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shRNA suppression of thymidylate synthase produces DNA mismatches and results in excellent radiosensitization

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Abstract

Purpose—To determine the effect of shRNA-mediated suppression of thymidylate synthase (TS) on cytotoxicity and radiosensitization and the mechanism by which these events occur.

Methods and Materials—shRNA suppression of TS was compared to FdUrd inactivation of TS $\pm$ ionizing radiation in HCT116 and HT29 colon cancer cells. Cytotoxicity and radiosensitization were measured via clonogenic assay. Cell cycle effects were measured by flow cytometry. Effects of FdUrd or shRNA suppression of TS on dNTP imbalances and consequent nucleotide mis-incorporations into DNA were analyzed by HPLC and as pSP189 plasmid mutations, respectively.

Results—TS shRNA produced profound (90%) and prolonged (8 days) suppression of TS in HCT116 and HT29 cells while FdUrd increased TS expression. TS shRNA also produced more specific and prolonged effects on dNTPs compared to FdUrd. TS shRNA suppression allowed accumulation of cells in S-phase, though its effects were not as long-lasting as FdUrd. Both treatments resulted in phosphorylation of chk1. TS shRNA alone was less cytotoxic than FdUrd, but was equally effective as FdUrd in eliciting radiosensitization (radiation enhancement ratio (RER): TS shRNA, 1.5 – 1.7; FdUrd, 1.4 – 1.6). TS shRNA and FdUrd produced a similar increase in the number and type of pSP189 mutations.

Conclusions—TS shRNA produced less cytotoxicity than FdUrd, but was equally effective at radiosensitizing tumor cells. Thus, the inhibitory effect of FdUrd on TS alone is sufficient to elicit radiosensitization with FdUrd, but only partially explains FdUrd-mediated cytotoxicity and cell cycle inhibition. The increase in DNA mismatches following TS shRNA or FdUrd supports a causal and sufficient role for the depletion of dTTP and consequent DNA mismatches underlying radiosensitization. Importantly, shRNA suppression of TS avoids FP-mediated TS elevation and its negative prognostic role. These studies support further exploration of TS suppression as a novel radiosensitizing strategy.

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Keywords

thymidylate synthase; radiosensitization; fluoropyrimidines; shRNA suppression; fluorodeoxyuridine

Introduction

Chemotherapeutic drugs used clinically to target thymidylate synthase (TS) directly include the fluoropyrimidines (FPs), 5-fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FdUrd). The FPs are the mainstay for colorectal cancer and other gastrointestinal malignancies, and the efficacy of these agents towards solid tumors can be improved when combined with concurrent radiotherapy (1). Chemoradiotherapy with 5-FU has been shown to improve local control for head and neck, esophageal, rectal, pelvic and gastrointestinal cancer and is considered the standard of care for these tumors.

Evidence suggests that the primary anticancer effects of FPs results from their inhibition of TS via activation to the potent inhibitor 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) (2). However, these drugs can also be incorporated into DNA following activation to fluorodeoxyuridine 5'-triphosphate (FdUTP) (3). 5-FU can also be incorporated into RNA following activation to 5-fluorouridine-5'-triphosphate (FUTP) (1). Because the FPs target TS at the same concentrations at which they can be incorporated into nucleic acids, it has been difficult to separate the contribution of each pathway to cytotoxicity (3) or to radiosensitization. Correlative studies with FPs have implicated dTTP depletion and S-phase arrest in radiosensitization and cytotoxicity, with an uncertain role for FdUTP incorporation into DNA (4, 5). Our prior studies demonstrated that the dNTP imbalances generated by FdUrd produced mismatches in DNA which, if not repaired, augmented cell death following irradiation (5). In these studies, nucleotide misincorporations in DNA occurred only at radiosensitizing concentrations of FdUrd. However, the use of FdUrd in these studies to assess the mechanism of radiosensitization by FPs makes it difficult to determine with certainty the effect that TS inactivation alone has on radiosensitization or to eliminate the contribution that DNA incorporation of drug may make to radiosensitization.

If TS is the primary target of the FPs, then suppressing TS protein should produce an effect equivalent to that observed with enzymatic inhibition of TS. TS suppression using TS antisense oligodeoxynucleotide (ODN) or siRNA produced modest inhibition of cell proliferation and tumor growth *in vitro* and *in vivo*, respectively (6, 7). However, these strategies resulted in only incomplete and transient decrease of TS protein. In the present study we have compared TS suppression using lentivirus delivered shRNA with FdUrd mediated inactivation of TS on cytotoxicity, radiosensitization and check point activation in two colon carcinoma cell lines. In addition, we have evaluated the contribution of each approach to producing mismatches in DNA for radiosensitization. This is the first report of the effects of TS shRNA in combination with ionizing radiation (IR) in tumor cells.

