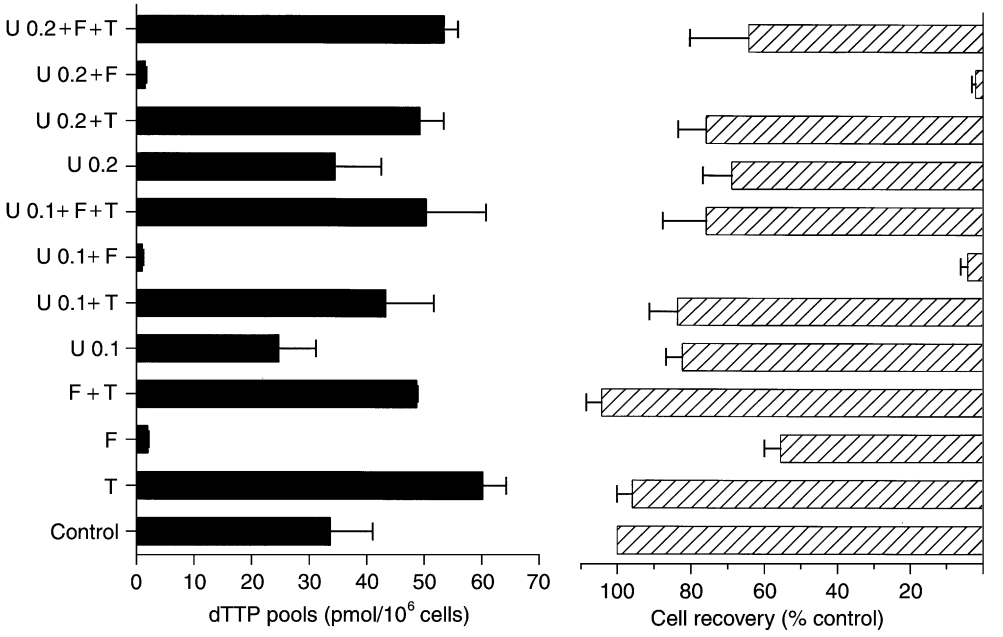

Figure 4. Thymidine protection and repletion of dTTP pools. A431 cells were exposed to no drug, UCN-01 or FdUrd as indicated. Deoxyribonucleotide levels were determined by an enzymatic DNA polymerase assay; each experiment was done in triplicate. The cells were harvested after a 4 h exposure to 10 nM FdUrd, 0.5 μM thymidine and 0.2 μM UCN-01, and a 24 h exposure to 0.1 μM UCN-01. The data, presented as the mean $\pm$ SE, are from at least four separate experiments. The cell recovery assays were performed after a 24-h exposure to 10 nM FdUrd, 0.5 μM thymidine and 0.2 μM UCN-01, and a 48-h exposure to 0.1 μM UCN-01.

cell recovery assay, but did not affect the toxicity of UCN-01 (Figure 4, right). However, thymidine exposure substantially reduced the toxicity of the combination of UCN-01 and FdUrd. Thymidine protection was associated with repletion of dTTP pools in cells treated with FdUrd alone or with UCN-01 (Figure 4, left).

Cell cycle effects

A 24-h exposure to 10 nM FdUrd led to S phase accumulation, while exposure to UCN-01 was accompanied by G_1 arrest (Table 3). With concurrent UCN-01 and FdUrd, an intermediate pattern was seen in G_1 and S phase distribution, but in all drug-treated cells the percent of cells in G_2/M was much lower than in control cells. A sub-G_0 peak was not seen in any of the drug-treated conditions.

Induction of DNA damage

A431 cells that were labeled with [^{14}C]thymidine during the final few hours of drug exposure were subjected to alkaline elution. A 24-h exposure to FdUrd 10 nM alone did not produce noticeable effects, while UCN-01 exposure was associated with a concentration-dependent increase in the fraction of nascent DNA eluting (Figure 5). Concurrent exposure to 10 nM FdUrd and UCN-01 produced a greater degree of nascent DNA damage compared to UCN-01 alone.

