Bcl-XL small interfering RNA suppresses the proliferation of 5-fluorouracil-resistant human colon cancer cells

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Abstract

5-Fluorouracil (5-FU) is commonly used to treat human colon cancers but resistance to this compound is frequently observed in clinics. To characterize mechanisms of resistance to 5-FU and to develop new strategies for overcoming it, we established two cell lines that were resistant to 5-FU but not other chemotherapeutic agents from parental 5-FU-sensitive cell lines. Western blot analysis revealed that these resistant cells overexpressed the proteins Bcl-XL, Bcl-Xs, and Bik, and further data showed that the cells were resistant to 5-FU-induced DNA damage and cell cycle disorder. However, in parental cells, enforced expression of Bcl-XL protein provided only limited protection from 5-FU-induced apoptosis and overexpression of Bcl-XL protein did not affect 5-FU-induced DNA damage or cell cycle changes; these findings suggested that overexpression of Bcl-XL protein was not the major contributor to 5-FU resistance in any of our cells lines. Even so, knockdown of Bcl-XL protein expression by Bcl-XL-specific small interfering RNA could inhibit proliferation more effectively in 5-FU-resistant cells than in 5-FU-sensitive cells, and the combination of Bcl-XL-specific small interfering RNA and 5-FU had additive effect on the inhibition of 5-FU-resistant cells. These results suggest that down-regulation of Bcl-XL protein expression might provide a new treatment strategy for human 5-FU-resistant colon cancer therapy. [Mol Cancer Ther 2005;4(3):451–6]

Received 7/1/04; revised 12/8/04; accepted 12/30/04.

Grant support: National Cancer Institute grants RO1 CAO92487-O1A1 (B. Fang), RO1 CAO98582-01A1 (B. Fang), and CA-16672.

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Introduction

In the past two decades, substantial advances have been made in the treatment of colon cancer (1–3), the second most common cancer in North America and other developed countries (1, 3). For example, 5-fluorouracil (5-FU)-based adjuvant chemotherapy has been efficacious in reducing mortality for lymph node–positive colon cancer and has become the standard of care (1). However, recurrence and metastasis due to drug resistance remain major obstacles. New strategies to overcome resistance to 5-FU and an improved curative rate for colon cancer are urgently needed.

An antimetabolite chemotherapeutic drug, 5-FU causes cell injury by inhibiting thymidylate synthase or by incorporating itself into DNA or RNA (4). High-level expression of thymidylate synthase (5), increased activity of deoxyuridine triphosphatase (4), methylation of the *MLH1* gene (6), and overexpression of Bcl-2 (7), Bcl-XL (7, 8), and Mcl-1 (9) proteins have all been reported to lead to resistance to 5-FU, which suggests that multiple factors might contribute to 5-FU resistance. However, the precise mechanisms of this resistance remain unclear.

We recently found that colon cancer cells resistant to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) could be resensitized by a combination therapy of TRAIL plus 5-FU (10). However, in that study, it remained unknown whether resistance to both TRAIL and 5-FU may develop after prolonged treatment and, if so, what the possible mechanism is and how this resistance can be overcome. To address these questions, we initiated a new study in which we exposed the parental human colon cancer cell line DLD1 and its TRAIL-resistant subclone DLD1-TRAIL/R with increasing concentrations of 5-FU. We found that prolonged exposure of 5-FU-sensitive cells resulted in the selection and expansion of 5-FU-resistant cells and TRAIL/5-FU double resistant cells, respectively. We also found different apoptotic and antiapoptotic protein levels in the sensitive and resistant cells. Furthermore, knockdown of Bcl-XL protein levels by small interfering RNA (siRNA) inhibited the proliferation more effectively in 5-FU-resistant cells than in 5-FU-sensitive cells. These findings may affect the design of treatment strategies for 5-FU-resistant colon cancer.

