Biochemical Pharmacology, Vol. 58, pp. 477–486, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00099-4

Sequence-Dependent Antagonism between Fluorouracil and Paclitaxel in Human Breast Cancer Cells

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ABSTRACT. The effects of 24-hr exposures to 5-fluorouracil (FUra) and paclitaxel in various sequences were studied in MCF-7 breast cancer cells to determine an optimal schedule for possible clinical use. In clonogenic assays, pre-exposure to FUra followed by paclitaxel resulted in marked antagonism, while sequential paclitaxel followed by FUra was optimal. Concurrent or pre-exposure to paclitaxel did not affect [^{3}H]FUra metabolism, [^{3}H]FUra-RNA incorporation, or the extent of FUra-mediated thymidylate synthase inhibition. Paclitaxel led to G_2/M phase accumulation that persisted for up to 24 hr after drug exposure, while a 24-hr FUra exposure produced S-phase accumulation. FUra pre-exposure diminished paclitaxel-associated G_2/M phase block, whereas subsequent exposure to FUra after paclitaxel did not. FUra exposure resulted in transient induction of p53 and p21, which returned to basal levels 24 hr after drug removal. p53 and p21 protein content also increased markedly during paclitaxel exposure, accompanied by phosphorylation of Bcl-2. Double-stranded DNA fragmentation (~ 50 kb) was seen at 48 hr when cells were exposed to paclitaxel for an initial 24-hr period. Paclitaxel-associated DNA fragmentation was not prevented by concurrent or subsequent exposure to FUra. Thus, paclitaxel-mediated G_2/M phase arrest appeared to be a crucial step in induction of DNA fragmentation. Since an initial 24-hr paclitaxel exposure did not interfere with subsequent FUra metabolism or thymidylate synthase inhibition, and delayed exposure to FUra did not impede either paclitaxel-mediated induction of mitotic blockade or DNA fragmentation, the sequence of paclitaxel followed by FUra is recommended for clinical trials. BIOCHEM PHARMACOL **58**;3:477–486, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. taxanes; fluorouracil; drug interactions; DNA damage; p53; p21; Bcl-2

Paclitaxel (Taxol®) is a member of a novel class of antitumor agents, the taxanes, derived from the bark of the Pacific yew tree, *Taxus brevifolia*. Paclitaxel binds to the β-subunit of tubulin in the microtubule and promotes the formation of extremely stable microtubules [1–7]. The impaired ability of paclitaxel-stabilized microtubules to disassemble in turn interferes with their normal functions, including formation of the mitotic spindle apparatus during cell division, maintenance of cell shape, motility, anchorage, mediation of signals between surface receptors and the nucleus, and intracellular transport. Antitumor activity has been shown in preclinical studies against several cell lines and murine xenografts, and clinical trials have confirmed its activity against a variety of epithelial malignancies.

FUra§, a fluorinated analog of the pyrimidine base uracil, is used in a variety of chemotherapeutic combinations in the treatment of solid tumors [8]. FUra requires intracellular activation to FdUMP and FUTP to exert its cytotoxic effects. Incorporation of FUTP into RNA can affect normal RNA processing and function. FdUMP competes with dUMP, the normal substrate, for binding to TS (EC 2.1.1.45), resulting in enzyme inhibition and depletion of thymidine triphosphate pools. Incorporation of the fraudulent nucleotides dUTP and FdUTP into DNA stimulates excision repair by uracil-DNA glycosylase. In the setting of decreased dTTP and increased dUTP, a futile cycle may ensue, accompanied by interference with nascent DNA synthesis and integrity.

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Received 6 July 1998; accepted 23 November 1998.

§ *Abbreviations*: FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FUTP, 5-fluorouridine triphosphate; FdUTP, 5-fluoro-2′-deoxyuridine triphosphate; PBS, PBS (pH 7.4) without calcium or magnesium; and TS, thymidylate synthase.

Paclitaxel has been combined with other active cytotoxic agents including cisplatin, doxorubicin, and cyclophosphamide, and prominent sequence-dependent interactions have been described. For example, the sequence of exposure to paclitaxel and cisplatin is a critical determinant of cytotoxicity. Administration of paclitaxel prior to cisplatin is more cytotoxic against cancer cells *in vitro* than the reverse sequence, whereas sequential paclitaxel followed by cisplatin is less myelosuppressive in clinical trials than the reverse sequence [9–11]. A greater degree of myelosuppression is seen if paclitaxel is given prior to cyclophosphamide, compared with giving the latter agent prior to paclitaxel [12]. More severe mucositis and myelosuppression occur when a 24-hr infusion of paclitaxel precedes doxorubicin given either by a 48-hr infusion or by bolus injection, compared with either concurrent infusion or the sequence of doxorubicin preceding paclitaxel [13]. The combination of FUra and paclitaxel represents a reasonable therapeutic strategy for the treatment of malignancies, including breast cancer and squamous cell cancers, in which both drugs are active, since their mechanisms of action and spectra of clinical toxicity are different. Before designing a clinical trial based on an empiric schedule, we wished to study the interaction of FUra combined with paclitaxel *in vitro* in MCF-7 human breast cancer cells to identify any potentially antagonistic schedules and select the optimal sequence. We found that pre-exposure to FUra for 24 hr followed by paclitaxel for 24 hr was antagonistic, whereas the sequence of paclitaxel followed by FUra appeared to be a better choice than simultaneous exposure. The basis for this sequence-dependent interaction is described herein.