Materials and Methods

Cell Culture, Plasmid, and Drug Preparation

HCT116 and HT29 colon carcinoma cells were maintained in Dulbecco's modified essential medium (DMEM) (Invitrogen), supplemented with 10% fetal calf serum (Invitrogen), and 2 mM L-glutamine (Fisher Scientific). FdUrd (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS.

Transduction with shRNA

Short hairpin RNA (shRNA) lentiviral plasmids (pLKO.1-purp) containing TS (GenBank accession number NM_001071) target sequences (Sigma MISSION®, SHCLNG-NM_001071; Sigma Chemical Co., St. Louis, MO) were used. HEK293T cells were transfected with lentiviral plasmid, virus collected 48 hr later, and cells were transduced at 37°C overnight (5). Selected cells (2 µg/ml puromycin) were harvested at the appropriate time and TS protein expression determined. Five TS shRNAs were tested and two were selected (TS1 shRNA, TRCN0000045665-1071.1.324; TS2 shRNA TRCN0000045667-1071.1.857), based on their strong and prolonged suppression of TS.

Western Blot Analysis

Cell lysates were prepared in RIPA (radioimmunoprecipitation assay) lysis buffer [0.05 M Tris-HCL, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, pH 7.4], with the addition of protease inhibitors (Roche), and proteins were separated by SDS-PAGE on 10% acrylamide gels. Membranes were probed with antibodies: TS (Santa Cruz Biotechnology, Santa Cruz CA); actin (albiochem, San Diego CA), followed by anti-mouse IgG (Millipore, Danvers MA) horseradish peroxidase (HRP) linked. Proteins were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Determination of nucleotide pools

Nucleotides were extracted and analyzed by strong anion exchange high pressure liquid chromatography (HPLC) as described previously (5, 8). Nucleotides were identified based on their UV absorbance spectrum and quantified at 254, 281, or 292 nm by comparison to the absorbance of authentic standards.

Cell Survival and Radiosensitization Assay

Cells were treated with IC₅₀ FdUrd (100 nM and 3.5 µM for HT29 and HCT116 cells respectively) for 24 h or with TS shRNA for 5 d alone or followed by IR [⁶⁰Co (AECL Theratron 80) at 1–2 Gy/min] only, and assessed for clonogenic survival (8). Radiation sensitivity is expressed as mean inactivation dose, which represents the area under the cell survival curve (D-bar) (9). Radiosensitization is expressed as a radiation enhancement ratio (RER), defined as the mean inactivation dose (untreated control + IR)/mean inactivation dose (drug or shRNA + IR). Radiation survival data from FdUrd and TS shRNA treated cells were corrected for plating efficiency by comparison to cells treated with drug or shRNA alone.

pSP189 Plasmid Mutation Assay

This assay detects mutations in the supF gene (B-galactocidase) in the pSP189 plasmid (10). Control cells (no shRNA, control/drug treated) and transduced cells (TS shRNA and NS shRNA,) were transfected with pSP189 plasmid overnight, then incubated with FdUrd (IC₅₀) or no drug (control and shRNA treated) (8). Replicated plasmid DNA was electroporated into MBM7070 *E. coli*, and transformants were grown on agar plates with ampicillin and X-gal. Mutation frequencies were calculated as # white colonies / # (white + blue) colonies. DNA from some control and all mutant clones was sequenced at the University of Michigan DNA Sequencing Core.

Results

Effect of FdUrd or TS shRNA on TS protein expression and dNTPs

Both TS1 and TS2 shRNAs produced nearly complete depletion of TS in HCT116 and HT29 cells by 2–4 d. While there was faint return of TS expression by day 7 or 8, it

remained decreased compared to control for at least 8 days (Figure 1). The non-specific (NS) shRNA did not alter the expression of TS protein compared to control. In contrast, FdUrd (IC_{50}) produced an early (24 h) and lasting band shift and increase in TS protein that persisted through 72 h, similar to reports by others (11).