PARP is involved in the repair of DNA and maintenance of the genome. Cleavage of the intact form of PARP into two smaller fragments ($\sim$85 and 25 kDa) results in loss of its function. As shown in Figure 6(A), there was minimal evidence of PARP cleavage exposure when cells were exposed to either 10 nM FdUrd from 20 to 24 h, UCN-01 0.2 μM from 20 to 24 h or 0.1 μM from 0 to 24 h, or the combination. When the duration of exposure was increased, a small amount of PARP cleavage was seen with the combination of UCN-01 0.1 μM 0–48 h plus FdUrd 10 nM 24–48 h (Figure 6B, lane E; 5.5% cleaved), while more prominent PARP cleavage was seen with UCN-01 0.2 μM and FdUrd 10 nM both given from 24 to 48 h (Figure 6B, lane f; 18.3% cleaved).

The effect of FdUrd and UCN-01 on high molecular mass DNA fragmentation was evaluated by pulsed field gel electrophoresis. In control cells (Figure 7,

Table 3. FdUrd and UCN-01 effects on cell cycle distribution

Condition	Cell cycle phase (%)		
	G_1	S	G_2/M
Control	66.2	18.3	15.5
FdUrd 10 nM × 24 h	58.5	38.7	2.7
UCN-01 100 nM × 24 h	79.3	14.0	6.7
UCN-01 200 nM × 24 h	83.0	9.6	7.4
UCN-01+FdUrd 10 nM × 24 h	68.7	25.9	5.4
UCN-01 200 nM+ FdUrd 10 nM × 24 h	78.1	17.3	4.6

The cell cycle distribution was determined after a 24 h exposure to no drug, FdUrd, UCN-01 or the combination at the indicated concentrations. The data represent information collected on 20 000 cells for each condition. Similar results were seen in separate experiments.

lanes 1, 8 and 15), virtually all of the DNA remained at the site of origin. Minimal high molecular mass DNA fragmentation was seen with either UCN-01 alone (lanes 2–4), FdUrd alone (lanes 5 and 9–11) or the combination (lanes 6 and 7) with exposure times of 16 and 24 h. When the duration of exposure to FdUrd was extended to 48 h, DNA migrating between the 48- and 1000-kb molecular weight markers was evident with 10, 50 and 100 nM (lanes 12–14). A similar DNA fragmentation pattern was seen with a 48-h exposure to 0.2 μM UCN-01 (lane 16), 10 nM FdUrd (lane 17) and the combination (lane 18). The degree of fragmentation was more prominent at 72 h (lanes 19–21). These results suggest a gradual onset of high molecular mass DNA fragmentation in A431 cells, consistent with delayed apoptosis. Taken together, these results suggest that FdUrd enhanced UCN-01-associated nascent single-strand breaks and parental DNA fragmentation, which appeared to be independent of acute changes in p53 protein content.

Discussion

Our studies were prompted by information from Kyowa Hakko Kogyo that a concurrent 96-h exposure to 50 nM UCN-01 reduced the IC_{50} of FUra in A431 cells from 71 to 7.4 nM. The A431 cells are a human epidermoid cancer developed from a patient with vulvar carcinoma. This cell line is of interest because it possesses a mutated p53 gene, but appears to be extremely sensitive to FUra. In contrast, cell lines with mutated p53 have been reported to be relatively insensitive to Fura.[24,25] UCN-01 enhances the cytotoxicity of a variety of anticancer agents with different intracellular targets and, in some cases, the interaction appears to be greater in cells that harbor mutant p53.[7,26,11]