MATERIALS AND METHODS

Materials

Moravek Biochemicals supplied the [^{3}H]paclitaxel (23 Ci/mmol), [^{3}H]FUra (20 Ci/mmol), and [5-^{3}H]dUMP (25 Ci/mmol). PBS was purchased from Biofluids Inc. Fetal bovine serum, RPMI 1640 tissue culture medium, and glutamine were obtained from Gibco BRL. Unless otherwise stated, chemicals were obtained from the Sigma Chemical Co. Paclitaxel was obtained from the Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, National Cancer Institute.

Growth and Clonogenic Assays

MCF-7 cells were grown in complete medium consisting of RPMI 1640 medium supplemented with 9% fetal bovine serum and 1% L-glutamine at 37° under a humidified, 5% CO_2 atmosphere. Exponentially growing cells were plated into 6-well tissue culture plates (cell growth, ~ 10,000 cells/well; colony formation, ~ 500 cells/well), and drugs or diluent were added the following day. After a 24-hr drug exposure, the medium was aspirated, the cells were gently washed twice with RPMI medium, and fresh drug-free medium was replaced. Either diluent, FUra, or paclitaxel then was added for another 24-hr exposure. The cells were washed again and incubated in drug-free medium for an additional day (cell growth) or 7 days (clonogenic assays). To assess the interaction of paclitaxel and FUra, multiple drug concentrations at a fixed ratio of paclitaxel to FUra were studied. Cell counts were determined after trypsinization with a Coulter® Multisizer II (Coulter Corp.). The doubling time of the cells was 19.8 ± 1.2 hr. Colonies were stained with 0.125% methylene blue in 50% methanol, and colonies of ≥ 50 cells were enumerated. The cloning efficiency for control cells was 42 ± 3%. The fractional inhibition associated with a range of concentrations was determined for each drug alone, and for the various combinations. The data were analyzed by CalcuSyn© version 1.1 software (Biosoft), assuming a mutually non-exclusive model. The combination index was used to signify antagonism (> 1), additivity (= 1), or synergism (< 1).

Measurement of FUra Metabolites and RNA Incorporation

Exponentially growing cells were exposed to 40 nM paclitaxel, 4 μM [^{3}H]FUra (final sp. act. 0.25 μCi/nmol), or the combination. After a 24-hr incubation, the cells exposed to [^{3}H]FUra were washed three times with ice-cold PBS and extracted with 3 mL of 0.5 N perchloric acid. The acid-soluble fraction was isolated, neutralized, and lyophilized. For the cells pre-exposed to paclitaxel, the medium was aspirated, and the cells were washed gently with RPMI medium, placed in fresh medium containing 4 μM [^{3}H]FUra, and harvested 24 hr later. The radioactivity in an aliquot of the reconstituted sample was determined, and the distribution of [^{3}H]FUra metabolites was determined by an ion-pairing reversed-phase HPLC method with in-line scintillation detection as described previously [14].

For measurement of FUra-RNA incorporation, [^{3}H]FUra was added for the final 2 hr (final sp. act. 0.125 μCi/nmol). The cells were washed three times with ice-cold PBS, and the RNA was isolated and purified with the RNA Stat-30™ kit (Tel-Test "B," Inc.) using RNAzolB/chloroform followed by isopropanol precipitation as recommended by the manufacturer. The RNA pellet was dissolved with 1 mL of 10 mM Tris/1 mM EDTA, pH 8.0; UV absorbance was measured at 260/280 nm, and TCA-precipitable counts retained on 0.45-μm HA filters (25 mm diameter; Millipore Corp.) were determined [15].

TS Catalytic Activity

MCF-7 cells were exposed to 4 μM FUra and 40 nM paclitaxel alone and in combination for 24 hr, or to sequential 24-hr exposures of FUra first followed by paclitaxel, FUra first followed by no drug (24 hr drug-free), or the sequence of paclitaxel followed by FUra. The cells were harvested, collected by centrifugation, and frozen at –70° until analysis. TS catalytic activity in cellular lysates was determined by tritium release from [5-^{3}H]dUMP [16, 17].

Protein concentration was assessed by the Bio-Rad protein assay kit (Bio-Rad Laboratories).

Cell Cycle Analysis

Permeabilized cells were incubated in 2 mL of Vindelov's propidium iodide containing DNase-free RNase, then gently filtered though 35-μm strainer caps into 12 × 75 mm polystyrene tubes. DNA histogram data were collected using a FACScan (Becton Dickinson). The list mode files were analyzed using "ModFit LT for Win32" version 2.0 software (Verity Software House, Inc.). In separate experiments, fluorescein-dUTP labelling of free 3′-OH DNA strand breaks was determined by flow cytometry using the ApopTag® Direct *In Situ* Apoptosis Detection Kit (Oncor) according to the manufacturer's instructions. The distribution of fluorescein staining was analyzed using WinMDI version 2.6 software ("Windows Multiple Document Interface Flow Cytometry Application" by Joseph Trotter, Scripps Institute).

Cellular Accumulation of Paclitaxel

Exponentially growing MCF-7 cells were pre-exposed to 4 μM FUra or diluent for 24 hr, and the cells were incubated in fresh, drug-free medium. [^{3}H]Paclitaxel (5 μCi/flask) then was added in the presence or absence of 4 μM FUra. After a 24-hr incubation, the medium was aspirated, and the cells were washed gently three times with ice-cold PBS. Cell monolayers were treated with 1 N sodium hydroxide to break up cell membranes and to dissolve the protein. Aliquots were added to a vial containing scintillation fluid, neutralized with 1 N hydrochloric acid, and the radioactivity was determined.