Materials and Methods

Cells and Cell Cultures

For our study, we used the human colon cancer cell line DLD1 and its derivatives DLD1-TRAIL/R, DLD1-5-FU/R, DLD1-TF/R, and DLD1/Bcl-XL. The latter were derived from DLD1 cells by stable transfection with the *Bcl-XL* gene as described previously (11). DLD1-TRAIL/R cells were selected from parental DLD1 cells (11). We derived DLD1-5-FU/R cells from parental DLD1 cells that were

treated with an increasing concentration of 5-FU (50–400 μmol/L) and survived up to 8 days after the end of this treatment. DLD1-TF/R cells were derived similarly from DLD1-TRAIL/R cells. All cells were maintained in RPMI 1640 supplemented with 7% (v/v) heat-inactivated fetal bovine serum, 1% glutamine, and 1× antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA). All cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

Chemicals and Reagents

We obtained 5-FU from The Pharmacy of the University of Texas M.D. Anderson Cancer Center and purchased Oligofectamine and OPTI-MEM I from Invitrogen. Two Bcl-XL siRNAs (XL1, targeting 5′-GGAGAUGCAGGUAUUGGUG-3′ specific for Bcl-XL, and XL2, targeting 5′-UGACCAGACACUGACCAUC-3′ specific for both Bcl-XL and Bcl-XS) and luciferase siRNA (targeting 5′-CGUACGCGGAAUACUUCGA-3′) were synthesized by Dharmacon (Dallas, TX) and were diluted in diethyl pyrocarbonate-treated water to 100 μmol/L as stock solution.

Cell Viability Assay

We seeded 1×10^6 cells into 10-cm dishes or 1×10^5 cells into six-well plates. Twenty-four hours later, the cells were treated with Bcl-XL siRNA, 5-FU, or both for desired courses. The cells were then trypsinized and stained with trypan blue. We counted viable cells microscopically using a hemocytometer.

Transfection of Small Interfering RNA

siRNA transfection was done according to the protocol supplied by Invitrogen. Briefly, 1×10^5 cells were seeded into six-well plates containing antibiotic-free medium and incubated overnight. For each well, we mixed together 1 μL each of XL1 and XL2 with 183 μL of OPTI-MEM I. The mixture was then combined with a solution prepared with 3 μL of Oligofectamine and 15 μL of OPTI-MEM I. After 15 minutes of incubation at room temperature, the final mixture was added to each well, which had been washed and contained FCS-free medium. The final concentration of siRNA was 200 nmol/L. We used the same concentration of luciferase-specific siRNA as a control oligonucleotide.

Flow Cytometry Assay

Cells were trypsinized, washed once with cold PBS, and fixed with cold 70% ethanol overnight at 4°C. About 30 minutes before assay, we did propidium iodide staining as described previously (11, 12). The flow cytometry assays were done in the Core Laboratory of M.D. Anderson Cancer Center.

Western Blot Analysis

We washed cells with cold PBS and lysed them with Laemmli's lysis buffer. Equal amounts of lysate were separated by 10% SDS-PAGE (12% gel for H2A.X) and transferred to Hybond enhanced chemiluminescence membranes (Amersham, Arlington Heights, IL). The membranes were blocked with PBS buffer containing 5% fat-free milk and 0.1% Tween 20 for 1 hour at room temperature and then incubated with primary antibodies for at least 1 hour at room temperature or overnight at 4°C. Finally, after washing the membranes thrice with PBS containing 0.1% Tween 20, we incubated them with peroxidase-conjugated secondary antibodies and developed with a chemiluminescence detection kit (Amersham Bioscience, Buckinghamshire, UK). Primary rabbit antihuman BAX, BCL-$X_{L/S}$, BCL-2, Bcl-W, Bik, Mcl-1, caspase-3, caspase-9; mouse antihuman multiple drug resistance (Mdr); and goat antihuman Bak antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We obtained mouse antihuman thymidylate synthase antibody from Lab Vision (Fremont, CA), mouse antihuman XIAP antibody from BD PharMingen (San Diego, CA), rabbit antihuman survivin and mouse antihuman ILP-2 antibodies from Alexis (San Diego, CA), and mouse antihuman phosphoryl H2A.X (Ser136) antibody from Upstate (Waltham, MA). Cox4 and β-actin were used as a loading control.

Statistical Analysis

Differences between treatment groups were assessed using unpaired Student's t test and a significance level of $P < 0.05$.

