Antisense Bcl-xl Down-Regulation Switches the Response to Topoisomerase I Inhibition from Senescence to Apoptosis in Colorectal Cancer Cells, Enhancing Global Cytotoxicity

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ABSTRACT

Purpose: To identify determinants of the effect of antisense-mediated Bcl-xl down-regulation (Bcl-xl knockdown) on the response of colorectal cancer cells to SN38, the active metabolite of irinotecan, a topoisomerase I inhibitor licensed for colorectal cancer chemotherapy.

Experimental Design: Using wild-type HCT116, p53 null, Bax null, or p21/WAF1 null isogenic derivatives, we measured expression of regulators of cellular response, and associated growth arrests or apoptosis, after SN38 treatment, with or without antisense-mediated Bcl-xl knockdown.

Results: A modified phosphorothioate antisense oligonucleotide (ISIS15999) reduced Bcl-xl protein expression by ~90%. SN38 induced p53, Bax, Bcl-xl, and p53-dependent p21/WAF1 protein accumulation. The Bax:Bcl-xl ratio changed little. In wild-type HCT116, but not in Bax null cells, Bcl-xl knockdown induced a shift in response from drug-induced senescence to apoptosis, and enhanced the global cytotoxicity of SN38. In p53 null or p21/WAF1 null cells marked apoptosis occurred after SN38 alone, and was additionally enhanced by Bcl-xl knockdown in p21/WAF1 null cells but not in p53 null cells.

Conclusions: Drug-induced senescence is associated with late relapse after therapy in transgenic models of cancer in vivo. We have shown that abolition of p21/WAF1-mediated drug-induced senescence or antisense-mediated Bcl-xl knockdown can both, independently, enhance the apoptotic response of colorectal cancer cells to SN38 in vitro. The growth arrest suppresses a p53-independent apoptotic pathway, whereas Bcl-xl induction suppresses a p53 and Bax-dependent apoptotic pathway. The combination of irinotecan and Bcl-xl antisense merits testing in models of colorectal cancer in vivo.

INTRODUCTION

It has been conclusively shown recently that downstream determinants of apoptosis (specifically Bcl-2 in a murine transgenic lymphoma model) can profoundly influence the response of malignant cells to chemotherapy in vivo, and that prolonged drug-induced growth arrest can suppress apoptosis, leaving a residual pool of viable malignant cells from which late relapsing clones may ultimately emerge with lethal results (1). Other solid tumors display similar properties in vitro. For example, colorectal cancer cell lines undergo a prolonged senescent-like growth arrest when treated with topoisomerase I inhibitors in vitro (2). A therapy which, when combined with a topoisomerase I inhibitor, could switch the chemotherapeutic response from growth arrest to apoptosis, might improve the outcome of colorectal cancer treatment. In this paper we demonstrate that antisense down-regulation of Bcl-xl (Bcl-xl knockdown) can enhance the apoptotic response in vitro to the topoisomerase I inhibitor SN38 in colorectal cancer cells otherwise destined to undergo drug-induced senescence. We identify p53 and Bax as important genetic determinants of this effect of Bcl-xl knockdown. Both p53 and Bax are frequent targets of genetic disruption in particular subtypes of human colorectal cancer (3, 4) so that elucidation of their role in chemotherapeutic response will help to guide more effective therapy.

Topo I inhibitors predominantly produce S phase-dependent DNA damage by stabilizing the cleavable complex of topo I with DNA and, thus, facilitate DNA double-strand breakage by the collision of a precessing replication fork with the cleavable complex. SN38 is the active metabolite of irinotecan, which is licensed for the treatment of patients with colorectal cancer (5).

Bcl-2 family members are of importance in colonic development and pathophysiology (6). Human colonic adenomas overexpress Bcl-2 relative to surrounding normal mucosa (7, 8). Associated with the transition to invasive malignancy, Bcl-2 expression tends to fall, and elevated Bcl-xL expression appears (9–11). Clinical studies have associated this transition with worsening prognosis in colorectal cancer (12). In some tumors the switch to Bcl-xL expression may relate to deletion of the Bcl-2 locus, by virtue of its close linkage to both the DCC and

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2The abbreviations used are: topo I, topoisomerase I; Bcl-xl knockdown, antisense-mediated Bcl-xl down-regulation; β-gal, β-galactosidase; 5FU, 5-fluorouracil; HCT116-wt, HCT116 wild-type; IOD, integrated optical density; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline/tween-20.
SMAD4 tumor suppressor genes on chromosome 18q (13). However, Bcl-2 has not been identified as a frequent target for mutational inactivation in colorectal cancer.