Measurement of p53, Bcl-2, and p21 Protein Content

MCF-7 cells were exposed to paclitaxel, FUra, or the combination for 24 hr in various sequences. Cellular lysates were prepared from control or drug-treated cells, and equal amounts of protein (300 μg) were resolved by SDS–PAGE (% polyacrylamide: 15% for p53; 12.5% for Bcl-2 and p21). Murine monoclonal antibodies (p53 Ab 2; Bcl-2 Ab 3; Waf-1 Ab 1; and α-tubulin) obtained from Oncogene Research Products (Calbiochem) were used as the primary antibodies at a dilution of 1:200. Horseradish peroxidase goat anti-mouse IgG was the secondary antibody, and was used at a dilution of 1:2000. The antigen/antibody complexes were visualized using the Enhanced Chemiluminescence™ kit (ECL Amersham). To correct for protein loading, the blots were stripped and probed for α-tubulin. The relative quantities of proteins on scanned images were analyzed by densitometry using Sigmagel™ for Windows version 1.0 (Jandel Scientifics).

Detection of Double-Stranded DNA Fragmentation

Intact cells were embedded in 0.5% low melting point agarose plugs (100,000 cells per plug), digested at 50° for 48 hr with buffer containing 0.5 M EDTA (pH 9.0), 1% sodium lauryl sarcosine, and 0.5 mg/mL of proteinase K, and then were washed and stored at 4° as recommended by the manufacturer (Bio-Rad Laboratories). Individual plugs were placed on the teeth of a gel comb, and a 1.0% chromosomal grade agarose gel (160 mL) was cast. A Chef Mapper™ (Bio-Rad Laboratories) program was employed: 120° angle, initial switch time 2.9 sec, final switch time 204.6 sec, and a gradient of 6 V/cm [18]. The total run time was 17.5 hr, during which Tris–borate–EDTA buffer (0.5x, 4 L) was recirculated at 14%. The gels were then stained with 0.5 μg/mL of ethidium bromide, destained, and photographed under UV illumination. For conventional agarose electrophoresis, DNA was purified by a salting-out method, and RNA was digested by DNase-free RNase [18]. Equal amounts of purified DNA (2–3 μg) were electrophoresed through a 1.5% agarose gel at 70 V for 4 hr, following which the gels were stained [19].

RESULTS

Sequence-Dependent Cytotoxicity

A 24-hr exposure to either 4 μM FUra or 40 nM paclitaxel inhibited cell growth over a 96-hr period by 50%, whereas 15 μM or 55 nM was needed to inhibit clonogenic capacity by 50%, respectively (Fig. 1). The influence of sequence of administration on cytotoxicity was studied by median-effect analysis with a fixed concentration ratio of 1:80 (paclitaxel: FUra). In cell growth studies, sequential FUra followed by paclitaxel was antagonistic (combination indices ranging from 1.1 to 3.9 in the fraction-affected range ≥ 0.20); simultaneous exposure led to nearly additive effects, whereas sequential paclitaxel followed by FUra was associated with additive or more than additive growth inhibition (combination index ranging from 1.04 to 0.71 when the fraction inhibited was ≥ 0.55). In clonogenic assays, pre-exposure to FUra was markedly antagonistic (Fig. 2), whereas paclitaxel followed by FUra was synergistic in the fraction-affected range of 0.30 to 0.95. Simultaneous exposure to FUra and paclitaxel led to less than additive effects when colony formation was determined. The apparent discrepancy with simultaneous exposure in the cell growth and clonogenic median effect studies may be attributed in part to the time at which the cytotoxic effect was assessed (2 days vs 7 days after final drug exposure, respectively).

FUra Metabolism, RNA Incorporation, and TS Inhibition

To elucidate the underlying basis for the sequence-dependent interaction, we selected concentrations of FUra and paclitaxel that inhibited cell growth by ~ 50%. The effects of various sequences of paclitaxel with FUra on the cellular

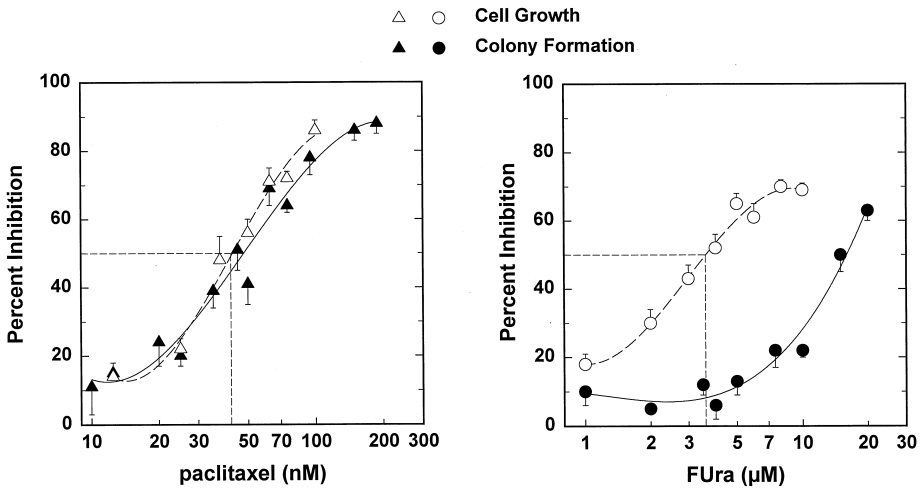

FIG. 1. Cytotoxicity of paclitaxel and FUra given individually. MCF 7 cells were exposed to the indicated concentrations of paclitaxel (Taxol) or FUra for 24 hr, then incubated in drug-free medium for an additional 48 hr (cell growth) or 7 days (colony formation). The data are compiled from ≥ six separate experiments, each done in duplicate. The data for cell growth are presented as percent of control cell number (mean ± SEM), and the control cell number on the day of enumeration averaged 3.82 ± 0.87 × 10^5 cells. The data for colony formation are presented as percent of control colony number (mean ± SEM), and the colony numbers for control cells averaged 166 ± 9. The error bars that are not visible are encompassed by the symbol.

