Combination Silencer RNA (siRNA) Targeting Bcl-2 Antagonizes siRNA against Thymidylate Synthase in Human Tumor Cell Lines

Aleksandra A. Pandyra, Randal Berg, Mark Vincent, and James Koropatnick

London Regional Cancer Program (A.A.P., R.B., M.V., J.K.) and Lawson Health Research Institute (R.B., M.V., J.K.), London Health Sciences Centre, London, Ontario, Canada; and Departments of Oncology (M.V., J.K.), Physiology and Pharmacology (J.K.), Microbiology and Immunology (J.K.), and Pathology (J.K.), Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada

Received October 11, 2006; accepted February 15, 2007

ABSTRACT

Nonspecific toxicity and resistance to traditional cytotoxic drugs are impediments to effective cancer therapy. Development of drugs targeting cellular molecules that mediate malignant characteristics may improve therapy. Antisense drugs that reduce mRNA and protein on which tumor cells depend for viability and treatment resistance are examples of such candidates. In particular, combining antisense drugs to simultaneously reduce multiple mRNAs/proteins is predicted to enhance antitumor effects. We hypothesized that combined treatment with silencer RNAs (siRNAs) targeting molecules mediating both proliferation (thymidylate synthase; TS) and survival (Bcl-2) would decrease proliferation and sensitize human tumor cells to nonantisense drugs in a greater-than-additive manner. We report that simultaneous treatment of human cervical carcinoma (HeLa) and breast tumor (MCF-7) cell lines with siRNAs targeting both TS and Bcl-2 had unexpected, nonreciprocal antagonistic effects. Two siRNAs targeting different Bcl-2 mRNA sequences reduced the capacity of TS siRNA to reduce TS mRNA and protein, with no evidence of converse effects by TS siRNA on Bcl-2 mRNA or protein. Moreover, treatment of HeLa cells with siRNA targeting Bcl-2 resulted in increased TS mRNA and protein. Pretreatment of HeLa and MCF-7 cells with TS siRNA sensitized cells to TS-targeting drugs, but addition of antagonistic Bcl-2 siRNA to the pretreatment regimen abrogated sensitization. Combined targeting of separate physiological pathways by antisense reagents may be a useful approach in treatment of cancer, but antagonistic interactions could abrogate advantages or reduce effectiveness of other antisense and nonantisense reagents.

Antisense compounds are valuable agents to explore the function of gene products through specific down-regulation of gene expression and to evaluate the consequences of that down-regulation on physiological events. Among their current applications, antisense molecules also have potential for use as anticancer therapeutics (Izquierdo, 2005). Antisense reagents [single-stranded antisense oligonucleotides (AONs) or siRNAs (siRNA); Elbashir et al., 2001] are in development as agents to specifically modulate processes on which tumor cells depend for viability and growth. They can have reduced nonspecific toxicity and improved antitumor activity compared with traditional cancer drugs (Tafech et al., 2006), and this has led to preclinical development of a large number of candidate antisense drugs. Several of these drugs (including Bcl-2, survivin, XIAP, and clusterin) are under investigation in phase I/II clinical trials (Gleave and Monia, 2005).

An important lesson emerging from early stage applications in preclinical and clinical models is that combined treatment with antisense drugs is likely to be a useful approach (Flaherty et al., 2001). The rationale is that blockage of multiple physiological events mediating malignancy and tumor cell survival, or blockage of a single critical path at multiple points to maximize inhibition of that path, will (Biroccio et al., 2003) result in greater-than-additive inhibition of tumor growth and viability. Combined treatment can consist of using antisense to reduce mRNAs encoding proteins that mediate treatment resistance (thus sensitizing tumors to the traditional treatments) (Tanabe et al., 2003), simultaneously treating with two antisense molecules that target separate pathways (Cioca et al., 2003), or targeting

This study was supported by grants from the Canadian Institute of Health Research (MOP-62636), Imperial Oil of Canada Ltd., and the Medical Oncology Research Fund (London Regional Cancer Program) (to J.K. and M.V.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.115394.

ABBREVIATIONS: AON, antisense oligonucleotide; siRNA, silencer RNA; TS, thymidylate synthase; 5-FUdR, fluorodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; SC, scrambled control; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT, room temperature.
different sequences in the same mRNA to result in greater-than-additive antisense effects.