The Bcl-2 family is implicated in the response of colorectal cancer cells to cytotoxic treatment in vitro, and this depends, in turn, on the presence or absence of the wild-type p53 tumor suppressor gene (14). In general, two competing p53-mediated responses to cytotoxic agents are observed, namely cell death or cell cycle arrest (14–16). Abrogation of cell cycle arrest by knock-out of p53, p21/WAF1, or 14–3–3σ genes shifts the balance of cellular response from arrest to mitotic death (17–19), and, in p21/WAF1 null cells for example, can lead to an enhanced therapeutic response to DNA damage in models of colorectal cancer (2, 16). On the other hand, in the presence of wild-type p53 and intact checkpoints, cell death can be mediated via the mitochondrial apoptotic pathway (15, 20). Indeed, colorectal cancer cells rendered null for p53 by targeted gene knockout are resistant to 5FU-induced apoptosis (14). Similarly, Bax null cells are highly resistant to apoptosis induced by nonsteroidal anti-inflammatory drugs, and are partly resistant to 5FU-driven apoptosis (21). Additional proapoptotic proteins regulated by p53 include Noxa, Bak, and PUMA, as well as Bax (20, 22, 23). Each of these, like Bax, localizes to mitochondria, and can heterodimerize with Bcl-xl through a BH3-containing domain (24–28). The relative ratio of pro- to antiapoptotic Bcl-2 family members is believed to determine the threshold for induction of mitochondrial-dependent apoptosis (27, 29, 30). Thus, overexpression of exogenous Bcl-xl suppresses mitochondrial-mediated apoptosis and enhances cancer cell survival in several cancer models (31–33). Therefore, down-regulation of Bcl-xl might be expected to enhance the apoptotic response to DNA damage in wild-type p53 containing cancer cells. Indeed, antisense targeting of Bcl-xl in colorectal cancer cells has been shown recently to enhance 5FU-induced apoptosis (34). Genetic determinants of this effect were not identified. Bcl-xl or Bcl-2 antisense oligonucleotides enhance the effect of chemotherapy in a variety of other tumor types in vitro and in vivo (35–38). Bcl-2 antisense has entered clinical trials in melanoma and lymphoma (39, 40).

Thus, Bcl-xl is highly expressed in colorectal cancer, correlating with invasiveness, regulates apoptotic responses to chemotherapy in several cancer models, and may provide a therapeutic target. We used a chemically modified Bcl-xl antisense oligonucleotide to down-regulate Bcl-xl protein expression (37). Using an isogenic set of cell lines derived from the HCT116 colorectal cancer cell line, we identified genetic determinants of response to SN38 alone or in combination with Bcl-xl knockdown. The parental HCT116 line carries wild-type p53, p21/WAF1, and Bax, and is mismatch repair-deficient (by virtue of silencing of the hMLH1 gene). The derivative lines we used were generated by selective knockout of p53, p21/WAF1, or Bax genes by targeted homologous recombination (21, 41).

**Materials and Methods**

**Cell Lines and Tissue Culture.** The parental HCT116-wt colorectal cancer cell line was obtained from the European Collection of Cell Cultures. The p53 null, p21/WAF1 null, or Bax null derivatives of HCT116 were a generous gift from Prof. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Immunoblots confirmed the expected lack of expression of p53, p21/WAF1, or Bax, respectively (data not shown). Cell lines were maintained in RPMI 1640 with 10% FCS and 1% penicillin/streptomycin (all from Life Technologies, Inc.) in a 37°C, 5% CO2, fully humidified incubator and passaged once or twice weekly.