Both FdUrd and shRNA mediated suppression of TS produced a similar decrease in dTTP in both cell lines (HT29 cells: maximal decrease 80%; HCT116 40%) with an expected reduction in dGTP (< 60% in both cell types) due to dTTP-mediated effects on ribonucleotide reductase (Figure 2). Whereas FdUrd increased dATP (up to 4-fold), which is typical following FP administration (5, 12), dATP and dCTP following TS shRNA were decreased or remained unchanged from control depending on the duration of time following transduction. The effects of FdUrd and TS shRNA on dNTPs were transient as the observed initial depletions or elevations, depending on the dNTP pool, showed signs of recovery toward control concentrations as early as 24 h after drug addition, and by 8 d post shRNA transduction. Thus, the observed pattern of changes in dNTPs paralleled the pattern of TS suppression or inactivation, and recovery by each treatment. The effects mediated by TS shRNA were specific, as all dNTPs were maintained at > 80% of control values with the non-specific shRNA.

The FP-mediated inactivation of TS and subsequent depletion of dTTP and dGTP slows DNA synthesis resulting in an accumulation of cells in S-phase (13). The majority of cells were in S-phase and strong inhibition of DNA synthesis was observed at 24 h and 4–6 d following FdUrd and TS shRNA, respectively. Consistent with the observed block in DNA synthesis and S phase progression, we also observed activation of Chk1 at Ser345 and Ser317 (14) following FdUrd and TS shRNA. The decrease in Chk1 phosphorylation at TS shRNA 8 d was coincident to removal of the S-phase checkpoint and cell cycle re-entry, as noted by an increase in cell number (data not shown).

Cytotoxicity and Radiosensitization by FdUrd or TS shRNA

Determination of whether the direct suppression of TS protein would be as effective as inactivation of TS by FPs as a radiosensitizing strategy necessitated that we choose time points when measures of parameters known to influence radiosensitization, namely dTTP depletion and S-phase accumulation (4, 5, 15), were similar. We chose 24 h FdUrd and 5d shRNA because: (1) majority of cells had accumulated in S-phase, (2) dTTP was substantially depleted, and (3) depletion of TS for at least 24 h occurred most reproducibly at 5d post-transduction, resembling the 24 h inhibition of TS by FdUrd.

Despite lengthy TS protein suppression and more sustained depletion of dTTP, the TS shRNAs killed only 20 – 30% of cells compared to approximately 50% cytotoxicity with FdUrd (Figure 1, Table 1). NS shRNA did not significantly alter cell survival compared to control in either cell line.

Radiation survival curves revealed similar radiosensitization with FdUrd in both cell lines ($RER = 1.4 - 1.6$). Both TS1 and TS2 shRNAs produced RER values that were at least as high as those produced by FdUrd ($RER = 1.5 - 1.7$) in both cell lines. In contrast, no radiosensitization occurred with NS shRNA (Table 1 and Figure 3).

Effect of FdUrd or shRNA-mediated suppression of TS on Mutation Frequency

pSP189 plasmid mutation frequencies in the untreated or NS shRNA treated controls from both cell lines were similar, and similar to reports by others (16). FdUrd and TS shRNA produced a 4 – 7-fold increase in plasmid mutation frequency in both cell lines compared to respective controls (Figure 4) under conditions that produced equivalent radiosensitization. Thus, although TS shRNA produced less cytotoxicity than FdUrd, it was equally capable of

producing DNA mutations. For all treatments, the increase in pSP189 plasmid mutations was primarily of single base substitutions, and their relative contribution to total replication errors was similar in both cell lines (~90%). The majority of mutations occurred at random sites in pSP189 for each condition. The most common base substitutions observed with FdUrd (IC₅₀) and TS shRNA were transversions with the largest increase observed at A:T sites, as expected in the presence of a decrease in dTTP.

Discussion

We have demonstrated for the first time that shRNA mediated TS suppression produced excellent radiosensitization that was at least as effective as that observed with FdUrd. Furthermore, these studies demonstrate that TS inhibition alone is less cytotoxic compared to FdUrd. FdUrd and other FPs target TS at the same concentrations at which they can be incorporated into nucleic acids. As a result, prior studies correlating the depletion of dTTP with radiosensitization by FPs were hampered by the concomitant presence of FP nucleotides (4, 5) making it difficult to separate the contribution of each pathway to radiosensitization. The shRNA mediated suppression of TS eliminates the possibility of fraudulent nucleotide incorporation into nucleic acids. Therefore, these results provide evidence that TS inhibition alone is sufficient for radiosensitization and also establish definitively that cytotoxicity with FdUrd requires mechanisms in addition to TS inhibition, such as DNA incorporation of FP nucleotides.