Results

Selection of 5-Fluorouracil-Resistant Cells

To test whether prolonged exposure of 5-FU-sensitive cells to 5-FU would lead to selection of cells resistant to this drug, we treated DLD1 parental cells and DLD1-TRAIL/R cells with this agent and selected colonies that survived up to 8 days after the end of the treatment (Fig. 1). 5-FU-resistant cells derived from DLD1 parental cells were designated DLD1-5-FU/R (resistant to 5-FU only) and those derived from DLD1-TRAIL/R cells were designated DLD1-TF/R (resistant to both TRAIL and 5-FU). Thus, 5-FU-resistant cells included both DLD1-5-FU/R (i.e., 5-FU/R) and DLD1-TF/R (i.e., TF-/R) cells, and 5-FU-sensitive cells included both DLD1 parental (i.e., parental) and DLD1-TRAIL/R (i.e., TRAIL/R) cells. Subsequent study revealed that the 5-FU concentration at which growth was inhibited in half of the cells (IC_{50}) was 2.5 μmol/L for parental cells and 210.6 μmol/L for 5-FU/R cells (an 84-fold difference) and 4.4 μmol/L for TRAIL/R cells and 105.7 μmol/L for TF/R cells (a 24-fold difference (Table 1).

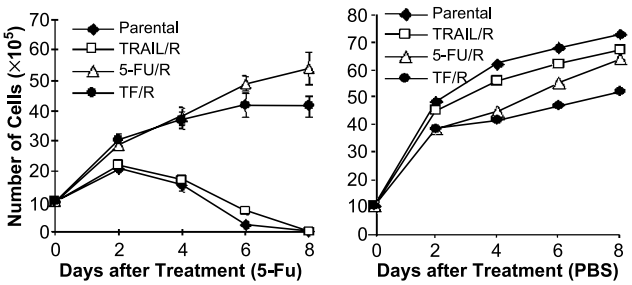

Figure 1. Cell growth after treatment with PBS or 5-FU. Parental, TRAIL/R, 5-FU/R, and TF/R cells were seeded into 10-cm dishes and treated with PBS or 400 μmol/L 5-FU. Cells were counted every 2 d. The two 5-FU-resistant cell lines (5-FU/R and TF/R) exhibited long-term survival after exposure to 5-FU. *Points,* mean; *bars,* SD (5-FU-treated cells).

To determine whether 5-FU-resistant cells were resistant to other chemotherapeutic agents as well, we evaluated the IC_{50} of the existing clinical chemotherapeutic drugs paclitaxel, cisplatin, and mitomycin. Both 5-FU-resistant cell lines remained sensitive to these agents (Table 1). Thus, resistance of these cells seemed to be specific to 5-FU.

Overexpression of Bcl-2 Family Members in 5-Fluorouracil-Resistant Cells

To evaluate the underlying mechanisms of resistance to 5-FU, we used Western blot analysis to evaluate the concentration of proteins purportedly related to resistance to chemotherapy, including Mdr (13), thymidylate synthase (5), Bcl-2 family members (Bax, Bak, Bik, Bcl-2, Bcl-w, and Bcl-XL; ref. 9), and inhibitor of apoptosis proteins (XIAP, survivin, and ILP-2; ref. 9). No obvious change of thymidylate synthase was detected in 5-FU-resistant cells with or without 5-FU treatment. Mdr protein expression was more pronounced in TF/R cells than in other cells, and Bik, Bcl-Xs, and Bcl-XL protein expression was higher in 5-FU/R cells than in parental cells and higher in TF/R cells than in TRAIL/R cells (Fig. 2). In other words, the expression of these three Bcl-2 family proteins was higher in 5-FU-resistant cells than in 5-FU-sensitive cells. Among these three proteins, Bcl-XL was the only one that is antiapoptotic. We observed no obvious change in protein expression level in other Bcl-2 family members, thymidylate synthase, or inhibitor of apoptosis proteins.

Lack of 5-Fluorouracil-Mediated S-Phase Arrest in 5-Fluorouracil-Resistant Cells

Because 5-FU has been shown to induce S-phase arrest (14), we evaluated cell cycle profiles after treatment with various concentrations of 5-FU for 3 days. We found that 5-FU induced S-phase arrest in 5-FU-sensitive cell lines but not in 5-FU-resistant cell lines (Fig. 3A). The expression level of the phosphorylated histone H2A.X, a hallmark of DNA damage (15), was high in 5-FU-sensitive cell lines within 24 hours of treatment with 200 μmol/L 5-FU but remained low in 5-FU-resistant cell lines even after 3 days of treatment (Fig. 3B). Our findings suggested that 5-FU-mediated DNA damage occurred in cells sensitive to 5-FU but not in cells resistant to it.