pharmacology of FUra were examined. Following a 24-hr exposure to 4 μM [^{3}H]FUra, FUra-ribonucleotides were the predominant metabolites (Table 1). Free FdUMP pools were very small. Concurrent or pre-exposure to paclitaxel did not affect FUra-ribonucleotide formation appreciably. FUra-RNA incorporation was not altered by concurrent paclitaxel exposure, and was slightly higher (1.5-fold vs FUra alone) with paclitaxel pre-exposure.

A 24-hr exposure to 4 μM FUra significantly inhibited TS catalytic activity compared with control ($P < 0.001$), and the degree of TS inhibition was comparable 24 hr after removal of FUra ($P = 0.009$) (Fig. 3). Thus, most of the FdUMP formed in the cells was bound in the ternary complex with TS. Paclitaxel given alone had no effect on TS activity compared with control, and had no substantial influence on FUra-mediated TS inhibition when given prior to, concurrent with, or following FUra exposure.

Paclitaxel Cellular Accumulation and Cell Cycle Effects

Following a 24-hr exposure to [^{3}H]paclitaxel, the cellular content of paclitaxel was 4.1 ± 0.7 pmol/million cells (mean ± SD, N = 3 experiments, each done in triplicate). Concurrent or pre-exposure to FUra did not affect [^{3}H]paclitaxel accumulation: 4.3 ± 0.7 and 4.3 ± 0.6 pmol/million cells, respectively.

The cell cycle distribution profile at 48 hr is shown in Fig. 4. Exposure to FUra alone from either hours 0 to 24 or hours 24 to 48 led to pronounced S-phase accumulation (~ 2.0-fold greater than in control cells), with a commensurate decrease in the fraction of cells in G_1 phase. Paclitaxel exposure for either the initial or final 24 hr produced a 3.0- and 2.7-fold increase in the percentage of cells in G_2/M phase compared with control, respectively. Concurrent exposure to FUra and paclitaxel from either hours 0 to 24 or hours 24 to 48 and pre-exposure of MCF-7 cells to FUra before paclitaxel each reduced the extent of paclitaxel-associated G_2/M phase accumulation. In contrast, no interference with paclitaxel-mediated G_2/M phase arrest was noted if FUra exposure followed an initial 24-hr paclitaxel exposure.

Paclitaxel and FUra-Mediated Induction of p53 and p21 Proteins

A 24-hr FUra exposure was accompanied by increased p53 protein expression (Fig. 5A, lane 3), which returned to basal levels 24 hr after drug removal (lane 2). Increased expression of p53 protein was also detected immediately after a 24-hr paclitaxel exposure (lane 5), and was sustained for at least 24 hr after drug removal (lane 4). The increased p53 protein content during and following paclitaxel exposure did not appear to be affected by FUra given either concurrently with, preceding, or following paclitaxel (lanes 6–9). With respect to Bcl-2 protein expression, a slower-migrating band (lane 5) was noted immediately following exposure to paclitaxel from hours 24 to 48, and is consistent with phosphorylated Bcl-2 [20]. The phosphorylated band was no longer evident when paclitaxel given for 24 hr was followed either by a 24-hr drug-free period (lane 4) or by FUra (lane 9). The phosphorylated Bcl-2 protein band (lane 7) was also evident when an initial drug-free period was followed by concurrent exposure to FUra and paclitaxel, but was much less prominent (lane 8) if FUra preceded paclitaxel. Similar results were seen in separate experiments. p21 protein expression closely paralleled p53 protein expression (Fig. 5B).

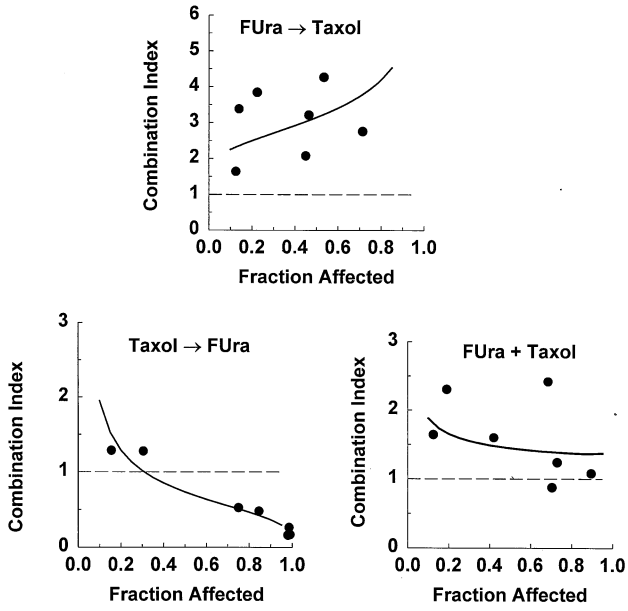

FIG. 2. Effect of sequence of exposure on lethality associated with paclitaxel and FUra. Exponentially growing MCF-7 cells were exposed to either no drug, paclitaxel (Taxol), FUra, or the combination for 24 hr, following which the cells were gently washed and fresh medium was added. Either diluent, FUra, or paclitaxel then was added for an additional 24 hr. Next the cells were incubated in drug-free medium for 7 more days, and then the colonies were stained and enumerated. Seven paclitaxel/FUra concentrations at a fixed molar ratio of 1:80 were evaluated (paclitaxel, 12.5 to 188 nM; FUra, 1 to 15 μM). The data represent a compilation of five or six experiments, each done in duplicate. The fractional inhibition for each drug in combination was analyzed in relation to the fractional inhibition observed with each drug alone using CalcuSyn© software with a mutually non-exclusive model. The symbols represent the observed data points for the seven drug combinations. The solid line reflects the combination index plot predicted by the software. A combination index of 1.0 (shown by the dashed line) reflects additive effects, whereas values above and below 1.0 indicate antagonism and synergy, respectively.