Combining antisense agents to simultaneously target mRNAs mediating both proliferation and prosurvival events is an attractive approach to maximize antitumor therapy (Elez et al., 2003). An appropriate prosurvival target for antisense therapy is Bcl-2, an inhibitor of the mitochondrial apoptosis pathway whose overexpression has been linked to drug resistance and poor prognosis in numerous malignancies (Jiang et al., 1995; Sup et al., 2005).

Thymidylate synthase (TS), a significant gene in cellular proliferation, is another important target in cancer chemotherapy due to its central role in DNA precursor synthesis and repair (Costi et al., 2005). Not only is TS a mainstay target of many currently used traditional chemotherapeutic drugs, such as the fluoropyrimidine fluorodeoxyuridine (5-FUdR) (Yawata et al., 2005), and the antifolate raltitrexed (Farrugia et al., 2003), but it is also of developing antisense therapies (Schmitz et al., 2004), including sensitization of tumor cells selected for resistance to those drugs (Ferguson et al., 2001).

In this study, we report that combined, simultaneous targeting of both TS and Bcl-2 mRNA unexpectedly antagonized the capacity of antisense TS siRNA to reduce TS mRNA and protein in two human tumor cell lines of diverse origin. Furthermore, Bcl-2 siRNA reduced the capacity of antisense TS siRNA to enhance the action of the TS-targeting drugs 5-FUdR and raltitrexed. The antagonism was associated with specific regions of Bcl-2 mRNA, because Bcl-2 siRNAs targeting two different regions of Bcl-2 mRNA had different antagonistic capacity in spite of equal ability to reduce Bcl-2 mRNA and protein. In addition, both Bcl-2 siRNAs had similar effects on cell cycle, reducing the likelihood that the antagonism was due to cell cycle effects. The antagonism of Bcl-2 siRNA to TS siRNA was not reciprocal: TS siRNA administration neither reduced nor enhanced the antisense effects of Bcl-2 siRNA, nor the capacity of Bcl-2 siRNA to sensitize HeLa and MCF-7 cells to the apoptosis-inducing drug docetaxel. These data reveal that combined antisense targeting of multiple mRNAs can have unexpected antagonistic effects dependent on the region of mRNA selected for targeting, and independent of the degree of mRNA down-regulation by the antagonizing siRNA. Such effects should be taken into consideration when selecting combination targets for antisense drugs.

Materials and Methods

Human Tumor Cell Lines. Human cervical carcinoma (HeLa) and human breast tumor (MCF-7) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Burlington, ON, Canada) with 10% fetal bovine serum (Invitrogen) in a humidified incubator at 37°C with 5% CO2 in air.

siRNAs and Transfections. siRNAs, obtained from Dharmacon RNA Technologies (Lafayette, CO) were as follows: 1) siRNA duplex targeting TS (targeting bases 763–871 from the translation start site): antisense (5'-GAUCAUACAAAAGAUAUGCUU-3') and sense (5'-GCAUAAUUCUAGUGCAAUU-3'); 2) siRNA duplex 1 targeting Bcl-2 (targeting bases 434–453 from the translation start site): antisense (5'-AAAGCCAGCAUCCUCCU-3') and sense (5'-GGAGGAUGUUGGCUCCUU-3'); 3) siRNA duplex 2 targeting Bcl-2 (targeting bases 35–54 from the translation start site): antisense (5'-PUACUUAUCACUACUCUU-3') and sense (5'-GGGAGAUGUGAAGAUAUU-3'); and 4) control (non-targeting) siRNA duplex (SC): designed by Dharmacon RNA Technologies to have four or more mismatches with all known human mRNAs.