Table 1. IC_{50} of four cell lines after treatment with five agents

Agent	Parental	TRAIL/R	5-FU/R	TF/R
5-FU (μmol/L)	2.4 ± 0.2	4.4 ± 0.2	202 ± 10.3	106.1 ± 4.6
Paclitaxel (nmol/L)	26.5 ± 0.3	16.0 ± 2.3	10.1 ± 1.5	25.7 ± 1.3
Cisplatin (μmol/L)	16.6 ± 0.2	20.0 ± 1.9	4.3 ± 0.4	19.9 ± 1.2
Mitomycin C (μmol/L)	10.9 ± 0.5	8.9 ± 0.1	5 ± 0.2	4.5 ± 0.1
TRAIL protein (ng/mL)	3.6 ± 0.6	>1,000	5.7 ± 0.5	>1,000

NOTE: Value represents mean ± SD of a quadruplet assay.

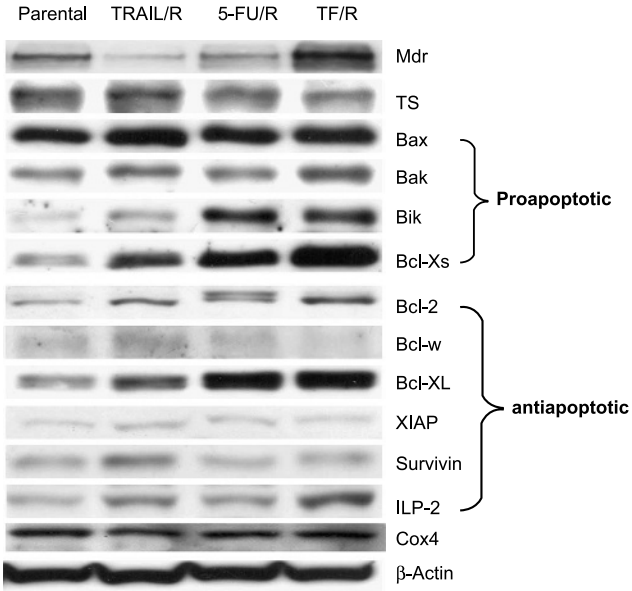

Figure 2. Expression of Mdr, thymidylate synthase (*TS*), Bcl-2 family members (Bax through Bcl-XL), and inhibitor of apoptosis proteins (XIAP, survivin, and ILP-2) in 5-FU-sensitive and 5-FU-resistant cells. As determined by Western blot analysis, Cox4 and β-actin were used as loading controls. Data from one of two independent experiments with similar results.

Effect of Overexpression of Bcl-XL Protein on Cell Sensitivity to 5-Fluorouracil

To evaluate whether the observed overexpression of the antiapoptotic Bcl-XL protein was sufficient for the resistance of 5-FU/R and TF/R cells to 5-FU, we tested the effect of 5-FU on DLD1 parental cells stably transfected with Bcl-XL (DLD1/Bcl-XL cells). Dose-effect analysis showed that the IC_{50} of 5-FU was 4.5 μmol/L in DLD1/Bcl-XL cells and 2.5 μmol/L in parental cells (a 1.8-fold difference; Fig. 4A). Cell cycle analysis showed that overexpression of Bcl-XL protein did not block 5-FU-induced S-phase arrest (Fig. 4B). Furthermore, according to Western blot analysis, 5-FU induced considerably higher expression of phosphorylated H2A.X in DLD1/Bcl-XL cells, although its level was partially attenuated on day 4 of treatment with 5-FU compared with that in parental cells (Fig. 4C). Subsequent analysis by fluorescence-activated cell sorting and Western blotting showed that 5-FU-mediated apoptosis in the DLD1/Bcl-XL cells was only partially attenuated (Fig. 4B and C). Our results suggested that overexpression of Bcl-XL protein affected 5-FU sensitivity but was not a major contributor to 5-FU resistance.