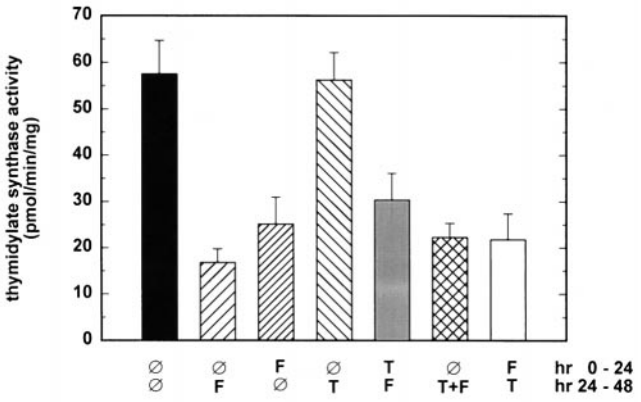

FIG. 3. FUra-mediated inhibition of TS. MCF-7 cells were exposed to 4 μM FUra (F) and 40 nM paclitaxel (T) alone and in combination for 24 hr, or to sequential 24-hr exposures of FUra first, followed by paclitaxel, FUra first followed by no drug, or paclitaxel followed by FUra. TS catalytic activity in cellular lysates was determined by tritium release from [5-^{3}H]2′-deoxyuridine 5′-monophosphate. The data, expressed as picomoles per minute per milligram of protein (means ± SEM), are from at least four separate experiments, each done in duplicate. All conditions except paclitaxel alone were significantly different from control (*t*-test): FUra alone and FUra plus paclitaxel, $P < 0.001$; FUra → 24 hr drug-free period and FUra → paclitaxel, $P < 0.01$; paclitaxel → FUra, $P = 0.014$.

Effects of FUra and Paclitaxel on DNA Integrity

Induction of DNA damage was examined after 48 hr of either no drug or FUra and paclitaxel alone or in combination for the first 24 hr followed by a 24-hr drug-free period, the same conditions given for the final 24 hr preceded by a 24-hr drug-free period, and the sequences of FUra followed by paclitaxel and paclitaxel followed by FUra. Classic oligonucleosomal DNA laddering was not identified with any of these conditions (data not shown). Flow cytometric analysis of fluorescein-nucleotide labelling of 3′-hydroxyl DNA ends by terminal deoxynucleotidyl transferase provides a more sensitive method to detect single- and double-strand breaks. As shown in Fig. 6, an inverse relationship was noted between surviving cell number at 48 hr and relative fluorescein-dUTP staining. Modest fluorescein staining was seen immediately following FUra exposure, although the intensity decreased after a 24-hr drug-free interval. The most intense staining patterns were evident with paclitaxel given for the initial 24 hr, whether followed by a 24-hr drug-free interval or FUra exposure. Simultaneous exposure to paclitaxel and FUra for 24 hr followed by a 24-hr drug-free period was associated with less extensive DNA damage. Minimal DNA damage was noted immediately after paclitaxel exposure from hours 24 to 48, regardless of whether it was given alone, concurrently with FUra, or preceded by FUra.

To detect high molecular weight DNA fragmentation, control and drug-treated cells were embedded in agarose plugs and then subjected to enzymatic digestion *in situ* to remove RNA and protein. The purified DNA was analyzed by pulsed-field gel electrophoresis. Parental DNA fragmentation was not evident in control cells (Fig. 7, lane B), or

TABLE 1. [^{3}H]FUra metabolism and RNA incorporation

	FdUMP	FUDP-sugars	FUDP + FUTP	FUra−RNA
FUra	0.5 ± 0.4	4.3 ± 1.2	13.2 ± 3.5	482 ± 148
FUra + paclitaxel	Not detected	5.2 ± 2.1	18.5 ± 6.2	451 ± 115
Paclitaxel → FUra	0.2 ± 0.2	4.6 ± 2.5	13.0 ± 7.2	744 ± 192

The units for FUra nucleotide metabolites are pmol/10^6 cells (mean ± SEM, N = 10 determinations). The data for FUra-RNA are expressed as fmol/μg RNA (FUra and FUra + paclitaxel, mean ± SEM, N = 6; paclitaxel → FUra, mean ± ½ range, N = 2).