All siRNAs were obtained in annealed and desalted form, then they were dissolved in siRNA buffer (supplied by Dharmacon RNA Technologies) to obtain a 10 μM working solution. Cells were transfected using Oligofectamine Reagent (Invitrogen) according to the manufacturer’s instructions. In brief, siRNA/agent complexes were formed in DMEM without FBS, and then they were serially diluted to generate desired transfection concentrations. siRNA complexes in DMEM were added to HeLa and MCF-7 cells grown to 50 to 60% confluence. Transfected siRNA concentrations were either 50 nM (in experiments to measure the effects of siRNA on specific TS and Bcl-2 mRNA and protein) or 100 nM (for experiments to measure siRNA effects on cell cycle, proliferation, and drug sensitivity). Regardless of the concentration of antisense Bcl-2 and/or TS siRNA applied to cells, the total concentration of siRNA applied in every case was kept constant at 50 or 100 nM by addition of SC siRNA duplex. For treatments with combined TS and Bcl-2 siRNA, the ratio of TS siRNA to Bcl-2 siRNA was kept at 1:1. When TS siRNA or Bcl-2 siRNA was used alone, SC siRNA was added (where necessary) so that the total siRNA concentration added to every aliquot of cultured cells was kept constant.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction. Cells were plated, and then they were transfected with siRNA(s) 24 h later. Cells were lysed in TRI Reagent (Sigma-Aldrich, St. Louis, MO) at the times indicated in the figure legends, RNA was extracted from cells, and it was quantified by UV-spectrophotometry. Complementary DNA (cDNA) was prepared from 1 μg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers according to the manufacturer’s recommendations. Relative gene expression (TS or Bcl-2 mRNA) was determined by real-time reverse transcription-polymerase chain reaction amplification of TS, Bcl-2, and 18S rRNA cDNA. TS primers were forward (5'-GGCCCTCGGTTGCTTCTTT-3'), reverse (5'-GATGGCCCATCTGACATGTTCTCTTG-3'), and the TaqMan probe was (5'-FAM-AGCCCTACACAGCCG-3'). Bcl-2 primers were forward (5'-TGGGCCCCGTTGCTTTCCT-3'), reverse (5'-GGTGTGGCTG-TACCCGTTGCTCT-3'), and the TaqMan probe was (5'-FAM-AGCCCTACACAGCCG-3'). Standard curves for Bcl-2, TS mRNA, and 18S rRNA were generated using serial dilutions of cDNA derived from the HeLa and MCF-7 cell lines. Bcl-2 and TS mRNA levels (relative to 18S rRNA levels) were normalized to the 18S rRNA control and then the untreated control.

Immunoblot. Cells were plated, transfected 24 h later, and lysed to obtain total soluble cellular protein. Total protein (10–30 μg) was separated using 12% SDS-polyacrylamide gel electrophoresis, followed by transfer onto nitrocellulose membranes (GE Healthcare, Baie-d’Urfé, QC, Canada). Membranes were blocked for 16 h at 4°C with 5% skim milk powder in Tris-buffered saline with 0.2% Tween 20, and then they were probed at 25°C using a rabbit anti-human TS polyclonal antibody (Flynn et al., 2006) at 0.0625 μg/ml or a monoclonal mouse anti-human Bcl-2 oncoprotein antibody (clone 24, 1:800; Dako Denmark A/S, Glostrup, Denmark) and an anti-actin monoclonal antibody (1:1000; Sigma-Aldrich). After incubation with primary antibodies, membranes were washed with Tris-buffered saline with 0.2% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:2000 and 1:3000, respectively) (GE Healthcare). Horseradish peroxidase activity was detected using ECL Plus (GE Healthcare) and X-ray film (Eastman Photograph). Band intensities were quantified by densitometry using a FluorChem 8900 digital image system and AlphaEase FC software (Alpha Innotech, San Leandro, CA). TS and Bcl-2 densitometric band intensities were divided by the intensity of actin bands in the same lanes to yield relative TS and Bcl-2 levels in cell.
lysates. Relative TS and Bcl-2 protein levels were normalized to relative TS and Bcl-2 protein levels in control cells. Protein from control cells (untreated with siRNAs) were routinely analyzed along with protein from siRNA-treated cells. Comparison of control and siRNA-treated cell protein revealed that actin levels were unchanged by siRNA treatment in all cases (data not shown). Actin was therefore adapted as a suitable loading control for analysis for siRNA-induced changes to TS and/or Bcl-2.

Flow Cytometry. Cells were plated in T25 flasks and transfected with siRNAs 24 h later as described above. Five hours later, they were trypsinized and replated in T75 flasks at lower densities (30% confluence). At the appropriate time point, cells were trypsinized, diluted in PBS, and collected by centrifugation (5 min; 1000g; RT). The cell pellet was washed in PBS, and then cells collected by centrifugation (5 min; 1000g; RT), suspended in 300 μl of PBS, and fixed in 95% ethanol for a minimum of 24 h at 4°C. The cells were precipitated by centrifugation (5 min; 1600g; RT) and then treated with ribonuclease and stained with propidium iodide to reveal cellular DNA content (0.01 mg/ml propidium iodide, 0.25 mg/ml RNase A, 0.25 mM Tris-HCl, and 0.38 mM NaCl, pH 7.5) for 30 min at 37°C. Cells were filtered, and flow cytometry was performed using an Epics XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA). The distribution of cells in G0/G1, S, and G2/M cell cycle phases was calculated using MultiCycle software version 3.0 (Phoenix Flow Systems, San Diego, CA).