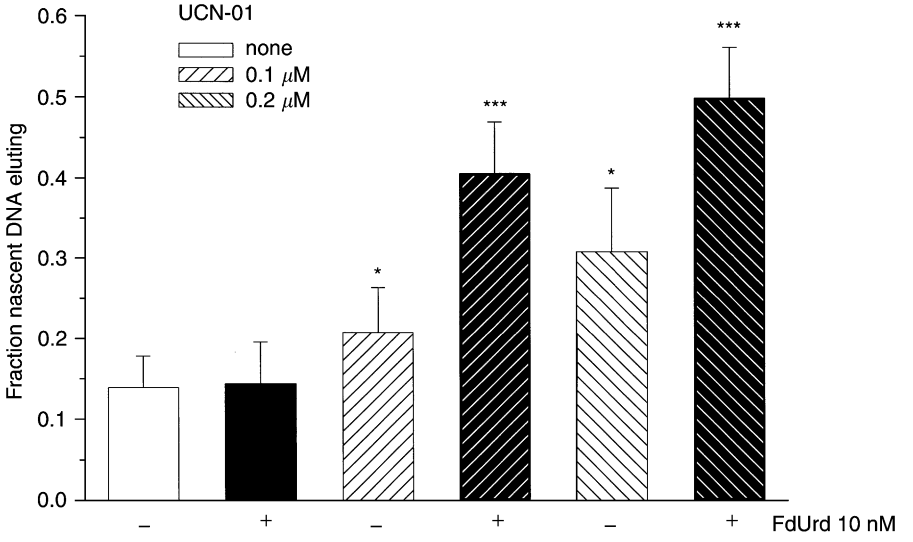

Figure 5. Damage of nascent DNA with UCN-01 and FdUrd. A431 cells were exposed to diluent, UCN-01, FdUrd or the combination at the indicated concentrations for 24 h. Cells were pulsed with [^{14}C]thymidine for the final 4 h of drug exposure and the DNA was eluted through nucleopore filters at a fixed, alkaline pH (12.1) over a 15-h period. The proportion of [^{14}C]thymidine that eluted relative to the total [^{14}C]DNA for each condition is presented as the mean ± SE from four separate experiments. The degree of nascent DNA damage was significantly different from control as follows: UCN-01 0.1 and 0.2 μM, $*p < 0.05$; FdUrd plus UCN-01 0.1 or 0.2 μM ($**p < 0.01$, paired *t*-test).

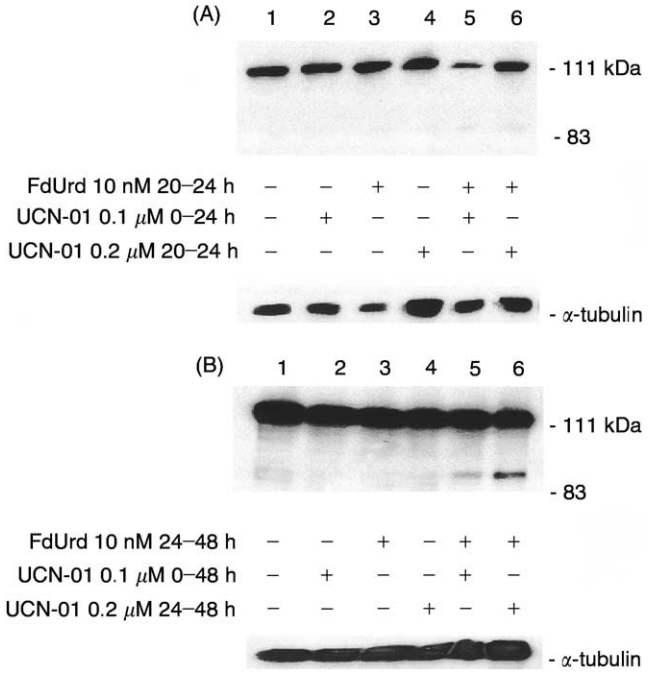

Figure 6. Effect of UCN-01 and FdUrd on PARP cleavage. A431 cells were exposed to diluent, UCN-01 for 24–48 h, FdUrd 10 nM from either 20 to 24 or 24 to 48 h, or the combination. Cellular lysates were prepared, and 40 μg protein were resolved on a 7.5% SDS–PAGE minigel. (A) Results with cells exposed to UCN-01 FdUrd 10 nM for 4 h, UCN-01 0.1 μM for 24 h or 0.2 μM for 4 h alone or in combination. (B) Results with a 24-h exposure to FdUrd, 0.2 μM UCN-01 for 24 h, 0.1 μM UCN-01 for 48 h alone and in combination. Cleaved PARP products are detected in lanes 5 and 6. The gels were stripped and re-probed for α-tubulin as a protein-loading control. Similar results were seen in other experiments.

