Therapeutic Synergy Between Irinotecan and 5-Fluorouracil against Human Tumor Xenografts

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ABSTRACT

Purpose: Although the combination of irinotecan and 5-Fluorouracil is clinically active, it is associated with significant toxicity and resistance. Studies were carried out to define the optimal dosage, sequence, and timing for the combination in mice bearing xenografted human tumors.

Experimental Design: The maximum tolerated dose of irinotecan and 5-Fluorouracil in combination was determined in nude mice. Therapeutic efficacy against established human colon carcinoma xenografts, HCT-8 and HT-29, and human head and neck squamous cell carcinoma xenografts, FaDu and A253, was determined using the rugs individually, simultaneously, and in sequence with various intervals in between. Treatments were i.v. weekly × 4. Immunohistochemical and reverse transcription-PCR measurements of relevant drug-metabolizing enzymes, apoptosis-related proteins, cell cycle distribution, cyclin A, and S phase fraction expression were carried out and compared with the therapeutic outcome.

Results: The maximum tolerated dose of irinotecan resulted in cure rates of 30% or less in all xenografts. No cures were achieved with FUra alone. Concurrent administration of irinotecan and FUra, or of FUra 24 h before irinotecan, resulted in cure rates of <20%, except for FaDu (60%). Administration of irinotecan 24 h before FUra resulted in the highest cure rates, 80% in HCT-8, 0% in HT-29, 100% in FaDu, and 10% in A253.

Conclusions: The optimal therapeutic synergy was achieved when irinotecan was administered 24 h before 5-Fluorouracil. Sensitivity to this combination was associated with poor differentiation status, higher cyclin A index, recruitment of cells into S phase, and induction of Bax expression and apoptosis.

INTRODUCTION

The clinical usefulness of chemotherapy against advanced solid tumors is limited by host toxicity and tumor resistance. Combination chemotherapy is an approach to meeting this challenge, and the timing and sequence in which the drugs are administered may be critical. Combining a topoisomerase-I (Topo I) poison, irinotecan, with a thymidylate synthase (TS) inhibitor, Raltitrexed, with a 24 h gap in between was reported to be optimum in vitro (1). Irinotecan and 5-Fluorouracil (FUra) are used clinically for patients with advanced colorectal cancer, resulting in an overall response rate of ~39%, with a median survival rate of 15.9 months (2). This combination is associated with significant diarrhea, myelosuppression, and mucositis. In this study, we examined the combination of irinotecan with FUra in vivo, varying sequence and timing, to define the best way to use these two agents in combination.

Several Topo I poisons are in clinical development, including irinotecan and topotecan. Irinotecan is enzymatically converted by carboxylesterase, in vivo, to its most active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38; Refs. 3–6). Studies in cell culture suggest that gene copy number, mRNA content, and protein expression of Topo I may predict tumor response to camptothecin derivatives (7–9). Furthermore, increased Topo I expression and activity were found in human tumor samples, especially in colon cancer, when compared with normal mucosa (10–12). However, interpatient Topo I expression varies widely (13–15). In vitro data suggest that induction of Topo I cleavable complex and tumor carboxylesterase activity may be better response predictors than Topo I expression (16, 17). Two major isoforms of carboxylesterase were identified, carboxylesterase-1 and carboxylesterases-2. Although conversion of irinotecan to SN-38 by liver carboxylesterase is an efficient process, clinical data indicate that irinotecan has significant antitumor activity. This raises the possibility that local conversion of irinotecan to SN-38 by carboxylesterases-2 in tumor tissues might occur (18).

Markers associated with poor clinical tumor response to FUra include a high level of TS and a high level of the FUra-degradative enzyme dihydropyrimidine dehydrogenase (DPD; Ref. 19). Preclinical data from our laboratory demonstrated that resistance to FUra and its active prodrugs could be reversed by the simultaneous administration of a DPD inhibitor, such as eniluracil or uracil (20, 21). FUra is an antimetabolite with a complex mechanism of action, including inhibiting DNA synthesis via TS inhibition and incorporation into cellular RNA. A
published report demonstrated a close inverse relationship between the level of TS, DPD, and response to FUra in patients with advanced colorectal cancer (22).