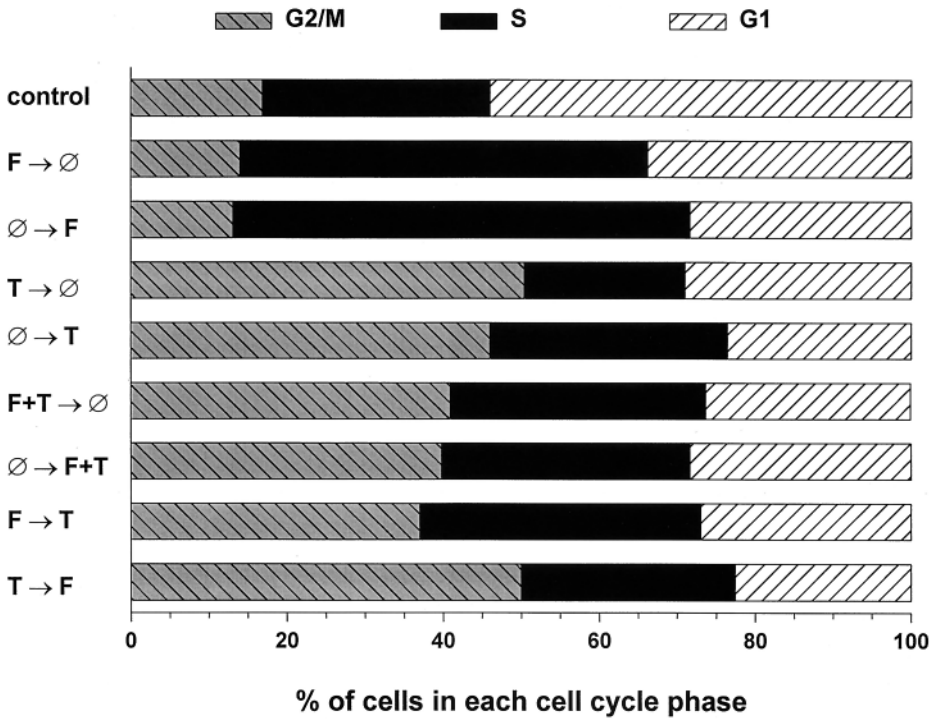

FIG. 4. Effect of paclitaxel and FUra on cell cycle distribution. Exponentially growing cells were exposed to either diluent (Ø), 4 μM FUra (F), or 40 nM paclitaxel (T) for 24 hr, following which the medium was aspirated, the flask was washed gently once with PBS, and the cells were incubated in fresh medium to which either diluent, FUra, paclitaxel, or the combination was added. The cells were harvested 24 hr later and prepared for cell cycle analysis. The results from 4 to 7 separate experiments were averaged, and the percentage of cells for each condition in either G_1, S, or G_2/M phase are shown in a horizontal stacked bar graph format. The standard errors (median and range) for each cell cycle phase for the nine conditions were as follows: G_1, 4.8 (2.4 to 9.4); S, 7.3 (2.3 to 10.2); G_2/M, 5.1 (2.4 to 9.9).

immediately after a 24-hr exposure to either 4 μM FUra (lane D), 40 nM paclitaxel (lane F), or the combination (lane H). Double-stranded DNA fragmentation was also absent following exposure to FUra for the initial 24 hr followed by either a 24-hr incubation in drug-free medium (lane C) or exposure to paclitaxel (lane G). In contrast, administration of paclitaxel for the initial 24 hr followed by a 24-hr drug-free period produced approximately 50-kb DNA fragments (lane E). Similar 50-kb DNA fragmentation was also evident with sequential paclitaxel followed by FUra (lane J), whereas the extent of DNA fragmentation appeared to be somewhat less prominent when FUra and paclitaxel were co-administered during the initial 24 hr followed by a 24-hr drug-free period (lane I). These latter results are similar to those observed with fluorescent dUTP-labelling: the extent of DNA damage appeared to be diminished with concurrent exposure to FUra and paclitaxel followed by a 24-hr drug-free interval, compared with either paclitaxel given alone for the initial 24 hr or sequential paclitaxel followed by FUra. The absence of double-stranded DNA fragmentation with FUra given for either the initial or final 24 hr of the 48-hr assay period suggests that the DNA damage detected with the ApopTag® assay likely represents single-strand DNA breaks.

DISCUSSION

We found a strong sequence-dependent interaction between paclitaxel and FUra in MCF-7 breast cancer cells. Sequential 24-hr exposures to FUra followed by paclitaxel led to profound antagonism, whereas the opposite sequence was associated with additive or more than additive cytotoxicity in clonogenic studies. To understand the possible basis for this sequence-dependent effect, we evaluated the potential effect of each drug on determinants of cytotoxicity to the other drug using the approximate 50% growth inhibitory concentrations. We documented that pre-exposure to paclitaxel did not interfere with FUra metabolism or inhibition of TS, whereas paclitaxel-mediated G_2/M phase arrest and induction of DNA fragmentation was not impeded when FUra exposure followed paclitaxel. FUra (4 μM) given alone for 24 hr was associated with S-phase accumulation, induction of p53 and p21 proteins, and modest generation of DNA strand breaks as detected by fluorescein dUTP-labelling. Following a 24-hr drug-free interval, however, p53 and p21 protein content returned toward basal levels, and the intensity of fluorescein dUTP-labelling diminished. No evidence of Bcl-2 protein phosphorylation was noted with FUra alone either immediately after drug exposure or 24 hr later. Since 4 μM FUra was growth inhibitory, but nonlethal, these results suggest the