We recently provided evidence for a novel mechanism of radiosensitization with FdUrd in which dTTP depletion, as a result of TS inhibition, produced mismatches in DNA that augmented cell death following irradiation (5). However, the contribution of FdUTP in producing mismatches could not be evaluated. The present study revealed a similar increase in both the extent and type of nucleotide misincorporation events following either FdUrd or shRNA suppression of TS under radiosensitizing conditions, despite the differences in cytotoxicity. Furthermore, the elevation of dATP characteristic of FdUrd treatment did not occur with TS shRNA, thus eliminating this imbalance as a source of mismatches. These results prove that the nucleotide misincorporation events are a direct result of the dNTP effects mediated by TS suppression, and cannot be explained by FdUTP incorporation into DNA. Furthermore, these results amplify and support our earlier findings, demonstrating that radiosensitization occurs as a result of mismatches in DNA due to TS inhibition (5).

Acute elevation in TS protein has been observed following exposure to FPs or analogs in preclinical and clinical studies (17, 18), as shown here (Figure 1). This increase in TS expression is clinically undesirable because high TS expression has been associated with poorer overall survival in colorectal cancer, while low TS expression is prognostic for better outcome in patients treated with 5-FU (18). Thus, use of small molecule TS inhibitors can actually antagonize therapy by promoting overexpression of TS. Previous studies evaluated suppression of TS with siRNA or ODNs on tumor cell proliferation, but not on radiosensitization (6, 7). Only modest effects were observed on cell growth when ODNs or siRNAs were used as single agents, with little to no additional effect when administered with a FP (19). Importantly, the combination of a FP or antifolate and TS siRNA or ODN still allowed overexpression of TS (7), rendering this combination clinically undesirable. These findings provide strong rationale for developing new approaches that inhibit TS without causing TS overexpression. The shRNA approach used in our studies provided a more profound (~90% decrease) and prolonged silencing of TS protein (~8 days). Although cytotoxicity was modest, the excellent radiosensitization suggests that combining TS shRNA with concurrent irradiation has potential to impact tumor growth *in vivo* while also eliminating negative clinical effects associated with elevation of TS.

Despite lengthy, nearly complete suppression of TS protein observed with TS shRNA, this approach produced less cytotoxicity than FdUrd. Previous studies have implicated FdUTP incorporation into DNA as an important contributor to cytotoxicity (3). Products of drug metabolism, such as FdUTP and its incorporation into DNA, are absent following shRNA suppression of TS, which likely accounts for its lower cytotoxicity (Table 1). ATR-mediated phosphorylation of Chk1 is a common response to DNA damaging drugs such as FPs and plays a role in the initiation of the S-phase checkpoint. Both FdUrd and TS shRNA induced S-phase arrest and ATR dependent phosphorylation of Chk1 (data not shown). Therefore, while FdUTP and/or its incorporation into DNA may elicit additional effects that contribute to an increase in cytotoxicity, TS suppression alone is sufficient to activate the Chk1 damage response.

The TS shRNA strategy allowed us to examine the effects of TS suppression without the confounding variables of FP metabolism and its associated effects. TS shRNA produced less cytotoxicity than FdUrd, but was equally effective at radiosensitizing tumor cells. This work marks the first demonstration of a shRNA strategy targeting TS to produce radiosensitization. Furthermore, this study has advanced the understanding of the lesion associated with radiosensitization by FdUrd. That compromised TS expression induced both mismatches and radiosensitization similar to FdUrd demonstrates a causal and sufficient role for the depletion of dTTP and consequent misincorporation of nucleotides into DNA in the underlying mechanism of action of FdUrd mediated radiosensitization. TS suppression may be particularly valuable as a radiosensitizing approach *in vivo* because concurrent irradiation with FPs is limited by normal tissue toxicity due to, at least in part, the toxic effects of the FPs and their catabolites (20). Furthermore, use of TS shRNA with radiotherapy may help to eliminate the negative prognostic role imparted by the increase in TS expression observed with traditional drug therapies and warrants further investigation.

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Correlative studies with fluoropyrimidines (FP) have implicated dTTP depletion and S-phase arrest in radiosensitization and cytotoxicity, with an uncertain role for the incorporation of FP nucleotides into DNA. We eliminated the possibility of fraudulent nucleotide incorporation into nucleic acids by comparing shRNA suppression to FdUrd mediated inactivation of TS on cytotoxicity and radiosensitization. We found that TS inhibition alone is sufficient for radiosensitization while cytotoxicity with FdUrd requires additional mechanisms, such as DNA incorporation of FP nucleotides.

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Figure 1. FdUrd mediated inactivation of TS versus TS shRNA mediated suppression of TS
Whole cell lysates from HCT116 and HT29 cells were analyzed by Western blotting for TS expression. (A) HCT116 and (B) HT29 cells were incubated with an IC_{50} for FdUrd (24, 48, and 72 h), left untreated (represented by the control lane in blots describing TS expression following FdUrd), or transduced with either shRNA targeted to TS mRNA (TS1, and TS2) for 1–8 days, or with non-specific shRNA (represented by the control lane in blots describing TS expression following shRNA transduction). A representative blot from a minimum of three separate experiments is shown.