Drug Sensitivity Assays. To assess the capacity of siRNA treatment to alter the ability of cells to proliferate in the presence of absence of drugs, cells were plated in T25 flasks and transfected 24 h later as described above. For all determinations of drug sensitivity (other than the sensitivity of HeLa cells to 5-FDUR or raltitrexed (2-[5-[methyl-[(2-methyl-4-oxo-1H-quinazolin-6-yl)methylamino]-thiophen-2-yl]carbonylaminopentanedioic acid; AstraZeneca Pharmaceuticals LP, Wilmington, DE), cells were replated in 96-well plates (VWR, Mississauga, ON, Canada) at 1700 (MCF-7) and 1200 (HeLa) cells per well in a volume of 100 μl of DMEM + 10% FBS. Cells were allowed to adhere to tissue culture plastic for 18 h before addition of drug. Drug was added as a 100-μl solution in DMEM + 10% FBS. Cells were then grown for 4 days, and cell numbers were assessed by the Alamar Blue fluorescence assay using a Wallac Victor2 multilabel counter (PerkinElmer Wallac, Gaithersburg, MD).

Because of relatively high background levels of Alamar Blue signals in HeLa cells exposed to 5-FDUR or raltitrexed, the number of those cells after growth in the presence and absence of 5-FDUR or raltitrexed was determined by direct cell counting. The number of HeLa cells after growth in the presence and absence of 5-FDUR or raltitrexed was determined by replating cells in T25 flasks following (9 × 10^5 cells/flask) transfection and direct cell counting after a 4-day drug exposure using an electronic particle counter (Beckman Coulter).

Statistical Analysis. Data are presented as means ± S.E. To determine the significance of differences between means, a Student’s t test was performed. The level of significance for all statistical analyses was chosen a priori to be p < 0.05.

Results

Effect of siRNA on TS and Bcl-2 mRNA and Protein Levels in HeLa and MCF-7 Cells. To reduce the potential for concentration-dependent, nonspecific toxicities to confound observations, both cell lines were treated with siRNA concentrations that yielded significant biological effects at the lowest practical concentrations.

TS siRNA (50 nM overall duplex RNA concentration, 25 nM TS siRNA + 25 nM SC siRNA) decreased TS mRNA levels by 70 to 80% in HeLa and MCF-7 cells, compared with the effect of SC siRNA (50 nM), 24 h following transfection (Fig. 1A). Both Bcl-2 siRNA 1 and Bcl-2 siRNA 2 (50 nM overall duplex RNA concentration, 25 nM of either Bcl-2 siRNA + 25 nM SC siRNA) decreased Bcl-2 mRNA levels by 60 to 70% in HeLa and MCF-7 cells compared with the effect of SC siRNA alone, 24 h post-transfection (Fig. 1B).

Changes in Bcl-2 protein levels 24 h after treatment of HeLa and MCF-7 cells with either of the two Bcl-2 siRNAs corresponded well, both quantitatively and qualitatively, with siRNA-induced reduction in Bcl-2 mRNA levels (Figs. 2 and 3). Likewise, TS siRNA treatment of HeLa and MCF-7 cells reduced TS protein, in accord with siRNA-mediated reduction in TS mRNA (Fig. 2). Reduction in TS protein levels mediated by TS siRNA in HeLa cells reached approximately 95% by 48 h and 80% at 72 h (Fig. 4A) post-TS siRNA treatment. Bcl-2 siRNA-mediated reduction in Bcl-2 protein was similarly down-regulated by approximately 80% at 48 to 72 h.

In addition to normalizing relative TS and Bcl-2 mRNA and protein values to untreated controls, the effects of SC treatment alone were assessed. There were no significant differences in either Bcl-2 and TS mRNA or protein in SC-treated HeLa and MCF-7 cells compared with untreated control cells.