Apoptosis is an orderly, characteristic sequence of structural changes resulting in programmed cell death. We have demonstrated that transfection of human head and neck squamous cell carcinoma (HNSSC; A253), de novo resistant to irinotecan, with Bax cDNA, increased sensitivity to several chemotherapeutic agents with different mechanisms of action (23, 24). Complementary to this data, it has been reported that Bcl-2, a BCL-2 family member, protects cells from a wide variety of apoptotic stimuli, acts in multiple positions within the cell cycle, and confers a multidrug resistance phenotype (25).

Data from our laboratory indicated that in the chemically induced Ward colorectal carcinoma, the combination of irinotecan/FUra is highly sequence dependent and that a sequence of irinotecan 24 h preceding FUra is superior, with a significant increase in the therapeutic index over the other sequences. The toxicity associated with high doses of irinotecan can be eliminated without loss of the antitumor efficacy by reducing the dose of irinotecan to 50% of its maximum tolerated dose (MTD), whereas the dose of FUra is kept at 50–75% of its MTD.

Using human tumor xenografts with different response rates to irinotecan and FUra, alone or in combination, studies were carried out to establish the optimal sequence and timing of irinotecan with FUra and identify markers associated with therapeutic synergy of the combination, with major focus on markers associated with cell cycle regulation and apoptosis.

MATERIALS AND METHODS

Mice. Eight- to 12-week-old female athymic nude mice (nu/nu, body weight 20–25 grams) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice were housed five mice per cage under specific pathogen-free conditions, with water and food ad libitum, according to an institutionally approved protocol.

Cell Lines and Tumors. The colon (HCT-8 and HT-29) and HNSCC cell lines (FaDu and A253) were purchased from American Type Culture Collection (Rockville, MD) and maintained as a monolayer in RPMI 1640, supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). The cell line was free from Mycoplasma as tested every 2 months with the Mycoplasma T. C. Rapid Detection System (Gen-Probe, Inc., San Diego, CA).

Four human tumor xenografts (two colon and two HNSCC tumors) were used in this study. Xenografts were initially established by implanting s.c. 10⁶ cultured cells and passed several generations by transplanting ∼50 mg non-necrotic tumor tissues before treatment, which began when the tumors were ∼200 mg in size (established tumors), ∼1 week after implantation.

Drugs. Irinotecan and SN-38 were supplied by Pharma- cia (Kalamazoo, MI), irinotecan as a ready-to-use clinical formulation solution in 5-ml vials containing 100 mg of drug (20 mg/ml), and SN-38 dissolved in DMSO with a final dilution made in growth medium. FUra was purchased from Hoffmann-La Roche, Inc. (Nutley, NJ) as a solution of 50 mg/ml in 10-ml vials. All drugs were diluted in sterile 0.9% NaCl.

Drug Doses and Schedules. Irinotecan and FUra were administered by i.v. injection via the tail vein of animals once a week for 4 weeks (weekly × 4, on days 0, 7, 14, and 21). The combination of irinotecan and FUra was administered on the same schedule and route with three different sequences: (a) irinotecan and FUra administered as a simultaneous injection (schedule 1); (b) FUra 24 h before irinotecan (schedule 2); and (c) irinotecan 24 h before FUra (schedule 3). We adapted similar schedules for in vitro study using SN-38 and FUra.

MTD and Toxicity Evaluation. The MTD was defined as the maximum dose that caused no drug-related lethality and produced <20% loss of initial animal weight. Drug-induced toxicities, including body weight loss and lethality, were determined daily for ≥3 weeks after treatment. IC₅₀ of drugs in vitro was determined using cell growth inhibition assay.