Figure 2. Both TS shRNA mediated suppression of TS and FdUrd mediated inactivation of TS elicit a decrease in dTTP

dNTPs were extracted from untreated (control) (A) HCT116 and (B) HT29 cells and following incubation with an IC₅₀ for FdUrd (4, 24, 48, and 72 hr), or transduction with shRNA targeted to TS mRNA (TS1, and TS2) for 2, 5, and 8 days, or with non-specific (NS) shRNA. The data are presented as a % of the corresponding control value and represent the mean $\pm$ SD from duplicate determinations. Control values (pmol dNTP/10⁶ cells): HCT116 0-1 cells, dCTP: 8.5 ± 3.0 , dTTP: 60 ± 17.6 , dATP: 8.8 ± 0.7 , dGTP: 3.6 ± 0.7 . HT29 cells, dCTP: 17.8 ± 10.0 , dTTP: 92 ± 33.0 , dATP: 20 ± 7.8 , dGTP: 5.3 ± 3.5 .

Figure 3. TS shRNA-mediated suppression of TS is as effective as inactivation of TS by FdUrd as a radiosensitizing strategy

The effect of FdUrd and TS shRNA on the sensitivity of (A) HCT116 and (B) HT29 cells to ionizing radiation compared to untreated (control) cells was examined by incubation of cells with an IC_{50} for FdUrd (24 h), or transduction with shRNA (5 d) targeted to TS mRNA (TS1, and TS2) or non-specific (NS) shRNA, followed by radiation. The surviving fraction was corrected for cell death attributable to FdUrd, TS shRNA or NS shRNA alone, respectively. Data shown are from a representative experiment. Each experiment was repeated at least three times in triplicate.

Figure 4. FdUrd mediated inactivation of TS or TS shRNA mediated suppression of TS produces an increase in DNA mismatches

pSP189 plasmid mutation frequency was determined in (A) HCT116 and (B) HT29. The mutation frequencies in plasmids replicated in the untreated control group of both cell lines were similar $0.09\% \pm 0.02$, and $0.07 \pm 0.03\%$; HT29 or HCT116 cells, respectively; $p=0.61$). Statistical significance was determined by the application of a one tailed t-test. * significantly different from 1.0 (control), $P<0.05$. A one-way ANOVA followed by post tests was used to test for differences between treatments. Bars marked with * are not significantly different from each other, $p<0.05$. Total number of colonies counted: HCT116,

Control = 44,128 FdUrd = 30,829, TS1 = 22,468, TS2 = 19,076; HT29, Control = 20,444, FdUrd = 16,287 , TS1 = 18.656, TS2 = 19,762.

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Table 1

Effect of FdUrd or the suppression of TS on the sensitivity of HCT 116 0-1 and HT29 cells to ionizing radiation.

Cell line	treatment	Radiation Enhancement Ratio	Cell survival (% control)	D-bar (no drug)
HCT116 0-1	FdUrd	$1.4 \pm 0.08^{*,a}$	46.6 ± 5.1^a	
	TS1-shRNA	$1.5 \pm 0.04^{*,a}$	68.0 ± 5.3^b	2.10 ± 0.13
	TS2-shRNA	$1.5 \pm 0.11^{*,a}$	69.5 ± 7.6^b	
	NS-shRNA	1.0 ± 0.05^b	104.0 ± 7.3^c	
HT29	FdUrd	$1.6 \pm 0.19^{*,a}$	48.6 ± 4.7^a	
	TS1-shRNA	$1.7 \pm 0.18^{*,a}$	70.0 ± 8.2^b	3.34 ± 0.09
	TS2-shRNA	$1.6 \pm 0.14^{*,a}$	69.2 ± 3.9^b	
	NS-shRNA	1.0 ± 0.04^b	99.7 ± 8.5^c	

Radiation enhancement ratios (RERs) (mean $\pm$ SE) are shown for all cell lines after 24 h drug incubation or 5 d after shRNA transduction, followed by varying doses of ionizing radiation. Sensitivity to radiation in control cells (no drug) is shown (D-bar) for each cell line. Each tabulated value is an average of at least 3 separate experiments. Statistical significance was determined by the application of a one sample t-test to determine two-tailed P values, *RER > 1.0, P<0.05. A one-way ANOVA followed by post tests was used to compare RER and cell survival between treatment groups within one cell line. Values not sharing a letter are significantly different, p<0.05.