Fig. 1. Down-regulation of TS and Bcl-2 mRNA by siRNA in HeLa and MCF-7 cells. HeLa and MCF-7 cells were treated with TS siRNA (25 nM) or SC siRNA (A) or Bcl-2 siRNA 1 or Bcl-2 siRNA 2 (25 nM) or SC siRNA (B). Relative TS and Bcl-2 mRNA levels were assessed 24 h thereafter, as described under Materials and Methods. Bars indicate mean ± S.E. (n = 3 independent treatments). Different from cells treated with SC siRNA (*, p ≤ 0.05, Student’s t test).
Bcl-2 siRNA 1 Antagonizes TS siRNA-Mediated Reduction of TS mRNA and Protein. TS siRNA, administered concurrently with Bcl-2 siRNA 1 to HeLa cells (Fig. 2) or MCF-7 cells (Fig. 3), reduced TS mRNA and TS protein less effectively after 24 h of treatment than when administered in combination with equal amounts of SC siRNA. TS siRNA alone reduced HeLa TS mRNA by 80% and TS protein by 50% at 24 h after administration. However, when delivered in combination with Bcl-2 siRNA 1, TS siRNA reduced TS mRNA by only 50% (leaving 3 times the TS mRNA in cells than after TS siRNA treatment alone) and protein by 30% (leaving approximately 30% more TS protein in cells than after TS siRNA treatment alone). Thus, coadministration of siRNA targeting a non-TS mRNA (Bcl-2) reduced the effectiveness of TS siRNA after 1 day of treatment. A similar effect was observed in MCF-7 cells, where coadministration of Bcl-2 siRNA 1 impaired TS siRNA-mediated reduction of
both TS mRNA and protein by leaving 50% more TS mRNA and protein in cells than after TS siRNA treatment alone (Fig. 3). Coadministration of Bcl-2 siRNA 2 with TS siRNA did not reduce the capacity of TS siRNA to decrease mRNA or protein in either cell line (Figs. 2 and 3).

No evidence of a converse inhibition of the antisense effect of TS siRNA on Bcl-2 siRNA 1 was evident in either HeLa cells (Fig. 2) or MCF-7 cells (Fig. 3). In both cell lines, Bcl-2 siRNA 1 at 25 nM reduced Bcl-2 mRNA by approximately 60% and Bcl-2 protein by 50 to 60%, regardless of the presence or absence of TS siRNA. Interestingly, treatment with Bcl-2 siRNA 1 alone for 24 h (in the absence of TS siRNA) led to a small but significant increase (approximately 15%) in both TS mRNA and TS protein in HeLa (Fig. 2) but not MCF-7 cells (Fig. 3), compared with cells treated with SC siRNA.

It was possible that the observed antagonism was due to delay or acceleration of TS siRNA activity such that changes apparent at 24 h of treatment would not be evident at other times. To test this, relative TS and Bcl-2 protein levels were measured in HeLa cells treated with TS siRNA or Bcl-2 siRNA 1, alone or in combination, at 12, 24, 48, or 72 h post-treatment. The capacity of Bcl-2 siRNA 1 to antagonize TS siRNA-mediated reduction of TS protein persisted to at least 48 and 72 h post-transfection (Fig. 4A). No converse effects of TS siRNA on Bcl-2 protein levels were evident at any time from 24 to 72 h (Fig. 4B).

The antagonism of TS siRNA effectiveness by Bcl-2 siRNA was not restricted to 1:1 ratios of administered siRNAs (Fig. 5A). Other TS siRNA:Bcl-2 siRNA 1 ratios (2:1, 5:1, and 1:2.5) also antagonized TS siRNA effectiveness in HeLa cells in a dose-dependent manner. The antagonism depended on Bcl-2 siRNA 1 dose (Fig. 5A). As observed after concurrent treatment with Bcl-2 siRNA 1 and TS siRNA, sequential treatment of HeLa cells, first with Bcl-2 siRNA 1 and then with TS siRNA 24 h later, antagonized TS siRNA effective-
ness (Fig. 5B). Bcl-2 siRNA 1 treatment in the reverse order (TS siRNA followed by Bcl-2 siRNA 1 24 h later) resulted in much less antagonism of TS down-regulation, although a small but significant antagonism was evident.

**Bcl-2 siRNAs Inhibit Proliferation and Induce Tumor Cell Accumulation in G1 Phase of Cell Cycle.** Treatment with antagonistic Bcl-2 siRNA 1 increased the fraction of cells in G1 at 24 and 48 h post-treatment in HeLa cells: a 10% increase (Fig. 6) and a 17% increase (Fig. 6B), respectively. Qualitatively similar increases were evident in MCF-7 cells: a 7% increase (Fig. 6C) and an 8% increase (Fig. 6D), respectively.