Tumor Measurement. Two axes of the tumor (L, longest axis; W, shortest axis) were measured using a Vernier caliper. Tumor weight (in milligrams) was calculated as: ½ (L × W²) (mm). Relative tumor volume (100%) was calculated by actual tumor weight (ATW) over initial tumor weight (ITW, day 0), as follows: ATW/ITW × 100%. Measurements were taken daily for the first 10 days and two to three times a week thereafter.

Antitumor Activity. Drug treatments were initiated 7–8 days after tumor transplantation when tumor weight was ∼200 mg. Antitumor activity was assessed by tumor growth inhibition, which is mean tumor weight (MTW) of the treated group (TG) relative to the untreated control group (CG) on day 12, calculated as: (MTW/G – MTWCG) MTWCG × 100%.

The tumor doubling time was defined as the mean time for the tumor to reach twice its initial weight (at treatment beginning, day 0). Tumor response was defined as partial response when tumor weight was temporarily reduced by ≥50% and complete response (CR) when tumor was undetectable by palpation for 90 days after treatment, at which time the mouse was sacrificed. The response rate was expressed as a percentage of animals in the group. In general, tumors in mice with partial responses regrew within 2 weeks after therapy. However, tumors rarely (<5%) regrew in mice with CRs (cured). As a general policy, animals were sacrificed when the tumor weight exceeded 2000 mg. Each experimental group had five mice, and each experiment was repeated at least twice.

Cell Cycle Analysis. Tumor samples were removed from mice bearing HCT-8 xenografts after various drug treatments (irinotecan, FUra, and irinotecan/FUra) and controls. Samples were immediately processed by mechanical disaggregation (27). Cells were stored in 70% alcohol. Approximately 10⁸ cells were filtered and resuspended in 1 ml of modified Krishan buffer with propidium iodide [Molecular Probes, Eugene, OR; 0.1% sodium citrate, 0.02 mg/ml RNase A, 0.37% NP40, and 0.05% mg/ml propidium iodide (pH 7.4)] and kept on ice and protected from light for 30–60 min. A FACScan flow cytometer was used to analyze DNA content. Analysis was carried out with Winlist 2.0 (Verity software). DNA analysis was performed with Multicyc for Windows (version 3.0) from the University of Washington.

Western Blot Analysis. Frozen tumor samples (100 mg) were broken into a fine powder and then lysed in RIPA buffer.
After determination of the MTDs of irinotecan/FUra combination, studies were initiated to determine the kinetics of tumor growth inhibition produced by irinotecan in all four xenografts, with or without FUra, at 50% of the MTD of each drug (50% of the MTD of each drug alone). However, the combination of the two drugs administered at a 2/3 dose of their MTDs and at their individual MTDs resulted in severe toxicity, with 80 and 100% lethality, respectively.

Kinetics of Antitumor Response to Irinotecan and FUra Alone and in Combination. After determination of the MTDs of irinotecan/FUra combination, studies were initiated to determine the kinetics of tumor growth inhibition produced by irinotecan in all four xenografts, with or without FUra, at 50% of the MTD of each drug (Fig. 1). The drug was given by i.v. injection every week for 4 weeks, starting when the tumor had been established (1 week after transplantation). The optimal response rate was achieved at ~2–3 weeks after cessation of the therapy (32–36 days after initiation of irinotecan/FUra treatment), and schedule 3 was superior to schedule 1 or 2.

Administration of FUra concurrently with (schedule 1) or 24 h before irinotecan (schedule 2) did not result in significantly increased cure rates (20 and 10% CR in HCT-8, respectively), but giving irinotecan 24 h before FUra (schedule 3) resulted in better CR (80% in HCT-8; P < 0.05). Data are shown in Fig. 2.

Optimization of Time for FUra Administration. The data in Fig. 3 demonstrate that when combining the two drugs at their new combination MTD, the antitumor efficacy was highly
time dependent. To determine the best time kinetics of the combination of irinotecan/FUra, we administered FUra at various time points after irinotecan. The highest therapeutic synergy resulting in 80% CR was achieved when irinotecan was administered 24 h before FUra, whereas 60% CR was achieved when irinotecan and FUra were administered 48 h apart and 40% when irinotecan was given 6 or 72 h before FUra.

