Thymidine Selectively Enhances Growth Suppressive Effects of Camptothecin/Irinotecan in MSI+ Cells and Tumors Containing a Mutation of MRE11

Rene Rodriguez, Lasse Tengbjerg Hansen, Geraldine Phear, Jennifer Scorah, Mogens Spang-Thomsen, Angela Cox, Thomas Helleday, and Mark Meuth

Abstract

Purpose: DNA synthesis inhibitors and damaging agents are widely used in cancer therapy; however, sensitivity of tumors to such agents is highly variable. The response of tumor cells in culture to these agents is strongly influenced by the status of DNA damage response pathways. Here, we attempt to exploit the altered response of mismatch repair (MMR)-deficient colon cancer cells and tumors to camptothecin or irinotecan and thymidine by combining them to improve therapeutic response.

Experimental Design: A panel of colon cancer cell lines was assayed for response to camptothecin-thymidine combinations by measuring colony formation, cell cycle distribution, and senescence. Cell strains defective in p53, p21, or Mre11 were used in these assays to investigate the role of these cell cycle regulators. The in vivo antitumor response of xenografts to irinotecan and thymidine combinations was assessed in nude mice.

Results: Camptothecin-thymidine combinations suppress colony formation of MMR-deficient tumor cells 10- to 3,000-fold relative to that obtained with camptothecin alone and significantly reduce the concentrations of the agents required to induce late S/G2 arrest and senescence. Sensitivity is not a direct result of MMR, p53, or p21 status. However, MMR-deficient cell lines containing an intronic frameshift mutation of MRE11 show greatest sensitivity to these agents. Increased sensitivity to this combination is also evident in vivo as thymidine enhances irinotecan-induced growth suppression of MMR-deficient tumors carrying the MRE11 mutation in mouse xenografts.

Conclusion: Irinotecan-thymidine combinations may be particularly effective when targeted to MSI+ tumors containing this readily detectable MRE11 mutation.
recombination (HR), and cells deficient in this repair pathway are sensitive to this agent (13, 14, 16).

Exposure of cells in culture to thymidine increases the intracellular pool of dTTP and depletes dCTP, as a result of the allosteric regulation of ribonucleotide reductase by dTTP (17). DNA replication is slowed, but not arrested, in thymidine-treated cells, leading to an accumulation of cells that slowly traverse S phase (an effect known as thymidine block; ref. 18). Thymidine induces little detectable DNA damage in the form of DSBs (19, 20), and its effects on DNA replication are readily reversible. However, HR-deficient cells are sensitive to thymidine (19), and thymidine triggers a rapid Ataxia-telangiectasia–mutated protein kinase cascade through Chk2 and the MRE11-RAD50-NBS1 complex similar to that induced by DSBs (20).

Considering that irinotecan is commonly used in the treatment of colon cancer, we sought to determine whether the growth suppressive effects of camptothecin on tumor cells could be enhanced by coadministration with thymidine. We reasoned that the prolonged S-phase arrest induced by thymidine in MMR-defective tumor cells (5) may enhance the ability of camptothecin to generate toxic DSBs at replication forks. Here, we show that MMR-deficient tumor cell lines are more sensitive to combined treatments with camptothecin/irinotecan and thymidine in tissue culture and in vivo. This increased toxicity is more pronounced in MSI+ tumor cells containing a mutation of MRE11 (21, 22).

Materials and Methods

Cell lines and cultures. The cell lines used in these experiments are presented in Table 1. The HCT116 human colon cancer cell lines (wild-type, p53–/–, and p21−/−) were generously provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The derivatives of SW480 expressing a dominant negative Mre11 that confers sensitivity to camptothecin and thymidine were reported previously (22). Remaining cell lines were obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% fetal bovine serum. For experiments using thymidine, dialyzed fetal bovine serum was used to remove deoxynucleosides in the serum that might alter the response to this agent. Cytotoxicity assays were done as described previously (5). Colonies consisting of >50 cells were counted. The surviving fraction was determined by dividing the average number of colonies for each treatment by the average number of colonies in the negative control siRNA was from Eurogentec. The siRNA was transfected into cells with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection of SW480 cells in 6-well plates, cells were replated on 10-cm plates and, after 4 h, were treated with camptothecin and thymidine.