Treatment with nonantagonistic Bcl-2 siRNA 2 had a similar effect in both cell lines at 48 h (Fig. 6, B and D), although the trend toward increased cells in G1 in MCF-7 cells at 24 h was not significant. As expected for G1 accumulation, there was a concomitant decrease in the number of cells in S phase after treatment with either Bcl-2 siRNA 1 or 2 at 48 h (Fig. 6, B and D) and at 24 h in all cases except in HeLa cells treated with Bcl-2 siRNA 2 (where the trend to decrease did not reach significance). As expected, both Bcl-2 siRNAs reduced the number of cells in G2 at 24 h and 48 h (Fig. 6, A and B), although only Bcl-2 siRNA 2 induced such a decrease in MCF-7 cells, and only at 48 h (Fig. 6D).

Treatment of HeLa and MCF-7 with TS siRNA induced a small reduction in proliferation (less than 5%) (Table 1). Bcl-2 siRNA 1 treatment in the reverse order (TS siRNA followed by Bcl-2 siRNA 1 24 h later) resulted in much less antagonism of TS down-regulation, although a small but significant antagonism was evident.

**Effect of TS and Bcl-2 siRNAs on Cytotoxicity of Raltitrexed, 5-FUdR, and Docetaxel.** TS siRNA sensitized HeLa and MCF-7 cells to the TS-targeting drugs raltitrexed and 5-FUdR (Fig. 7). TS siRNA sensitization to raltitrexed and 5-FUdR (Fig. 7) was almost completely abrogated by concurrent treatment with Bcl-2 siRNA 1 in both HeLa and MCF-7 cells (Fig 7, A–D). In HeLa cells, at low raltitrexed concentrations (1–4 nM) and at all tested 5-FUdR concentrations, some abrogation of TS siRNA sensitization was also observed following concurrent treatment with Bcl-2 siRNA 2, although not to the same degree as concurrent treatment with Bcl-2 siRNA 1. Bcl-2 siRNA 1 and 2, regardless of whether they were coadministered with TS siRNA, sensitized MCF-7 (but not HeLa) cells to docetaxel (Taxotere) (Fig. 7, F and E). Treatment of HeLa cells with Bcl-2 siRNA 1 alone reduced sensitivity to both 5-FUdR and raltitrexed, compared with SC siRNA (Fig. 8, A and C). Bcl-2 siRNA 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation</th>
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<tbody>
<tr>
<td>A) SC siRNA</td>
<td>HeLa Cells</td>
</tr>
<tr>
<td>B) TS siRNA</td>
<td>100 ± 1.1</td>
</tr>
<tr>
<td>C) Bcl-2 siRNA 1</td>
<td>77 ± 2.8*</td>
</tr>
<tr>
<td>D) Bcl-2 siRNA 2</td>
<td>85 ± 2.5*</td>
</tr>
<tr>
<td>E) TS siRNA + Bcl-2 siRNA 1</td>
<td>68 ± 0.8*</td>
</tr>
<tr>
<td>F) TS siRNA + Bcl-2 siRNA 2</td>
<td>71 ± 1.8*</td>
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<tr>
<td>G) SC siRNA</td>
<td>98.5 ± 6.0</td>
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<tr>
<td>H) No siRNA (control)</td>
<td>100 ± 2.5</td>
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* Different from untreated cells (p < 0.05, Student’s t test).

**Table 1**

Proliferation of HeLa and MCF-7 cells is inhibited by Bcl-2 and TS siRNA treatments.
also reduced sensitivity to raltitrexed (Fig. 8A). In MCF-7 cells, there was no clear change in sensitivity to raltitrexed induced by either of the two Bcl-2 siRNAs, compared with the SC siRNA (Fig. 8B). However, each of the Bcl-2 siRNAs reduced sensitivity to 5-FUdR (compared with SC siRNA), to a small degree.

**Discussion**

Targeting multiple mRNAs mediating more than one mechanism of resistance to chemotherapeutic drugs is hypothesized to enhance cancer therapy. To assess this possibility, we studied the consequences of combined treatment of human tumor cell lines with siRNAs targeting TS and Bcl-2. TS and Bcl-2 mRNA and proteins were down-regulated by treatment with either TS siRNA or Bcl-2 siRNAs in both HeLa and MCF-7 cells, with no detectable nonspecific toxicity (Figs. 1–4). The degree of siRNA-mediated reduction was consistent with other reports where antisense reagents of several types were used to target Bcl-2 (Chawla-Sarkar et al., 2004; Lima et al., 2004; Basma et al., 2005; Ocker et al., 2005) or TS (Ferguson et al., 1999; Schmitz et al., 2004) protein in several human tumor cell lines.