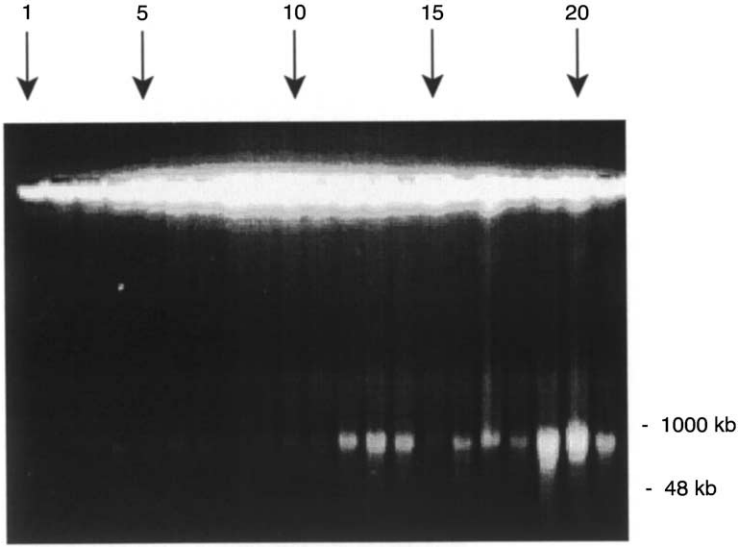

Figure 7. Delayed induction of high-molecular mass DNA fragmentation. At various times, adherent and non-adherent cells were harvested. Intact cells were embedded in agarose plug, and digested *in situ* to remove protein and RNA. The DNA plugs were then subjected to pulsed field gel electrophoresis. Control or drug-treated A431 cells are in the following lanes: lanes 1–8, 16 h exposure: control, lane 1; UCN-01 0.1, 0.2 or 0.5 μM, lanes 2–4; FdUrd 10 nM alone, lane 5; FdUrd+UCN-01 0.1 or 0.2 μM, lanes 6–7; lanes 8–11, 24 h exposure: control, lane 8; FdUrd 10, 50 and 100 nM, lanes 9–11; lanes 12–18, 48 h exposure: FdUrd 10, 50 and 100 nM, lanes 12–14; control, lane 15; UCN-01 0.2 μM alone (lane 16), FdUrd 10 nM alone (lane 17), or the combination of UCN-01+FdUrd (lane 18); lanes 19–21, 72 h exposure: UCN-01 0.2 μM alone (lane 19), FdUrd 10 nM alone (lane 20), or the combination of UCN-01+FdUrd (lane 21). Similar results were seen in independent experiments.

We found that the combination of UCN-01 with either FUra or FdUrd produced more than additive cytotoxicity. Overall, the greatest effects were seen with concurrent exposure for 24 h, with an average combination index of around 0.09 for FdUrd and around 0.28 for FUra. With each of the UCN-01/fluoropyrimidine schedules evaluated, the combined effects were greater with FdUrd, a more selective inhibitor of TS.

The metabolism studies using the acid-soluble cellular extract indicated that FdUMP was the predominant metabolite, while FdUrd accounted for the balance. Macromolecules, including FdUMP covalently bound to TS protein, are precipitated by the acid. Although free FdUMP pools tended to be lower in cells treated with UCN-01, the crucial issue is the extent of TS inhibition. Assessment of inhibition of TS activity by biochemical assays proved difficult, since the TS activity in control cells was very low. However, immunoblot was useful in assessing free and bound TS content, and summation of these two TS bands in FdUrd-treated cells permitted an estimate of any possible increases in total TS protein content compared to control cells. TS ternary complex accounted for about 50% of the total TS in cells exposed to 10 nM FdUrd for either 4 or 24 h and concurrent or pre-exposure to UCN-01 did not appear to affect the percent of bound TS. Total TS protein content increased by several fold after a 24-h exposure to 10 nM FdUrd, which is of a similar magnitude to that reported with FUra treatment in both preclinical and clinical studies.[27–31] The basis for this phenomenon is generally attributed to increased synthesis of TS protein,[30,32] although increased stability of TS protein may also play a role.[33] Exposure to UCN-01 alone did not affect total TS protein content, nor did it diminish total TS protein content in A431 cells treated with FdUrd.