Cell cycle analysis. Cell cycle analysis of floating and adherent cells was carried out as previously described (23). For measurement of BrdUrd incorporation by flow cytometry, cells were incubated with 10 µmol/L BrdUrd for 30 min, fixed with 70% ice-cold ethanol, and stored at −20°C. Cells were washed twice with PBS, incubated for 30 min in 2 mol/L HCl, and washed twice with PBS and once with PBS-T (PBS/0.1% bovine serum albumin/0.2% Tween 20). Two micro-liters of anti-BrdUrd (Dako) were added to the cell pellet for a 20-min incubation. After two washes with PBS-T, cells were incubated with a FITC-conjugated anti-mouse IgG (1:50; Dako) for 20 min, washed in PBS, and treated with propidium iodide and RNase. After 15 min of incubation, cells were analyzed by flow cytometry. Phosphorylated histone H3 was measured as previously described (23). DSBs were analyzed by pulsed field gel electrophoresis as described previously (20).

Senescence-associated β-galactosidase staining. After the indicated treatment, cells were fixed and incubated overnight with x-gal solution (pH 6.0) as previously described (24).

Western blotting. Cell extracts were prepared as described previously (20), resolved on 10% SDS-PAGE gels, and blotted onto nitrocellulose (Schleicher & Schuell). Proteins were detected with the enhanced chemiluminescence detection system (Amersham) using anti-p53 (DO-1; Santa Cruz), anti–phospho-p53 (Ser15; Oncogene), anti-p21 (PharMingen), anti-Mre11 (Oncogene and Bethyl Labs, Inc.), or anti–β-actin (Sigma).

Analysis of p53/p21 expression and cell cycle. After the indicated treatments, cells were fixed with 70% ice-cold ethanol, washed twice with PBS, and incubated for 10 min in PBS-T before incubation with a 1:50 dilution of the primary antibody in PBS-T [anti-p53 (DO-1; Santa Cruz) or anti-p21 (PharMingen)] for 30 min. After two washes with PBS-T, cells were incubated with FITC-conjugated anti-mouse IgG (1:50; Dako) for 30 min, washed again with PBS, and resuspended in PBS containing 50 µg/mL propidium iodide (Sigma) and 100 µg/mL RNase A (Sigma). After a 15-min incubation, cells were analyzed by flow cytometry.

Mice, tumor xenografts, and treatments. Male 6-wk-old athymic nude mice (NMRI-nu/nu) were obtained from Taconic. Mice were kept in individually ventilated Sealsafe cages (Scanbur BK A/S) and received sterile food pellets and water ad libitum. Institutional guidelines for animal welfare and experimental conduct were followed. SW480 and HCT116 tumor xenografts were established by s.c. injection of 5 × 10⁶ tumor cells, and maintained by serial transplantation. Tumor blocks of 1 mm³ in size were s.c. transplanted bilaterally into each flank of recipient mice. Tumor growth was measured five times a week, by two orthogonal diameters. Tumor volume and therapeutic efficacy were calculated as previously described (25).

Mice were treated i.p. once daily for 5 d with 12.5 mg/kg irinotecan (CAMPITOSAR; Pfizer Inc.) either alone or in combination with thymidine (Sigma). Thymidine was dissolved in sterile saline (50 mg/mL). Alzet pumps that slowly releases the thymidine solution at a rate of 1 µL/h (Model 2001; Scambur BK A/S) was loaded with 200 µL. At the first day of treatment, one Alzet pump was s.c. implanted into each mouse. Subsequently, mice received a single i.p. injection of 200 µL thymidine and each thymidine-treated mouse hence received a total amount of 20 mg thymidine. Control mice were treated i.p. once daily for 5 d with 200 µL sterile saline.

Table 1. Cell lines used in these experiments

<table>
<thead>
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<th>Cell line</th>
<th>MMR defect</th>
<th>Other damage response</th>
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<tr>
<td>HCT116</td>
<td>hMLH1</td>
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<td>p53</td>
<td></td>
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<tr>
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<td>p53, Mre11</td>
<td>(22)</td>
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<tr>
<td>SW480/SM1.6</td>
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<td>p53, Mre11</td>
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Results

Thymidine enhances the inhibitory effect of camptothecin on colony formation by MMR-deficient tumor cells. We first tested
the sensitivity of colon cancer cell lines (Table 1) to camptothecin-thymidine combinations in colony-forming assays. Two hMLH1-deficient colon cancer cell lines (HCT116 and SW48) showed a strong suppression of colony formation in these combinations. In the presence of 5 nmol/L camptothecin and 200 μmol/L thymidine, colony formation by HCT116 cells was 3,000-fold lower than that obtained in camptothecin alone (Fig. 1A), whereas colony formation by SW48 was 100-fold lower (Fig. 1B). Colony formation by the MMR-deficient tumor cell lines DLD-1 and LS411N (deficient in hMSH6 or hMSH3 respectively) was also lower in this combination, but this effect (~10-fold) was not as pronounced as that seen in hMLH1-deficient lines (Fig. 1C and D). In the MMR-proficient tumor cell lines SW480 and HT29, there was no significant effect on plating efficiency in 5 nmol/L camptothecin/200 μmol/L thymidine. In fact, thymidine only had consistent suppressive effects on colony formation in camptothecin at high concentrations in these cell lines (500 μmol/L; Fig. 1E and F).