Effect of Bcl-XL-Specific Small Interfering RNA on 5-Fluorouracil-Resistant Cells

To further evaluate the role of overexpression of Bcl-XL protein in cell resistance to 5-FU, we tested the effect of Bcl-XL knockdown by Bcl-XL-specific siRNA. The Bcl-XL protein level was greatly reduced at 24 and 48 hours after treatment with Bcl-XL siRNA but not after treatment with a control siRNA specific for luciferase (Fig. 5A).

The expression of Bcl-XS was not affected (data not shown). The cell growth assay done by cell counting showed that Bcl-XL siRNA inhibited cell proliferation more effectively in 5-FU-resistant cells than in 5-FU-sensitive cells. For example, at 72 hours, compared with treatment with luciferase siRNA, treatment with Bcl-XL siRNA inhibited growth by 45.8% in 5-FU/R cells but only by 6.6% in parental cells ($P < 0.05$) and by 52.3% in TF/R cells but only by 28.1% in TRAIL/R cells ($P < 0.05$; Fig. 5B). In comparison, treatment with luciferase siRNA did not lead to a significant change in cell growth when compared with that of mock treatment with PBS. Furthermore, cell cycle analysis showed that Bcl-XL siRNA induced apoptosis more effectively in 5-FU/R cells than in parental cells ($P < 0.05$) and more effectively in TF/R cells than in TRAIL/R cells ($P < 0.05$; Fig. 5C); these results were consistent with our results from the cell growth assay (Fig. 5B).

We also analyzed the viability of 5-FU-resistant cells treated with Bcl-XL siRNA alone, high concentration of 5-FU that was around IC_{50} for resistant cells, or both. Bcl-XL siRNA and high concentration of 5-FU both inhibited the growth of 5-FU-resistant cells after 4 days of treatment;

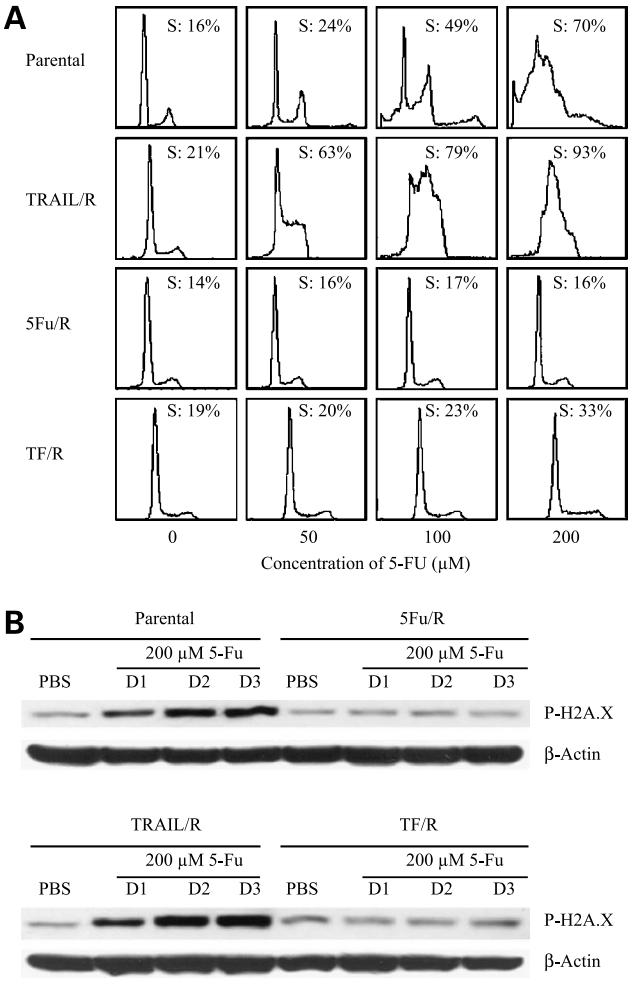

Figure 3. 5-FU-mediated S-phase arrest and DNA damage in 5-FU-resistant cells. **A,** histograms derived from fluorescence-activated cell sorting analysis. Cells were treated for 3 d with various concentrations of 5-FU. This agent induced obvious S-phase arrest in 5-FU-sensitive cells but not in 5-FU-resistant cells. Data from one of two experiments with similar results. *S,* percentage of cells in S phase. **B,** detection of the phosphorylated histone H2A.X (*P-H2A.X*) by Western blot analysis. Cells were treated with 200 μmol/L of 5-FU for 1 to 3 d (*D1–D3*). Cells treated with PBS were used as controls. Data from one of two experiments with similar results.