**Oligonucleotides.** Oligonucleotides with 2′O-methoxyethyl modification of the ribose ring in flanking nucleotides and a fully phosphorothioate backbone were provided by ISIS Pharmaceuticals. The antisense agent ISIS 15999 (TCCTGGTT-GCTCTGAGACAT) is complementary to the first 20 Bcl-xl coding nucleotides. An 8-β mismatch oligonucleotide ISIS 16971 (TCACATGGCCTTAGGCGGT) provided a missense control, of which the single agent cytotoxic effects were similar to those of the antisense agent over a wide dose range (data not shown). Oligonucleotides were transfected using lipofectin (Life Technologies, Inc.), and antisense-specific Bcl-xl knockdown sought for a variety of lipofectin:oligonucleotide ratios. For experiments combining oligonucleotide and SN38 treatment, the transfection protocol was optimized to minimize nonspecific cytotoxic oligonucleotide effects. Thus OPTI-MEM I was preincubated for 45 min at room temperature with lipofectin, in the ratio of 3 μl/ml lipofectin per 400 μl final oligonucleotide concentration. Oligonucleotides were added and the mixture incubated for an additional 15 min (room temperature) before transfections were performed in triplicate on six-well trays containing 0.75–1.25 × 106 cells/well, by washing with 1 ml PBS at pH 7.5, adding 1 ml of transfection mix and incubating for 6 h. It proved essential to closely control the number of cells present on the day of treatment, to obtain quantitatively reproducible results.

**Cytotoxic Agents.** Stock solutions of SN38 (gift of Aventis) were prepared in DMSO at 100 μg/ml and stored frozen. Treatment solutions were made by serial dilution in growth medium such that the final concentration of DMSO was always <0.02%, inactive in the assays described. Drug-containing medium was added to washed cells and incubated without replacement for a maximum of 72 h or until cells were harvested.

**Growth Assays by Coulter Counting.** Adherent cells were washed in PBS, harvested in trypsin/EDTA (Life Technologies, Inc.), and counted in a Z2 Coulter counter.

**Immunobots.** For actin, p53, p21/WAF1, Bax, and Bcl-xl, immunoblot pellets of adherent cells were washed with cold PBS and stored at −20°C, resuspended in 100 μl lysis buffer [50 mm HEPES (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 150 mm sodium chloride, 5 mm EDTA with protease inhibitors pepstatin A 2 μg/ml, aprotinin 10 μg/ml, leupeptin 10 μg/ml, and phenylmethylsulfonyl fluoride 100 μg/ml] per million cells and mixed (25 min at 4°C). Lysate supernatants were subjected to a Bradford assay for protein concentration. Twenty μl aliquots of total protein were denatured in loading buffer (95°C, 5 min), electrophoresed on a 10% SDS-PAGE gel, and transferred to a polyvinylidene fluoride Immobilon-P transfer membrane (Millipore). Protein loadings were checked by Ponceau staining. Membranes were blocked in...
5% fat-free milk powder (Marvel; Premier Brands) in TBS (pH 7.5) with 0.1% TBS-T for 1 h at room temperature, incubated with primary antibody in 5% Marvel in TBS-T (1 h at room temperature for actin, p53, Bcl-xl, and Bax, or overnight at 4°C for p21/WAF1), washed in TBS-T, and incubated with secondary antibody conjugated to horseradish peroxidase (1 h at room temperature). After additional washing in TBS-T and TBS, blots were visualized by chemiluminescence (Santa Cruz Biotechnology) and photographed. Actin immunoblots confirmed near equal protein loading per lane. Expression for each protein was quantified by measuring the appropriate IOD using Labworks software (Ultra-Violet Products). The IOD for each band was corrected for variations in protein loading (which were small) using the corresponding IOD for the actin band. For each independent experiment and for each protein band of interest, the corrected IOD derived for that band was expressed as a proportion of the total IOD obtained for that band summed for all of the samples. The mean and SEs for the proportion of protein expression in drug-treated compared with untreated cells were, thus, derived from at least three independent experiments, each in duplicate. These results were normalized to give a mean value of 1 for each protein in untreated cells.

PARP immunoblots were used to detect caspase-mediated PARP cleavage during apoptosis. Floating and adherent cells were pooled and counted, washed in PBS, then ultrasonicated on ice in a volume of reducing buffer proportional to the cell count [62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, and 5% 2-mercaptoethanol] and electrophoresed on a 7.5% gel. Ponceau staining and actin immunoblotting confirmed near equal protein loadings. PARP immunoblotting followed the protocol above (substituting PBS for TBS at each step) using the antibodies outlined below. The IOD of uncleaved and cleaved PARP bands was measured using Labworks software (Ultra-Violet Products) and extent of cleavage expressed as a ratio of cleaved to total PARP. The mean and SEs for this ratio were derived from at least three independent experiments for each treatment condition.