**Prolonged exposure to thymidine or camptothecin induces late S/G2 arrest and senescence.** To examine the mechanism underlying the suppression of plating efficiency by combinations of thymidine and camptothecin, we first examined the effects of these agents on cell cycle distribution (Fig. 2A). HCT116 cells treated with camptothecin accumulated in G2 after 24 hours, whereas those treated with thymidine initially accumulated in S phase but arrested in G2 at later times. Cells treated with the camptothecin-thymidine combination showed an initial accumulation in S-phase but arrested in late S-G2 at later times. This arrest was poorly reversible as only 17% to 18% of HCT116 cells treated with either of these agents for 96 hours re-entered the cell cycle within 72 hours of removal of the agents (as measured by BrdUrd incorporation; Fig. 2B). When these agents were combined, a similar level of arrest was achieved with lower concentrations (Fig. 2C). DSBs were detected on pulsed field gels in cells treated with 20 nmol/L camptothecin but not in cells treated with 2 mmol/L thymidine after 96 hours (Fig. 2D). When cells were exposed to camptothecin-thymidine, DSBs were also detected after a 96-hour treatment; however, the level of these breaks was not enhanced relative to cells exposed to the camptothecin alone. DSBs seem to develop late after exposure as they were not detectable in cells treated with camptothecin or thymidine alone or in combination for 48 hours (data not shown).

Phosphorylated histone H3 was not detected in cells treated with thymidine or camptothecin for 96 hour (data not shown), indicating that cells arrest in G2 before entry into mitosis. These cells showed a clear increase in size with prominent and enlarged nuclei characteristic of senescent cells (Fig. 2E). Additionally, a high proportion of cells (~90%) treated with thymidine or
Fig. 2. Thymidine and camptothecin combinations enhance the induction of G2 arrest and senescence. A. HCT116 cells were treated during the indicated periods of time with 2 mmol/L thymidine (left), 20 mmol/L of camptothecin (middle), or a combination of both (right) and then analyzed for cell cycle distribution. Cell cycle profiles and percentage of cells in each phase are shown. B and C, analysis of the effect of the removal of drugs on the re-entry of thymidine or camptothecin-treated cells into cell cycle. HCT116 cells were treated for 4 d with the indicated concentrations of thymidine and/or camptothecin. Cells were then washed with PBS and fresh medium was added for 2 d to allow restart of progression through the cell cycle. BrdUrd (10 μmol/L) was added 24 h before fixation and analysis of BrdUrd incorporation by flow cytometry (see experiment diagram, B, top). D. DSBs were visualized by pulsed field gel electrophoresis of DNA obtained by gentle lysis of HCT116 cells in agarose plugs after the indicated treatments for 96 h. Agarose plugs treated with 10 Gy of IR were loaded as positive controls for DSBs. Regions of the gel showing DSBs and other DNA damage are indicated. E and F, HCT116 cells were left untreated or were treated for 4 d with the indicated concentrations of thymidine and/or camptothecin and then stained for senescence-associated β-galactosidase activity. E, cell enlargement and induction of senescence-associated β-galactosidase activity (black) after thymidine or camptothecin treatment. F, summary of the percentage of cells positive for senescence-associated β-galactosidase activity after treatment with thymidine, camptothecin or a combination of both.
Fig. 3. Effect of p53/p21 and MMR status on thymidine- and camptothecin-induced toxicity. A, whole extracts were prepared after treating HCT116 cells with 2 mmol/L thymidine or 20 nmol/L camptothecin for the indicated times. Total p53, ser15-phosphorylated-p53, p21, and β-actin expression were analyzed by Western blotting. B and C, HCT116 cells were ethanol fixed after treatment 2 mmol/L thymidine or 20 nmol/L camptothecin (only in B) for the indicated times. p21 (B) and p53 (C) expression were then analyzed together with DNA content (as determined by propidium iodide staining) by flow cytometry. The percentage of cells positive for p21 (R3) or p53 (R2) for each condition is shown. D, p53-/- and p21-/- HCT116 cells and SW480 cells treated with 2 mmol/L thymidine for the indicated times were fixed and analyzed for cell cycle distribution. E, wild-type HCT 116, HCT 116 p53-/-, and HCT 116 p33-/- cells were plated in the presence or absence of 50 μmol/L thymidine and the indicated concentrations of camptothecin. The fractions of the indicated cells forming colonies are presented. F, HCT116+3 cells were left untreated (control) or were treated for 4 d with 2 mmol/L thymidine or 20 nmol/L camptothecin and then stained for senescence-associated β-galactosidase activity. The percentage of cells showing positive staining is represented.
We also tested the sensitivity of HCT116 cells corrected for the MMR deficiency (29) to camptothecin-thymidine combinations. Consistent with previous reports that MMR status did not affect sensitivity to thymidine or camptothecin (5, 6), we found that the HCT116 cells corrected for the hMLH1 defect (HCT116 +3) did not differ from HCT116 in their ability to form colonies (Fig. 3E) or senesce (Fig. 3F) in the camptothecin-thymidine combination. Thus, in HCT116 cells, neither MMR nor p53 status account for the increased sensitivity to camptothecin or thymidine.