Although both Bcl-2 siRNAs were equally effective at reducing Bcl-2 mRNA and protein, only Bcl-2 siRNA 1 antagonized the capacity of TS siRNA to down-regulate TS mRNA and protein in HeLa (Figs. 2, 4, and 5) and MCF-7 cells (Fig. 3). Direct interaction between the two siRNAs, if it occurred, might mediate such antagonism: such interaction, however, was unlikely. First, no complementary sequences capable of mediating direct hybridization between the TS and Bcl-2 siRNAs exist. Second, TS siRNA did not impede the ability of Bcl-2 siRNA 1 to down-regulate Bcl-2 mRNA and protein in HeLa or MCF-7 cells (a converse antagonism expected if direct interaction occurred) (Figs. 2–4).
Therefore, it seems that antagonism was a downstream consequence of events mediated by down-regulation of Bcl-2 mRNA and/or protein.

Bcl-2 siRNA 1, when administered alone, increased TS mRNA and protein in HeLa cells (Figs. 2 and 4A). Such an increase might conceivably act counter to the down-regulatory activity of TS siRNA and thus contribute to antagonism of antisense effect. This, however, is unlikely to be a major contributor, because it was evident only in HeLa (Figs. 2 and 4) and not MCF-7 cells (Fig. 3). Antagonism of TS siRNA by Bcl-2 siRNA 1, and increased TS expression induced by Bcl-2 siRNA 1 as a single agent, were sustained phenomena evident at all times from 24 to 72 h post-treatment (Fig. 4A), suggesting that they are unlikely to be easily overcome simply by prolonged treatment with TS siRNA (although a lower dose of Bcl-2 siRNA 1 did diminish antagonism; Fig. 5A). Sequential treatment with Bcl-2 siRNA 1 followed by TS siRNA also resulted in antagonism, suggesting that a direct interaction of the siRNAs was not responsible for the effect.

Both Bcl-2 siRNAs induced cell cycle changes in HeLa and MCF-7 cells by increasing the fraction of cells in G1 (Fig. 6). TS is regulated post-transcriptionally, and in a cell cycle-dependent manner, such that TS protein levels increase in S phase (Chu and Allegra, 1996). The observed decrease in numbers of cells in S, with increased numbers in G1 in response to Bcl-2 siRNAs, is not consistent with an explanation of antagonism based on differential accumulation of cells in specific cell cycle compartments. Therefore, cell cycle effects are unlikely to be responsible for either antagonism of TS siRNA activity or up-regulation of TS levels by Bcl-2 siRNA 1.

To our knowledge, the cell cycle changes following treatment with Bcl-2 siRNAs in HeLa and MCF-7 cells have not been reported previously. However, there has been an asso-
cation noted between differences in cell cycle and altered levels of Bcl-2. Elevated Bcl-2 has been associated with a more slowly proliferating human ovarian carcinoma (SKOV3) cell that exhibits prolonged transit through S phase (Bélanger et al., 2005). Bcl-2 has also been shown to retard G1 entry progression (Huang et al., 1997; Vairo et al., 2000).

The physiological function of these reported changes is not clear. Bcl-2 siRNA-mediated down-regulation of Bcl-2 did not, in our hands, induce an opposite effect, suggesting that the cell cycle-associated accumulation of Bcl-2 reported by others might be an effect rather than a modulator of cell cycling.

Targeting different regions of a particular message using antisense reagents can have different physiological consequences, regardless of message or protein reduction (Berg et al., 2003). Targeting the 3′-untranslated region of TS mRNA in HeLa cells with antisense has been shown to reduce proliferation and induce G2/M arrest (Berg et al., 2001). In the current investigation, targeting the coding region of the TS mRNA had little or no effect on proliferation or cell cycle in HeLa and MCF-7 cells, despite both agents being comparable in their ability to decrease mRNA and protein levels. In view of this, differential antagonistic effects of the Bcl-2 siRNAs raise the possibility that Bcl-2 mRNA and possibly protein mediate noncanonical functions additional to their well-characterized regulatory roles in pro- and antiapoptotic signaling pathways (Shore and Viallet, 2005).