It is possible that analysis of ternary complex formation by Western analysis may underestimate the extent of inhibition of TS in intact cells if some disruption of the ternary complex were to occur during the interval between the preparation of the cellular lysate prior to loading on the SDS–PAGE gel. Since the functional consequence of TS inhibition is depletion of dTTP, we compared dTTP levels in drug-treated cells with basal levels in control cells using a sensitive enzymatic assay. As anticipated, dTTP pools were profoundly decreased during FdUrd exposure. UCN-01 exposure by itself did not appreciably alter dTTP pools and the combination of UCN-01 with FdUrd did not lead to further depletion of dTTP; these findings are consistent with the immunoblot results. Thus, despite apparent UCN-01-associated decrease in free FdUMP formation, no adverse impact on TS inhibition was evident by either ternary complex formation or dTTP depletion. Exposure to FdUrd alone or with UCN-01 was accompanied by a pronounced increase in the ratio of dATP to dTTP and such deoxyribonucleotide triphosphate imbalances may promote DNA damage in some models.[34–38] Using a quantitative real-time RT-PCR method, we found that the level of TS mRNA relative to β-actin was very low in A431 cells compared to our experience with dozens of other cancer cell lines.[23] No change in TS mRNA expression was observed following either a 4- or 24-h exposure to 10 nM FdUrd compared to control cells. Relative TS mRNA expression with UCN-01 given in combination with FdUrd from 20 to 24 h was lower than that seen with UCN-01 given alone. When cells were harvested at 48 h, relative TS mRNA expression appeared to be lower than the comparable conditions at 24 h, including the control cells, suggesting that TS mRNA expression decreased with increasing time in culture. The lowest TS mRNA levels at 48 h were observed with both UCN-01/FdUrd conditions. The decreases in mRNA expression were not accompanied by noticeable changes in TS protein content, indicating a discontinuity between TS mRNA and protein expression in drug-treated A431 cells. The decreases in TS mRNA may be due to cell cycle perturbations and/or enhanced DNA damage, as summarized below.

Our conclusions are different than that of a previous report[13] in which the SK-GT5 human gastric cancer cell line was employed. In this model, a 24-h exposure to 1 μM UCN-01 led to a marked decrease in TS protein content by Western blot, which appeared to be due to a decrease in TS mRNA expression as assessed by Northern blot. Further, a 24-h exposure to 50 μM FUra followed by a 24-h exposure to 1 μM UCN-01 was associated with greater induction of apoptosis compared to either concurrent exposure or the opposite sequence. This sequence-dependent toxicity to UCN-01 mediation was attributed to suppression of TS mRNA expression. The SK-GT5 cell line is relatively insensitive to FUra compared to the A431 cell line and higher concentrations of UCN-01 were employed; both factors may contribute to the different findings in the two models. Since our studies were based on the observation that concurrent exposure to low concentrations of UCN-01 potentiated FUra toxicity in A431 cells, we did not explore sequential FUra followed by UCN-01.

Although UCN-01 decreased FdUrd incorporation into DNA, the increased cytoxicity and DNA damage seen with the combination suggests that FdUrd-incorporation is not essential for these events in this cell line. In agreement with other studies, we found that UCN-01 led to profound G_1 phase accumulation.[7,26,39–41] Moreover, UCN-01 attenuated FdUrd-mediated S phase accumulation. Hsueh reported that UCN-01 exposure was accompanied by down-regulation of E2F1, which is known to play a pivotal role in regulating the transcription of genes required for DNA synthesis including thymidine kinase.[42–44] The cell cycle effects of UCN-01 that result in G_1 arrest likely account for the decreased formation of FdUMP and incorporation of FdUrd into DNA.

A FdUrd concentration that did not induce strand breaks in nascent DNA or fragmentation of parental DNA increased UCN-01-associated DNA damage and a likely explanation is FdUrd-mediated effects on deoxynucleotide pools with the potential for interference with DNA repair. This hypothesis is supported by the observation that exposure to thymidine at concentrations sufficient to replete dTTP pools protected against the cytotoxicity of FdUrd given alone and in combination with UCN-01. Induction of programmed cell death following drug exposure in these cells appeared to be a delayed event. A431 cells are p53 mutants, which may partially account for the relative inability of moderate genotoxic stress to trigger rapid induction of programmed cell death.

In summary, we found that both concurrent and pre-exposure to UCN-01 with FdUrd and FUra was associated with enhanced cytotoxicity, although the effects were more pronounced with FdUrd. In contrast to other reports in which relatively non-toxic concentrations of UCN-01 augmented the cytotoxicity of DNA-damaging agents, in this model FdUrd appears to potentiate the DNA damage associated with UCN-01 exposure. Clinical trials evaluating the combination of UCN-01 with 5-FU or FdUrd may be of interest, particularly if biochemical and/or molecular endpoints are integral components of the trial.

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