The Effect of Treatment Schedule on Cure Rate. We measured the partial response rate and CR with various treatment schedules, as shown in Table 1. The highest CR was achieved in HCT-8 (80% CR) and FaDu (100%) with schedule 3. The lowest CR was noticed with all implemented schedules in HT-29 (0%). There was no difference in the CR (10%) in A253 xenograft if irinotecan was administered concurrently with or 24 h before FUra.

To determine whether the schedule 3 superiority was specific to irinotecan, we used half the MTD of topotecan (7.5 mg/kg) 24 h before FUra (50 mg/kg) instead of irinotecan. This
The four human xenograft tumors were analyzed histologically for drug-specific dependency with irinotecan/FUra combination, and accumulation of cells in S phase was evaluated at 24-h post-i.v. drug treatment. The data indicate that maximum accumulation of cells in S phase (57%) occurs at 24-h postirinotecan, but there was no change on S phase after FUra treatment (23%). Treatment with the MTD of irinotecan (100 mg/kg) yielded a similar profile, with ~70% of cells in S phase at 24 h posttreatment (data not shown). These data suggest that administration of an S phase-specific agent (FUra) at 24 h after irinotecan correlates with the maximum therapeutic benefit (Fig. 3). In fact, administration of FUra 24 h after irinotecan resulted in 80% complete tumor regression compared with 40, 60, and 40% when FUra was administered at 6, 48, and 72 h after irinotecan.

To determine whether the accumulation of cells in S phase is specific to irinotecan, the effect of FUra and Topo I poison, topotecan, at their respective 1/2 MTDs on HCT-8 cell cycle distribution was evaluated at 24-h post-i.v. drug treatment. The data in Fig. 4 summarize the cell cycle distribution (S phase accumulation) at various times after i.v. injection of the therapeutic dose of irinotecan alone (50 mg/kg), followed 24 h later by i.v. injection of FUra (50 mg/kg). The data indicate that maximum accumulation of cells in S phase (57%) occurs at 24-h postirinotecan, but there was no change on S phase after FUra treatment (23%). Treatment with the MTD of irinotecan (100 mg/kg) yielded a similar profile, with ~70% of cells in S phase at 24 h posttreatment (data not shown). These data suggest that administration of an S phase-specific agent (FUra) at 24 h after irinotecan correlates with the maximum therapeutic benefit (Fig. 3). In fact, administration of FUra 24 h after irinotecan resulted in 80% complete tumor regression compared with 40, 60, and 40% when FUra was administered at 6, 48, and 72 h after irinotecan.

Table 1 shows that expression of these markers in the untreated tumors does not correlate with the differential response rates, except for differentiation status, doubling time, and cyclin A index. Poorly differentiated tumors, with less doubling time and higher levels of cyclin A (HCT-8 and FaDu), were more sensitive to the combination therapy of irinotecan/FUra. The effects of irinotecan and FUra on cell cycle distribution and on markers associated with apoptosis were investigated further to determine whether these data could provide the basis for understanding the mechanism of the optimal therapeutic schedule for the irinotecan/FUra combination.

**Specificity of Cell Cycle Perturbation.** We studied the effect of irinotecan and FUra on the cell cycle in HCT-8. The data in Table 1 summarize the cell cycle distribution (S phase accumulation) at various times after i.v. injection of the therapeutic dose of irinotecan alone (50 mg/kg), followed 24 h later by i.v. injection of FUra (50 mg/kg). The data indicate that maximum accumulation of cells in S phase (57%) occurs at 24-h postirinotecan, but there was no change on S phase after FUra treatment (23%). Treatment with the MTD of irinotecan (100 mg/kg) yielded a similar profile, with ~70% of cells in S phase at 24 h posttreatment (data not shown). These data suggest that administration of an S phase-specific agent (FUra) at 24 h after irinotecan correlates with the maximum therapeutic benefit (Fig. 3). In fact, administration of FUra 24 h after irinotecan resulted in 80% complete tumor regression compared with 40, 60, and 40% when FUra was administered at 6, 48, and 72 h after irinotecan.