Tumor cell lines carrying a mutation in MRE11 show enhanced sensitivity to camptothecin-thymidine combinations. Recent work has shown that HCT116 cells encode a mutant transcript of MRE11 that lacks exons 5 to 7 as a result of a frameshift mutation in a run of 11T residues in intron 4 (21, 22). This mutant transcript confers sensitivity to both camptothecin and thymidine when expressed ectopically in cultured cells (22). To determine the effect of the mutant Mre11 on camptothecin-thymidine combinations, we examined the survival of SW480/SN3 cells expressing this transcript (SM1.3 and SM1.6; Fig. 4A; Table 1) in these agents. SM1.3 and 1.6 cells plated in 200 μmol/L thymidine showed increased sensitivity to all concentrations of camptothecin tested. At 5 nmol/L camptothecin/200 μmol/L thymidine, the cells were 40- to 100-fold more sensitive relative to cells plated in camptothecin alone (Fig. 4B and C). Importantly, SM1.3 and 1.6 were significantly more sensitive...
Table 2. Effect of thymidine on irinotecan activity

<table>
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<tr>
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<tr>
<td></td>
<td>n</td>
<td>SGD</td>
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<tr>
<td>Control</td>
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<tr>
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<tr>
<td>Irinotecan</td>
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<tr>
<td>Thymidine + Irinotecan</td>
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NOTE: Subcutaneous SW480 and HCT116 tumor xenografts were established in nude mice (Taconic) as previously described (25). Growing tumors were randomized into four treatment groups, i.e., control (saline), thymidine, irinotecan, and thymidine + irinotecan. One cycle of irinotecan (12.5 mg/kg; CAMPTOSAR; Pfizer, Inc.) was administered i.p. once daily for 5 d. Thymidine (20 mg/mice in sterile saline; Sigma) was administered as a single bolus injection (10 mg/mice) on day 1 combined with a slow release by s.c. implanted Alzet 2001-pumps. Therapeutic efficacy, assessed as SGD and time until a tumor volume of 300 mm³ was reached, was calculated as previously described (25). Number of mice in each group (n) and statistical difference (P, Student’s t test) of individual tumor SGD values between control versus thymidine or irinotecan and between irinotecan versus thymidine+irinotecan are indicated. Similar results were obtained in a subsequent study.

Discussion

The objective of this investigation was to determine whether the effectiveness of camptothecin/irinotecan could be improved by combining it with thymidine in the treatment of MMR-deficient tumor cells in vivo. Here, we show that combinations of these two agents substantially enhance their suppressive effects on growth and colony formation of MMR-deficient tumor cell lines, reducing plating efficiencies of such cells from 10- to 3,000-fold relative to those obtained in the same concentration of camptothecin alone. The two tumor cell lines carrying a mutation of MRE11 (HCT116 and SW48) show greatest sensitivity to the combination, and ectopic expression of a mutant MRE11 transcript found in HCT116 cells confers sensitivity to camptothecin and thymidine alone and in combination. Although HCT116 and SW48 are also hMLH1 deficient, hMLH1 does not seem to be a major determinant of sensitivity because correction of the defect does not alter sensitivity to thymidine, camptothecin, or the combination. We further show that coadministration of thymidine and irinotecan enhances in vivo growth inhibition of MMR-defective tumors carrying the mutant MRE11. Moreover, even at these relatively high irinotecan doses, compared with doses recommended for clinical use, no diarrhea or other toxicities were observed. This supports the notion of a relatively selective cytostatic effect exerted on the MMR-deficient tumor cells versus normal stromal cells. Furthermore, clinical antitumor response to irinotecan is substantially increased in MSI+ tumors (7, 8). Because the mutation of MRE11 that seems to confer sensitivity to these agents can be found in over 80% of MSI+ colon cancers (21), this strategy for further improving the effectiveness of irinotecan may be widely applicable to the treatment of MSI+ colon cancers. MMR-deficient tumor cell lines that do not carry this mutation (e.g., DLD1 and LS411N) show an intermediate sensitivity to this combination. Other mutations carried by these cell lines (e.g., the Chk2 mutations found in DLD-1; ref. 31) may contribute to increased sensitivity.