Antibodies used were: ascites antiactin IgM CP01 (at 1:120,000 dilution), anti-p53 IgG OP43 (1:200), and anti-p21/WAF1 IgG OP64 (1:100; all mouse monoclonals from Oncogene Research Products); anti-Bcl-xl IgG sc-634 (1:200) and anti-Bax IgG sc-493 (1:500; both rabbit polyclonals from Santa Cruz Biotechnology); and mouse monoclonal anti-PARP C2-10 (1:10,000; R&D Systems). Secondary antibodies were goat antiamine IgM 401225 (1:4,000) from Calbiochem, and goat antirabbit IgG sc-2030 (1:2,000), and goat antimouse IgG sc-2031 (1:2,000), both from Santa Cruz Biotechnology.

Cell Cycle Analysis. One million cells were washed in cold PBS, fixed in 70% ethanol (at least 1 h at 4°C), washed, resuspended in 25 µg/ml PI with 100 µg/ml RNase A (Sigma), and incubated for 30 min at 37°C. Fluorescence was measured on a Becton Dickinson FACSCaliber flow cytometer within 1 h. Data were analyzed using the MODFIT 2.0 program (Verity Software). The mean and SE for the percentage of cells in each phase of the cell cycle were derived from at least three independent experiments, each in duplicate. For analysis of growth arrest, adherent cells only were harvested. For determination of sub-G1 fraction, adherent and floating cells were pooled.

β-Gal Staining and Photography. Adherent cells treated in triplicate in six-well trays were washed with PBS, fixed for 3 min in 1 ml 3% paraformaldehyde, washed, and incubated at 37°C in air overnight in 1 ml X-Gal staining solution [5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-Gal (Sigma) from stock at 20 mg/ml in dimethylformamide, 150 mM sodium chloride, 2 mM magnesium chloride, 34.25 mM citric acid, and 117.5 mM sodium hydrogen phosphate (pH 5.6)]. β-Gal-expressing cells develop a blue color. Morphological effects of treatment were also evident. Representative regions of each well were photographed using a digital camera (Kodak MDS 120) linked to Adobe Photoshop software.

Annexin V Assays. One million cells were washed in cold PBS and suspended in 100 µL Annexin-V binding buffer containing 5 µg/µl PI and 0.5 µg/ml Annexin-V-FITC, incubated for 15 min at room temperature in the dark and diluted in 400 µL Annexin-V binding buffer (R&D Systems). Fluorescence was measured on a Becton Dickinson FACSCaliber flow cytometer within 1 h. The collected events were gated on the forward and side scatter plots to exclude cellular debris. Three discrete cell populations identified using standard cutoffs in each experiment represented viable (unstained), early apoptotic (Annexin-V but no PI staining), and late apoptotic/necrotic cells (Annexin-V and PI staining). Thus, the mean and SEs for the proportion of early apoptotic cells were derived from at least three independent experiments, each in duplicate.

Definition of Supra-Additive Enhancement of Response in Growth Inhibition Assays. Single agent dose response curves were measured in at least three independent experiments, each in duplicate for antisense oligonucleotide (6-10h exposure to 0, 400, 800, 1600, or 4800 nM) or for SN38 (0, 15, 30, or 60 nM for 72 h), with counts of adherent cell numbers at 72 h. The dose response curves were concave upwards. Therefore, in the nomenclature of Steel and Peckham, the mode I expected combination effect defines the appropriate limit of the envelope of additivity at every level of effect and is calculated by multiplication of the corresponding single agent effects (42).

Combination experiments were performed at fixed antisense exposure (0 or 400 nM for 6 h) followed by SN38 (0, 15, or 30 nM for 72 h) with counts at 72 h. Therefore, for each experiment the mode I expected combination effects could be calculated from the single agent effects and compared with the measured combination effects. The mean measured or expected combination effects, with SEs, were thus derived from four independent experiments, each in triplicate. The measured combination effect was classified as supra-additive if greater than the expected combination effect by a statistically significant margin. Controls lipofectin/SN38 or missense/SN38 combination experiments were included to exclude supra-additive interactions among these agents.