To determine whether the accumulation of cells in S phase is specific to irinotecan, the effect of FUra and Topo I poison, topotecan, at their respective 1/2 MTDs on HCT-8 cell cycle distribution was evaluated at 24-h post-i.v. drug treatment. The data in Fig. 5 indicate that 57% of HCT-8 tumor cells were in S phase after treatment with irinotecan, whereas 23 and 18% of cells were in S phase after FUra and topotecan, respectively.
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The apoptotic index was calculated in vivo and a moderate, yet gradual, increase of apoptosis level was observed at 24 h (1%), 48 h (3%), and the highest (5%) at 72 h after the administration of irinotecan 24 h before FUra (P = 0.22). To confirm this induction of apoptosis, we applied the same in vivo schedules (1, 2, and 3) to the HCT-8 cell line in vitro, with both drugs at their perspective IC50. Induction of apoptosis was at the highest (14%) when SN-38 was administered 24 h before FUra (P < 0.0001). No significant induction of apoptosis was detected after administration of the reverse sequence (FUra/SN-38) or concurrent administration of both drugs (3 and 4%). The level of apoptosis was 1% and 4% after FUra or SN-38 alone (Fig. 6).

DISCUSSION

Irinotecan, a Topo I poison, and FUra, a TS inhibitor, are active agents in the treatment of patients with advanced colorectal cancer. The drug combination of irinotecan/FUra is Food and Drug Administration approved for colorectal cancer, with an overall response rate of ~39%. Diarrhea, myelosuppression, and mucositis are dose-limiting toxicities in 20–30% of patients treated, depending on the schedule used. Resistance and lack of therapeutic selectivity limit effectiveness of chemotherapy with irinotecan alone and in combination with other anticancer drugs. Thus, there is a critical need to study the molecular changes induced by this combination that would allow us to increase cure rate and overcome drug resistance without significant toxicity.

We chose four human xenografts to evaluate this combination therapy. Two of them were colon carcinoma (HCT-8 and HT-29), and the other two were HNSCC (FaDu and A253). In this study, we demonstrated the superiority of administration of irinotecan 24 h before FUra over any other schedule. We showed that the combination of irinotecan/FUra is sequence (irinotecan first), time (24 h apart), dose (50 mg/kg), and drug (irinotecan not topotecan) dependent. Although the concurrent combination of irinotecan/FUra (clinically approved schedule) used at their optimal doses and schedules is active in xenografts bearing human HCT-8 (20% CR) and FaDu, (60% CR), giving irinotecan 24 h before FUra resulted in an increase in CR to 80 and 100% in HCT-8 and FaDu, respectively. In contrast, x-
nografts bearing HT-29 and A253 had 0 and 10% CR, respectively, with either schedule.

After establishing the best schedule for this combination therapy, we studied the four untreated xenografts searching for any major changes that could be related to the different response rates among those xenografts. We investigated the differentiation status, doubling time, level of TS, DPD, p53, Bax, Bcl-2, carboxylesterase-2, and cyclin A index. We found no correlation between the level of those proteins and the different response rates of the four untreated xenografts, except for the differentiation status, doubling time, and cyclin A index. Poorly differentiated tumors with less doubling time and higher cyclin A index (HCT-8 and FaDu) were more sensitive to the combination therapy of irinotecan/FUra.

Because the best improvement in response rate by adding FUra sequentially after irinotecan occurred in HCT-8 (4-fold increase in CR, from 20 to 80%), the HCT-8 xenograft was used to further investigate other markers that could predict the response rate.