Both camptothecin and thymidine cause MMR-deficient cells to arrest in G₂ and enter an irreversible senescence-like state, dependent on p53 and p21. Coadministration of these two agents produces the same end point; however, much lower levels are able to induce this response than when the agents are used alone. Initially, we proposed that thymidine might potentiate growth inhibitory effects of camptothecin by arresting cells in S phase where DSBs occur. However, the pattern of induction of p21 and p53 and the onset of G₂ arrest in the presence of thymidine and camptothecin resembles that of thymidine rather than camptothecin. Moreover the combination does not increase the level of DSBs. Thus, we to camptothecin/thymidine combinations than the parental SW480 cells. In 10 nmol/L camptothecin/200 μmol/L thymidine, the cells expressing the mutant Mre11 have a 15- to 50-fold lower plating efficiency than the parental SW480 (see Figs. 1E and 4B and C). Thus, the mutant Mre11 enhances the cytotoxic effects of these agents singly and in combination.

It is notable that HCT116 and SW48 cells, such as many other MSI+ tumor cells with this MRE11 frameshift, have a reduced level of the wild-type Mre11 protein relative to MMR-proficient cells (Fig. 4A; refs. 21, 30). To determine whether reduced levels of Mre11 contribute to the sensitivity, we measured colony formation in camptothecin and thymidine by SW480 cells depleted of Mre11 after siRNA treatment (Fig. 4D and E). Cells depleted of this protein showed a small effect on sensitivity to these agents singly or in combination (Fig. 4E). Thus, our data suggest that cellular survival after treatment with these agents is particularly sensitive to the mutant protein.

Thymidine enhances irinotecan efficacy in MRE11 mutant HCT116 tumors. To test of the therapeutic potential of combinations of the clinically relevant form of camptothecin (irinotecan) and thymidine, we evaluated the effect of these agents in a preclinical in vivo model of SW480 and HCT116 tumor xenografts established in nude mice. In SW480 tumors, thymidine increased the irinotecan-induced specific growth delay (SGD) by 17% (P = 0.23) and time to a tumor volume of 300 mm³ by 5 days (Table 2). In HCT116 tumors, the SGD was increased by 75% (P = 0.01) and time to 300 mm³ by 12 days. Hence, consistent with results obtained in vitro indicating a role for Mre11 in tumor susceptibility to thymidine and irinotecan combinations, this regimen is far more potent in HCT116 tumors compared with SW480 tumors. Importantly, with the selected dose regimen, no deaths, weight loss, diarrhea, or apparent toxicity were observed in any of the treatment groups, supporting clinical investigation of this treatment modality.

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speculate that intermediates generated at DNA replication forks by the thymidine treatment of tumor cells carrying the Mre11 mutation require DNA topoisomerase I function for efficient progression through S-phase. Mutant forms of Mre11 may block the resolution of such intermediates by suppressing HR-mediated rescue of replication forks stressed by thymidine treatment (22). We further propose that an inability to resolve such replication blocks may result in a permanent G2 arrest and senescence in p53+/+ cells or apoptosis in p53-/- cells.

Irinotecan is commonly used in the treatment of colorectal cancers. We show that growth suppressive effects of this agent can be substantially enhanced by coadministration with thymidine. A potentially attractive feature of this strategy is that it may reduce the adverse side effects caused by administration of high levels of irinotecan alone. Phase I trials using thymidine in combination with carboplatin have been attempted previously with some success (32). Interestingly, the levels of thymidine required to potentiate the growth inhibitory effects of irinotecan are much lower than those achieved in previous phase I trials (32, 33). Consistent with reports showing that MSI+ is a predictive factor for tumor response to irinotecan (7, 8), our results indicate that the combination with thymidine may be far more effective and less toxic when targeted to patients with MSI+ tumors that contain this readily detectable mutation of MRE11.

Disclosure of Potential Conflicts of Interest

A patent including some of the data presented here was filed by The University of Sheffield with M. Meuth and T. Helleday as inventors.

References

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