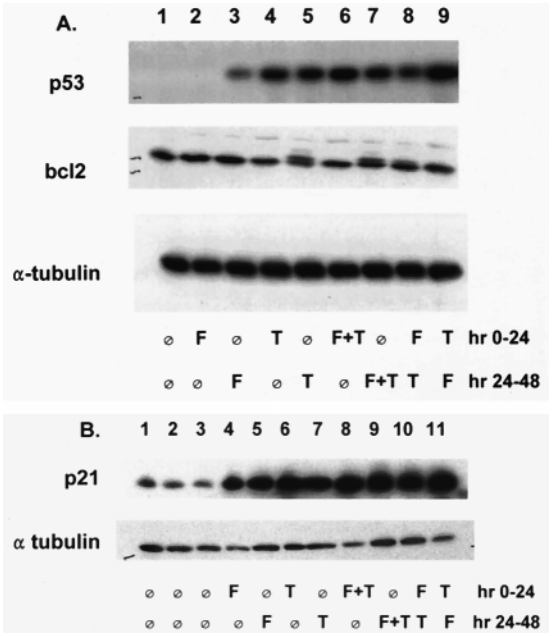

FIG. 5. Induction of p53, Bcl-2, and p21 by FUra and paclitaxel. Cells were treated with no drug, 4 μM FUra (F), or 40 nM paclitaxel (T) for two consecutive 24-hr periods as indicated. Equal amounts of protein (300 μg) were resolved by SDS–PAGE. Representative immunoblots of p53 and Bcl-2 are shown in Fig. 5A, whereas a p21 immunoblot is shown in Fig. 5B. Protein loading was verified by stripping the blot and re-probing for α-tubulin. Similar results were obtained in three (Bcl-2, p21) or four (p53) separate experiments.

occurrence of a transient FUra-mediated genotoxic insult, presumably a consequence of TS inhibition, that was insufficient to trigger programmed cell death pathways. In contrast, paclitaxel (40 nM) given for 24 hr led to Bcl-2 phosphorylation and a sustained increase in p53 and p21 protein content. Phosphorylation of Bcl-2 has been reported with antimicrotubule agents that produce G_2/M-phase arrest, and it has been postulated that this modification represses its anti-apoptotic properties [21]. Persistent paclitaxel-associated increases in p53 and p21 protein content 24 hr after drug removal might represent either continued genotoxic stress or decreased protein degradation. These phenomena were accompanied by DNA fragmentation as revealed by both the ApopTag® assay and pulsed-field gel electrophoresis. While the former assay detects free 3′-OH ends resulting from both single- and double-strand breaks, the latter methodology reflects induction of double-strand breaks consistent with apoptosis. These findings are consistent with the observation that the 50% growth inhibitory and 50% lethal concentrations of paclitaxel are similar in this cell line.

Previous investigators have reported that exposure of epithelial cancer cell lines to various DNA damaging agents produces high molecular weight DNA fragmentation in the absence of nucleosomal laddering [18, 22–26]. The absence of oligonucleosomal laddering in the previously reported epithelial malignancies and in the MCF-7 cells in the present report may signify that the cells either do not contain or do not activate the classic apoptosis-associated calcium- and magnesium-dependent endonuclease under the experimental conditions used and/or at the time points examined.

Two prior reports have described antagonism between paclitaxel and FUra *in vitro*. Kano and colleagues [27] noted that sequential 24-hr exposures to paclitaxel followed by FUra led to additive effects in four different human cancer cell lines, using the MTT (tetrazolium salt) assay. Sequential exposure to FUra followed by paclitaxel led to subadditive effects in three of the four cell lines, but possible mechanisms of interaction were not explored. Johnson *et al.* [28] found that concurrent continuous exposure of both BCap37 breast cancer cells and KB human epidermoid cancer cells to 100 nM paclitaxel and 10 μM FUra inhibits the customary oligonucleosomal DNA fragmentation seen with paclitaxel alone at 48 and 72 hr. In their model, although FUra given alone did not produce noticeable changes in the cell cycle profile, it diminished the ability of paclitaxel to produce G_2/M blockade and prevented apoptosis. The two cell lines used in these prior reports appear to be less sensitive to both paclitaxel and FUra than the MCF-7 cells in our model, since higher concentrations and longer exposures were needed to produce DNA fragmentation. Neither of these studies evaluated the potential impact of paclitaxel exposure on determinants of FUra-mediated cytotoxicity.

The time course of paclitaxel-mediated DNA fragmentation has been described in other model systems. In HeLa S3 cells treated with five times the 50% inhibitory concentration of paclitaxel, oligonucleosomal laddering was evident by 16–24 hr [29]. In two human myeloid leukemia cell lines, a 24-hr exposure to 100 nM, 1 μM, and 10 μM paclitaxel produced classic signs of apoptosis and was associated with down-regulation of Bcl-2 RNA expression [30]. Phosphorylation of Bcl-2 was seen in PC3 and LNCaP prostate cancer cells after a 24-hr exposure to 5–10 μM paclitaxel [20]. Oligonucleosomal DNA fragmentation was observed in both cell lines after a 48-hr exposure to 10 μM paclitaxel, although the phosphorylation state of Bcl-2 at the time of DNA fragmentation was not reported. Compared with the time course of paclitaxel-mediated apoptosis in HeLa S3, HL-60, and KG-1 cells, induction of double-stranded fragmentation was delayed in our model, which may in part be explained by our use of a lower concentration of paclitaxel for 24 hr. Phosphorylation of Bcl-2 was evident after a 24-hr paclitaxel exposure in MCF-7 cells, but was not seen 24 hr after paclitaxel removal, the time at which DNA fragmentation was seen.