Statistical Analysis. The significance of differences between experimental conditions was determined using the two-tailed Student’s t test.

RESULTS

SN38 Induced Effects in HCT116-derived Cell Lines. A brief dose-dependent pulse of apoptosis occurred among HCT116-wt cells on day 2 after SN38 treatment, as demon-
SN38-induced apoptosis was Bax dependent but p53 and p21/WAF1 independent. Cells were treated with SN38 as indicated. Floating and adherent cells were harvested at indicated times and pooled. A, early apoptotic changes as determined by Annexin V apoptosis assay on day 2. B, representative histograms demonstrating a population with sub-G1 DNA content as indicated by flow cytometry on day 2. Each graph records on the Y axis (linear scale) the number of fluorescent events with the given fluorescence intensity (X axis, linear scale, arbitrary units). C, onset of PARP cleavage: cells were counted and ultrasonicated on ice in a volume of reducing loading buffer proportional to the count. Lysates were immunoblotted for actin or PARP. Blots were visualized using chemiluminescence and photographed. Results for actin and Ponceau staining confirmed equal protein loadings. The graph displays mean for three independent experiments for which the ratio of cleaved to total PARP integrated absorbance was quantified using Labworks Software. Representative photographs are shown; bars, ±SE.
treatment to 85% ± 9% (mean ± SE; n = 3; not significant) of untreated control levels. Bax expression was unaffected (Fig. 4). Antisense mediated Bcl-xl knockdown was equally effective in p53 null HCT116 (data not shown).

Effect of Prior Bcl-xl Knockdown on SN38-induced Apoptosis and on Global SN38 Cytotoxicity. In HCT116-wt cells antisense Bcl-xl knockdown but not missense treatment enhanced the extent of SN38-induced apoptosis, as demon-

Table 1 Mean (SE) for ratio of cleaved:total PARP

<table>
<thead>
<tr>
<th>Cell line</th>
<th>A Opti + 30 nM SN38</th>
<th>B Antisense + 30 nM SN38</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2 Wild-type</td>
<td>0.11 (0.04)</td>
<td>0.41 (0.03)</td>
<td>0.004</td>
</tr>
<tr>
<td>P53 null</td>
<td>0.22 (0.04)</td>
<td>0.25 (0.04)</td>
<td>0.49</td>
</tr>
<tr>
<td>P21 null</td>
<td>0.08 (0.02)</td>
<td>0.48 (0.11)</td>
<td>0.002</td>
</tr>
<tr>
<td>Bax null</td>
<td>0.002 (0.001)</td>
<td>0.03 (0.008)</td>
<td>0.06</td>
</tr>
<tr>
<td>Day 3 Wild-type</td>
<td>0.05 (0.01)</td>
<td>0.23 (0.04)</td>
<td>0.007</td>
</tr>
<tr>
<td>P53 null</td>
<td>0.20 (0.05)</td>
<td>0.28 (0.03)</td>
<td>0.17</td>
</tr>
<tr>
<td>P21 null</td>
<td>0.28 (0.15)</td>
<td>0.74 (0.10)</td>
<td>0.002</td>
</tr>
<tr>
<td>Bax null</td>
<td>Not done</td>
<td>Not done</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 SN38-induced prolonged senescent-like growth arrest is p53 and p21/WAF1 dependent but Bax independent. Cells were treated in triplicate for 72 h with 0 or 30 nM SN38. A, growth curves for 30 nM treated cells; adherent cells were harvested and counted at times indicated. B, DNA content of adherent cells determined by flow cytometry on day 7. Cells were also fixed in parafomaldehyde, washed, and stained in X-Gal staining solution. β-gal-expressing cells develop a blue color. Morphological effects of treatment were also evident. Representative photographs are shown.
Software, corrected for the corresponding actin IOD, and normalized to
The graph shows mean for three or four independent experiments for
results for actin and Ponceau staining confirmed equal protein loadings.
Blots were visualized using chemiluminescence and photographed. Re-
times, were immunoblotted for actin, p53, p21/WAF1, Bcl-xl, or Bax.