It has been reported that higher S phase fraction is associated with improved drug response in cancer (29). We measured the effect of irinotecan and FUra alone or in combination on the cell cycle distribution and S phase fraction in HCT-8 xenografts by flow cytometry. Our data showed that 57% of cells were accumulated in S phase at 24 h post-i.v. injection of irinotecan. This effect is specific for irinotecan and was not seen with another Topo I poison, topotecan, or FUra alone. Accumulation of cells in S phase may have sensitized the cells to FUra (DNA-damaging agent) given 24 h after irinotecan administration, which correlated with 80% CR with irinotecan/FUra. In the reverse schedule (irinotecan given 24 h after FUra), there was no accumulation of S phase after treatment with FUra (23% same as untreated control) that corresponded with only 10% CR. Thus, there was a correlation between S phase accumulation and response rate.

Bax protein level and Bax:Bcl-xL ratio were increased after irinotecan treatment. The ratio increased from 1.3 in the untreated control to 3.2 after irinotecan alone and to 5.4 after the combination of irinotecan/FUra, whereas there was no change in the ratio after FUra alone. The apoptosis level increased in both in vivo (from 1 to 5%) after irinotecan/FUra (P = 0.22) and in vitro (0% in the control to 14% after SN-38/FUra treatment in HCT-8 cells; P < 0.0001) that corresponded with the activation of Bax and 80% in vivo CR. The apoptotic index in the reverse schedule in vitro of FUra/SN-38 was 3% that correlated with 10% in vivo CR.

**Fig. 5** Cell cycle distribution of HCT-8 tumor after various drug treatments. HCT-8 xenograft was treated with various drugs via i.v. push (irinotecan, FUra, and topotecan). Samples were collected at 24 h postdrug treatment and stained with propidium iodide for DNA content analysis. Cell cycle distribution percentage was calculated using Multicycle for Windows (version 3.0) from the University of Washington. S phase accumulation is specific for irinotecan.

**Fig. 6** Irinotecan is associated with increased levels of Bax and Bax:Bcl-xL ratio and induction of apoptosis. Western blot analysis was performed in HCT-8 for Bax at 0, 4, and 24 h. Bax:Bcl-xL ratio was calculated after measuring the protein band densities at 24 h after the last drug treatment irinotecan and/or FUra. Bands were detected with anti-human Bax pAb and anti-Bcl-xL monoclonal antibody. Apoptotic index was detected in HCT-8 cells 24 h after the last drug treatment SN-38 and/or FUra. A, apoptosis; N, necrosis; –, 24 h later; CR, complete response.
In conclusion, results generated in mice bearing human HCT-8 xenograft tumors indicate that irinotecan, but not FUra, induced molecular changes associated with recruitment of cells into S phase, Bax activation, and induction of apoptosis in a dose- and time-dependent manner. Administration of a DNA synthesis inhibitor, FUra, after optimal molecular alterations have been induced by irinotecan, results in a significant increase in the cure rates (from 20 to 80% cures) of mice bearing human HCT-8 tumors. The high cure rates achieved with sequential weekly administration of irinotecan followed 24 h later by FUra could not be achieved when FUra was administered before or concurrently with irinotecan (the most common clinical practice) or with topotecan replacing irinotecan.

Although the sequential combination of irinotecan, followed 24 h later by FUra, yielded high cure rates in drug sensitive, poorly differentiated HCT-8 and FaDu human tumor xenografts, two well- and moderately differentiated human tumor xenografts, A253 (HNSCC) and HT-29 (colon carcinoma), are less responsive to cure with this combination. Future studies with the other sensitive xenograft FaDu would determine whether the mechanism of sensitivity of FaDu is similar to HCT-8 or could reveal other new mechanisms, as well as future studies with the less sensitive xenografts (A253 and HT-29) could unveil the mechanism of resistance to this combination therapy.

Clinical trials based on these preclinical findings are under way in our institute to evaluate the clinical benefits of the best schedule (schedule 3).

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