It must be acknowledged that current statistical models for assessing drug interactions have certain inherent limitations [31]. Further, *in vitro* systems cannot faithfully reproduce the complexity of an *in vivo* biological environment. There are examples in which preclinical assessment of a chemotherapy doublet predicted possible antagonism [32], yet the two-drug combination has proven to be

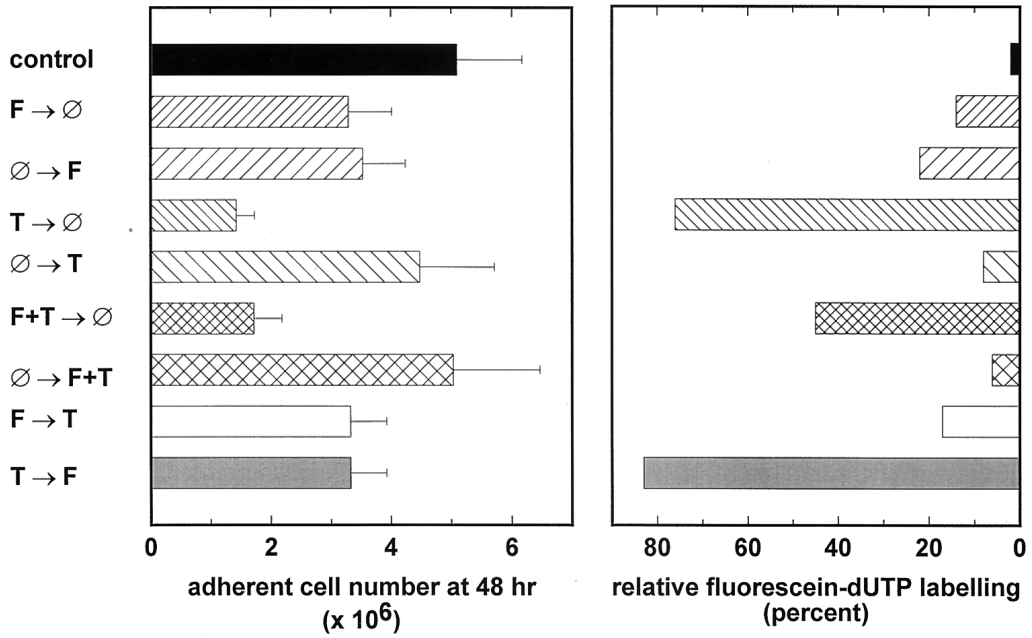

FIG. 6. Inverse correlation between cell growth and DNA strand breakage at 48 hr. In the left-hand panel, the number of adherent cells was determined at 48 hr following various sequences of either no drug, 4 μM FUra (F), or 40 nM paclitaxel (T) as shown. The data (means ± SEM) are from 5 separate experiments. The right-hand panel shows fluorescein-dUTP labelling of 3′-hydroxyl DNA ends by terminal deoxynucleotidyl transferase using the ApopTag® flow cytometry assay. A boundary was established for control cells, and excess fluorescent labelling is presented as percent of total fluorescent distribution.

clinically useful (e.g. cisplatin/etoposide for lung and testicular cancers). A previous clinical trial that evaluated opposite sequences of paclitaxel and cisplatin did not substantiate a suggestion of improved efficacy utilizing the optimal sequence predicted by *in vitro* models [10, 11]. Indeed, while no difference in clinical efficacy was noted, the incidence of adverse toxic effects differed substantially between the two sequences. In clinical trials involving paclitaxel/doxorubicin, the duration of drug exposure appears to influence potential sequence-dependent clinical toxicity. For example, a 24-hr exposure to paclitaxel followed by a 1-hr exposure to doxorubicin in three human cancer cell lines led to less than additive cytotoxicity [33]. One clinical study comparing sequential paclitaxel for 24 hr followed by doxorubicin for 48 hr versus the opposite sequence reported that the clinical toxicity was more severe when paclitaxel was given first, presumably due to a paclitaxel-associated decrease in doxorubicin clearance [13]. In contrast, no apparent differences in clinical toxicity were observed in a clinical study that explored doxorubicin given as a 15-min infusion prior to or following paclitaxel administered as a 3-hr infusion [34].

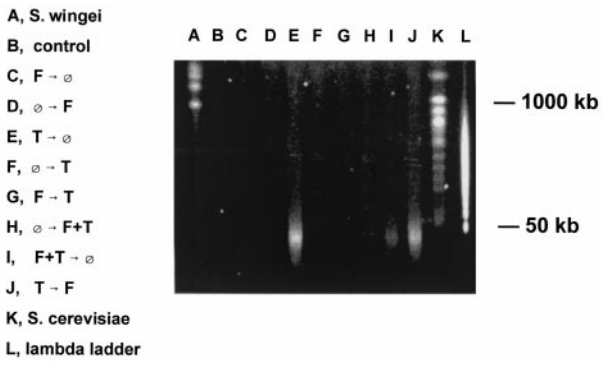

FIG. 7. Paclitaxel-mediated induction of parental DNA fragmentation. Cells treated with diluent, 40 nM paclitaxel (T), 4 μM FUra (F), or the combination for 24 hr in various sequences were harvested at 48 hr, embedded in agarose, digested *in situ*, and subjected to pulsed field gel electrophoresis. The conditions for each lane are indicated to the left of the gel. Similar results were obtained in separate experiments.

Therefore, it seems prudent to recognize the constraints in the ability of a preclinical model to predict clinical outcome. Nonetheless, experimental information that provides a mechanistic explanation for potential antagonism or synergism does present a rational alternative to empiric designs for combination chemotherapy regimens. Our preclinical data suggest that pre-exposure of MCF-7 breast cancer cells to FUra followed by paclitaxel was antagonistic, whereas sequential administration of paclitaxel followed by FUra was preferable. Moreover, our data provide a plausible explanation for this striking sequence-dependent interaction. Therefore, we designed a Phase I study of paclitaxel given on day 1 with leucovorin-modulated FUra given on days 2–6. The regimen appears to be well tolerated and is clinically active [35]. Additional clinical studies clearly will be needed to determine if the sequence of paclitaxel and FUra administration influences toxicity and response to therapy.

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