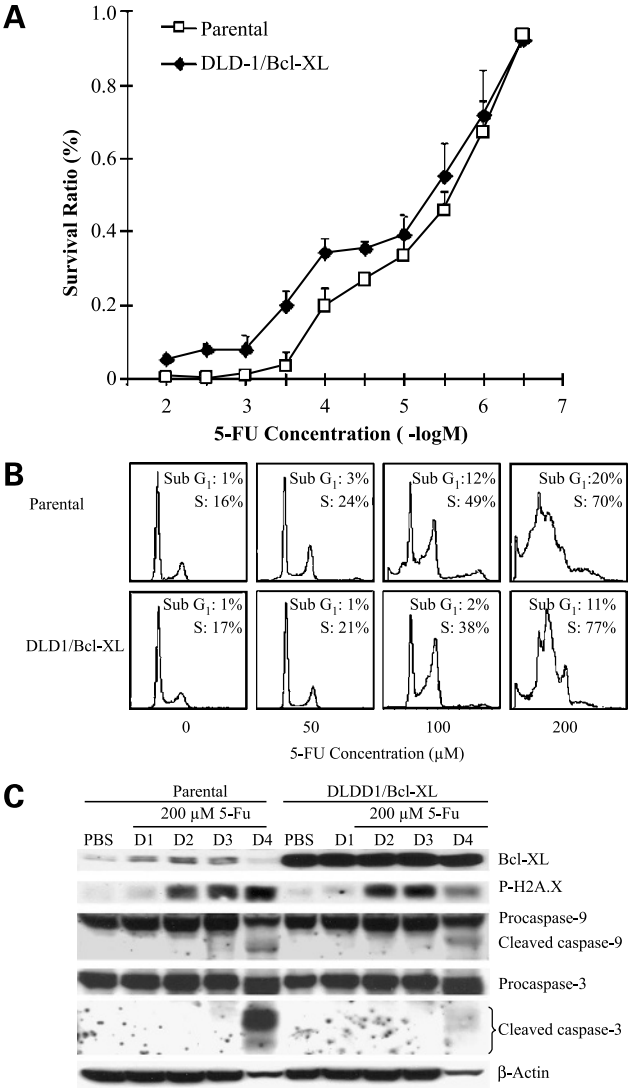

Figure 4. Effect of Bcl-XL protein overexpression on cell sensitivity to 5-FU. **A,** 5-FU dose-effect response in parental cells and DLD1/Bcl-XL cells. *Points,* mean of a quadruplet assay; *bars,* SD. **B,** for cell cycle analysis, the same cells were treated with 5-FU for 4 d. Bcl-XL protein overexpression did not block 5-FU-induced sub-G_1 or S-phase arrest. *Sub-G 1,* percentage of cells in Sub-G_1 phase; *S,* percentage of cells in S phase. Data from one of two experiments with similar results. **C,** detection of phosphorylated H2A.X (P-H2A.X), caspase-9, and caspase-3 by Western blot Analysis. Cells were treated with 200 μmol/L 5-FU for 1 to 4 d (*D1–D4*). Cells treated with PBS were used as controls. Data from one of two experiments with similar results.