Moderate (but clinically relevant) concentrations of SN38 (44, and, therefore, in a well-controlled fashion. We chose to use
moderator (but clinically relevant) concentrations of SN38 (44,
Figure 3 Induction of p53, and p53 dependence of Bax and Bcl-xl in-
duction after SN38 treatment. Lysates from HCT116-wt or p53 null
cells, treated with 0, 15, or 30 nM SN38, and harvested at indicated
times, were immunoblotted for actin, p53, p21/WAF1, Bcl-xl, or Bax.
Blots were visualized using chemiluminescence and photographed. Re-
sults for actin and Ponceau staining confirmed equal protein loadings.
The graph shows mean for three or four independent experiments for
which the IODs of the relevant bands were measured using Labworks
Software, corrected for the corresponding actin IOD, and normalized to
the untreated state; bars, ±SE.

strate d by an increase in early apoptotic changes in Annexin V
assays (Fig. 5A), an increase in the population of cells with sub-
G1 DNA content (Fig. 5B), and an increase in the extent of PARP
cleavage (Fig. 5C). In Bax null cells, SN38 treatment after Bcl-xl knockdown induced a very minimal degree of
apoptosis only, significantly less than observed in HCT116-wt (Fig. 5; Table 1). As noted above, SN38 alone
induced marked apoptosis in p53 null or in p21/WAF1 null
cells, but this was significantly enhanced by Bcl-xl knockdown
only in p21/WAF1 null cells (Fig. 5; Table 1). Thus, Bcl-xl
knockdown enhanced SN38-induced apoptosis to a degree de-
pendent on p53 and Bax status. Finally, Bcl-xl knockdown
produced a supra-additive enhancement of the global cytoto-
icity of SN38 in HCT116-wt as assayed by surviving cell counts
at 72 h (Fig. 6; n = 4; P < 0.01 for expected versus observed
combination effect by the method of Steel and Peckham; Ref. 42).

Bcl-xl Antisense Treatment Given 7 Days after Treat-
ment with 15 nM SN38-Induced Apoptosis in Growth-
Arrested HCT116-wt. To test the hypothesis that Bcl-xl ex-
pression continued to protect HCT116-wt cells from apoptosis
after SN38-induced senescence was fully developed, we used
Bcl-xl antisense treatment (800 nM for 24 h) to down-regulate
Bcl-xl expression, 7 days after SN38 treatment (15 nM for 72 h).
Again, Bcl-xl antisense but not missense treatment induced
apoptosis in a significant proportion of arrested cells, as shown
by the onset of PARP cleavage (P < 0.002; n = 3; Fig. 7).

DISCUSSION

Using HCT116-wt cells or p53 null, p21/WAF1 null, or
Bax null HCT116 derivatives (21, 41), we have studied genetic
determinants (p53, p21/WAF1, or Bax) of the effect of SN38,
and of Bcl-xl knockdown, on an otherwise isogenic back-
ground, and, therefore, in a well-controlled fashion. We chose to use
moderate (but clinically relevant) concentrations of SN38 (44,
45) to avoid a pitfall of in vitro work, namely the generation
of dramatic effects by the use of drug concentrations so high as to
be irrelevant to clinical practice (2). Nevertheless, we have been
able to draw statistically sound conclusions by careful quanti-
tation of our data. For precise quantitation of the extent of
apoptosis we used the PARP cleavage assay (46) in preference
to measurement of the sub-G1 fraction or to the Annexin V
assay. The sub-G1 assay does not readily detect cells dying from
the G2 phase of the cell cycle. The Annexin V assay detects
early apoptotic changes specifically but cannot discriminate late
apoptotic from necrotic cells. Nevertheless, both of these apo-
ptosis assays provided useful confirmation of our more detailed
PARP cleavage results. Indeed, the PARP cleavage assay has
been shown to correlate well with other measures of apoptosis in
vitro and in vivo (46).