Accumulation in G1 can contribute to inhibition of proliferation. As expected, both Bcl-2 siRNA 1 and 2 reduced the capacity of HeLa and MCF-7 cells to increase in number (Table 1). Interestingly, combined treatment with siRNAs targeting both TS and Bcl-2 mRNA had at least additive effects on inhibition of human tumor cell proliferation, with no evidence of antagonism. This was not unexpected in view of the low capacity of TS siRNA to inhibit proliferation on its own, but it left open the question of other physiological consequences of the antagonism. Therefore, we investigated potential changes in sensitivity to TS-targeting drugs.

As reported previously using different AONs or siRNAs (Ferguson et al., 2001; Schmitz et al., 2004), treatment with TS siRNA sensitized HeLa and MCF-7 cells to 5-FUdR and raltitrexed (Fig. 7). The nearly complete abrogation of this sensitization upon concurrent treatment with the antagonistic Bcl-2 siRNA 1, but not Bcl-2 siRNA 2, demonstrates that antagonism, regardless of its cause, has consequences for a physiological event of potential therapeutic benefit. Because both Bcl-2 siRNAs are equal in efficacy when judged by their capacity to down-regulate Bcl-2 mRNA and protein, empirical assessment of physiological consequences of siRNA reagents targeting different mRNA regions is essential.

Treatment with a combination of TS siRNA and Bcl-2 siRNA 2 was less effective in sensitizing HeLa cells to 5-FUdR and raltitrexed than TS siRNA alone. Furthermore, Bcl-2 siRNA 1 treatment, as a single agent, decreased HeLa cell sensitivity to raltitrexed and 5-FUdR (Fig. 8, A and C); Bcl-2 siRNA 2 induced a minor decrease in sensitivity, and only to raltitrexed. Treatment of MCF-7 cells with either Bcl-2 siRNA did not increase TS protein levels, and, as a result, such treatment had a lesser effect on sensitivity to 5-FUdR and no clear effect on raltitrexed sensitivity (Fig. 8, B and D). Because both 5-FUdR and raltitrexed cause cell cycle arrest at G1, it is possible that agents that induce prearrest in G1 (in this case, both Bcl-2 siRNAs) will reduce subsequent sensitization (Johnson et al., 1999).

The mechanism underlying abrogation of sensitization following combination treatment, or treatment with Bcl-2 siRNAs alone, does not seem to be induction of general cellular resistance to cytotoxic agents, because addition of Bcl-2 siRNA in combination with TS siRNA enhanced, rather than antagonized, the capacity of the tubule depolymerization-inhibiting drug docetaxel to inhibit MCF-7 proliferation (Fig. 7F). Furthermore, the combination of TS and Bcl-2 siRNA sensitized rather than antagonized MCF-7 cells to 5-FUdR and raltitrexed, compared with pretreatment with TS siRNA alone. It is therefore likely that the mechanism of antagonism of TS sensitization to TS-targeting drugs involves both cell cycle effects and the antagonism/up-regulation of TS protein after concurrent or single agent use of Bcl-2 siRNAs.

Previous reports have shown that overexpression of Bcl-2 plays an important role in the survival of cells undergoing thymineless stress induced by TS-targeting drugs such as 5-FUdR (Fisher et al., 1993; Houghton et al., 1997). Therefore, it might be predicted that treatment with Bcl-2 siRNAs would lead to increased sensitivity to TS-targeting drugs. This was not the case. However, in view of the prearrest in G1 induced by Bcl-2 siRNAs and the increase in TS protein following treatment with Bcl-2 siRNA 1 alone, our observations are not necessarily in conflict with previous reports. Overexpression of Bcl-2 did not induce cell cycle or TS protein expression changes (Fisher et al., 1993) unlike the consequences of Bcl-2 down-regulation reported here. It is apparent, therefore, that overexpression and down-regulation of Bcl-2 will do not necessarily yield converse physiological effects.

Overall, Bcl-2 siRNA 1 exerted unexpected, nonreciprocal antagonism of down-regulation of TS mRNA and protein by TS siRNA, in two human tumor cell lines, concomitantly reducing potentially therapeutic sensitization to TS-targeting drugs. Combining antisense targeting different mRNAs to generate greater-than-additive therapeutic effects should be approached cautiously and carefully assessed.

References


Address correspondence to: Dr. James Koropatnick, London Regional Cancer Centre, 790 Commissioners Rd. East, London, ON, Canada N6A 4L6. E-mail: jkoropat@uwo.ca