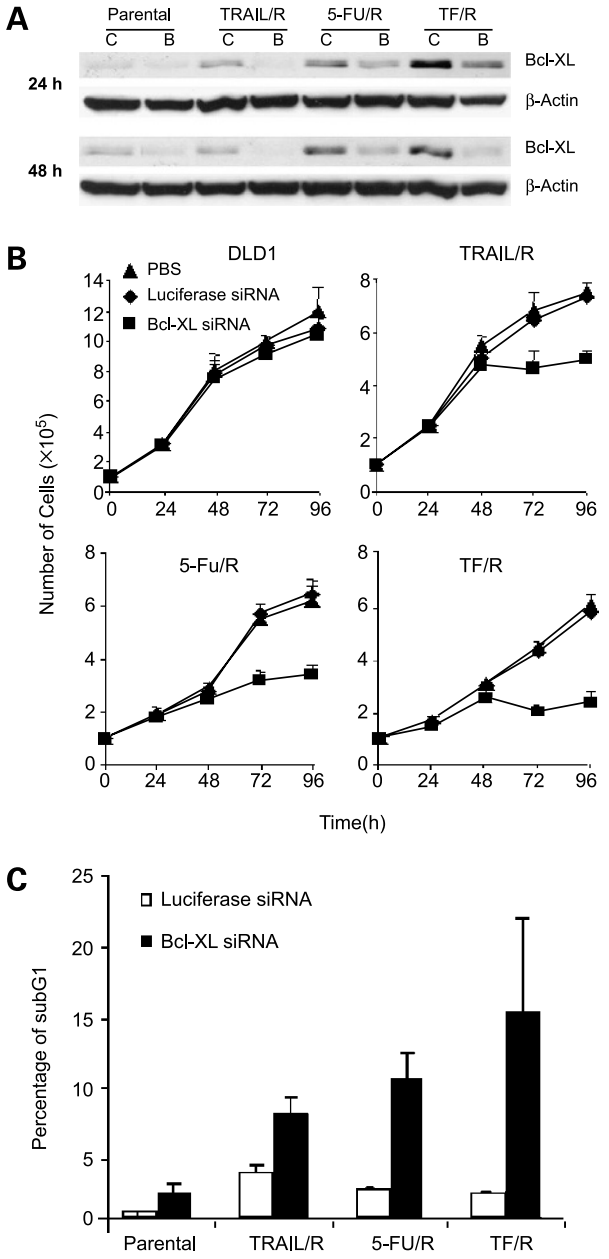

Figure 5. Effects of Bcl-XL-specific siRNA on 5-FU sensitive and resistant cells. **A,** cells treated with 200 nmol/L Bcl-XL siRNA (**B**) or a control siRNA specific for luciferase (**C**) for 24 or 48 h. Subsequently, presence of Bcl-XL protein was determined with Western blot analysis. β-Actin was also used as a loading control. **B,** cell growth after treatment with 200 nmol/L Bcl-XL siRNA as detected by cell count assay. Cells were counted every 24 h. Seventy-two hours after treatment, 5-FU/R cells were more sensitive to Bcl-XL siRNA-induced inhibition than parental cells ($P < 0.05$) and TF/R cells were more sensitive than TRAIL/R cells ($P < 0.05$). Data from one of two experiments with similar results. *Points,* mean; *bars,* SD. **C,** induction of apoptosis by Bcl-XL siRNA. Cells were treated with 200 nmol/L Bcl-XL or luciferase siRNA for 72 h. Apoptosis was determined by the percentage of cells identified with fluorescence-activated cell sorting to be in the sub-G_1 phase of the cell cycle. 5-FU/R cells were more sensitive to Bcl-XL siRNA-induced apoptosis than parental cells (*, $P < 0.05$) and TF/R cells were more sensitive than TRAIL/R cells (*, $P < 0.05$). Data from one of two experiments with similar results (*points,* mean; *bars,* SD).

combination treatment resulted in even stronger inhibition (Fig. 6). Our results suggested that simultaneous knockdown of Bcl-XL protein expression and 5-FU treatment had an additive effect on the suppression of 5-FU-resistant cells.

Discussion

In this study, prolonged exposure to the chemotherapeutic drug 5-FU resulted in selection of cells resistant to this agent, and Bcl-XL protein expression was increased in these cells. The latter result is consistent with reports by other researchers that Bcl-XL protein or mRNA levels could be evaluated after a long-term exposure to 5-FU (7, 8). However, we also found that overexpression of Bcl-XL protein alone was not sufficient for resistance to 5-FU. Although overexpression of Bcl-XL reportedly leads resistance to paclitaxel, cisplatin, and mitomycin C (16), such a resistance was not observed in 5-FU- and/or TRAIL-resistant cells, presumably because proapoptotic proteins, such as Bik and Bcl-XS, were also dramatically increased in these cells. Thus, overall ratio of Bcl-XL/Bcl-XS or Bcl/XL/Bik may be an important factor for apoptosis induction by these compounds.

We also detected an increase in Mdr protein expression in TF/R cells. Even so, these cells remained sensitive to paclitaxel, a chemotherapeutic agent of which sensitivity can be greatly reduced by overexpression of Mdr (17, 18), indicating that the increase in Mdr expression was not enough to cause the drug resistance we observed. The precise mechanisms involved in 5-FU remain unclear. Further characterization of molecular changes in these resistant cells may provide insights to the underlying resistant mechanisms.

Knockdown of Bcl-XL protein expression inhibited the proliferation of 5-FU-resistant cells in our study. Bcl-XL

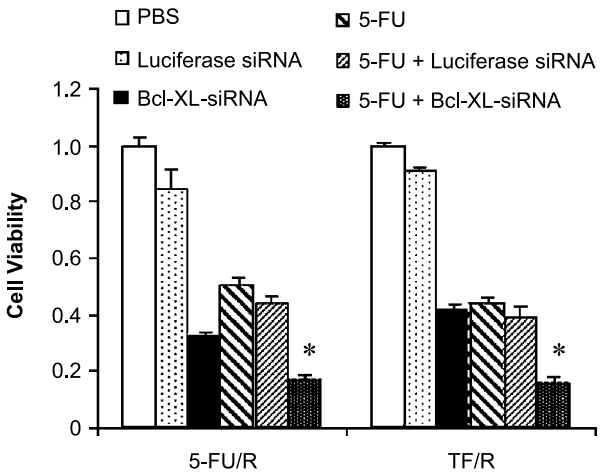

Figure 6. Effect of Bcl-XL siRNA and 5-FU treatment on cell viability. Cells were pretreated with 200 nmol/L Bcl-XL siRNA or luciferase siRNA for 24 h. 5-FU (200 μmol/L) was added for an additional 72 h. The number of viable cells was counted and then normalized to results from cells treated with PBS. Combined Bcl-XL siRNA and 5-FU treatment provided stronger inhibition than 5-FU alone or Bcl-XL siRNA alone. *, $P < 0.05$ versus Bcl-XL siRNA or 5-FU alone treatment.

protein is known to promote cell survival by regulating the electrical and osmotic homeostasis of mitochondria in response to various stimuli (19, 20), and down-regulation of Bcl-XL protein by chemicals or antisense oligonucleotides can induce subsequent mitochondrion-based cell death (21, 22). Although our data suggested that enforced overexpression of Bcl-XL protein was not sufficient to render cells resistant to 5-FU, suppression by Bcl-XL-specific siRNA led to stronger growth inhibition in 5-FU-resistant cells. The different genetic background of these cells may help explain these observations.

We noted that the concentrations of both antiapoptotic proteins (e.g., Bcl-XL) and proapoptotic proteins (e.g., Bcl-Xs and Bik) were higher in 5-FU-resistant cells than in 5-FU-sensitive cells, which may also help explain why the 5-FU-resistant cells were more sensitive to Bcl-XL siRNA than the latter cells were. It is possible that a different balance between antiapoptotic and proapoptotic proteins may be maintained in 5-FU-resistant cells and 5-FU-sensitive cells. Specific knockdown of Bcl-XL protein by siRNA might have changed the balance and induced stronger mitochondrion-mediated apoptotic signaling in 5-FU-resistant cells. This scenario may also explain why Bcl-XL specific siRNA induced more cell death in TRAIL/R cells than in parental cells.

The development of siRNA technology has made it possible to suppress the function of specific molecular targets. This technology will be very useful in developing new treatment for cancer (23, 24) because our knowledge of molecular targets that demarcate the difference between normal and malignant cells is increasing. Our study results on human colon cancer cells resistant to 5-FU suggested Bcl-XL protein as a good target for cancer therapy, especially for cancers resistant to conventional chemotherapy. Nevertheless, *in vivo* delivery and tumor specificity are challenging issues for the use of Bcl-XL siRNA as an anticancer therapeutics agent (25). Development of genetic vectors or formulations for *in vivo* delivery of siRNA will be necessary before siRNA can be used as a therapeutic agent.

Acknowledgments

We thank Elizabeth L. Hess (D) for editorial review and Alma Vega, Carrie A. Langford, and Jesse Null for assistance in preparing the manuscript. This article represents partial fulfillment of the requirements for the Ph.D. degree for J.J. Davis.

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