In these and in all of the other assays, the lack of effect of
missense controls argues that the observed antisense-specific
effects are because of Bcl-xl down-regulation and are not be-
cause of nonspecific effects of oligonucleotides on SN38 sensi-
tivity. However, our experiments were optimized to reduce
such nonspecific effects. In addition, nonspecific effects have
been described for the combination of irinotecan with oligonu-
cleotides in vivo (47). In these circumstances our use of p53 null
or Bax null isogenic cell lines provide important additional
controls. Because Bax is a major mediator of p53-dependent
apoptosis, and a proapoptotic heterodimeric partner for Bcl-xl,
we reasoned (and ultimately observed) that the specific effect
of Bcl-xl knockdown on SN38-induced apoptosis should be atten-
uated in p53 or Bax null cells, whereas nonspecific effects
should be p53 or Bax independent (because they are not known
or expected to be mediated solely through p53 or Bax-dependent
pathways).

SN38 induced the accumulation of p53 and of the cell cycle
inhibitor p21/WAF1 in a p53-dependent manner. Accumulation of
the proapoptotic protein Bax was also induced to a greater
degree in HCT116-wt than in p53 null HCT116. Bax expression
is under the transcriptional control of p53 (48), and Bax can
induce mitochondrial-mediated apoptosis (21, 49, 50). Despite
Bax induction in our system, the dominant cellular response to
SN38 in HCT116-wt was a prolonged growth arrest (at least 10
days), and drug-induced senescence developed.
The maintenance of viability during the growth arrest after SN38 treatment was dependent on wild-type p53 and on p21/WAF1, such that apoptosis supervened as the major cellular response in p53 or p21/WAF1 null HCT116 after day 2, despite the initial onset of a G2 growth arrest in these cells. Thus, maintenance of a stable p53- and p21/WAF1-dependent growth arrest contributes to the protection of HCT116-wt cells from SN38-induced cell death, as has been observed with chemotherapeutics of various cytotoxic mechanism in similar model systems (2, 14, 16–19, 41, 43, 51–53). Clearly, in p53 null HCT116 cells, the apoptosis observed from day 2 onwards must occur via a p53-independent apoptotic pathway.

In HCT116-wt cells Bcl-xl was induced in parallel with Bax after SN38 treatment, such that the Bax:Bcl-xl ratio changed little. The accumulation of both Bax and Bcl-xl after exogenous p53 expression has been reported in colorectal cancer cells (54). In our study, prior Bcl-xl knockdown markedly enhanced the apoptotic response of HCT116-wt cells to SN38, switching the ultimate cellular fate from drug-induced senescence to apoptosis in a significant proportion of cells, as demonstrated by Annexin V assays, reflecting an early and reversible step in the apoptotic pathway (55), and by PARP cleavage assays, reflecting caspase activation in late apoptosis (46). Even after SN38-induced senescence was well established in HCT116-wt cells, 7 days after the initial chemotherapeutic insult, Bcl-xl antisense treatment could still switch arrested cells to apoptosis. Thus, Bcl-xl expression suppresses apoptosis during the initial establishment of senescence and during its maintenance after topoisomerase I inhibition by SN38 in HCT116-wt cells.

In contrast, SN38-induced apoptosis after Bcl-xl knockdown was markedly attenuated in Bax null cells. In p53 null cells the marked SN38-induced apoptosis occurring from day 2 onwards was not enhanced significantly by Bcl-xl knockdown, consistent with activation of a mitochondrial independent, and, hence, Bcl-xl independent, apoptotic pathway in p53 null HCT116 (56). On the other hand, in p21/WAF1 null cells, SN38-induced apoptosis was markedly enhanced by Bcl-xl
knockdown, consistent with p53-mediated activation of the mitochondrial-dependent apoptotic pathway in these cells, which retain wild-type p53 function.

These results demonstrate that Bcl-xL induction contributes to the protection of HCT116 cells from p53- and Bax-dependent apoptosis, in keeping with a model in which a critical determinant of mitochondrial mediated apoptosis is the ratio between pro- and antiapoptotic Bcl-2 family members, rather than their absolute expression levels (27, 29, 30). In addition, however, our data imply that at least one other DNA damage-induced signal cooperates with the increased Bax:Bcl-xL ratio to drive apoptosis, because basal Bax protein levels were insufficient to induce apoptosis in HCT116-wt cells in the absence of SN38 treatment, even when the Bax:Bcl-xL ratio had been increased ~10-fold by antisense-mediated Bcl-xL knockdown. The nature of this additional apoptotic signal remains to be determined.

As well as a qualitative shift in cell fate from growth arrest to apoptosis, antisense Bcl-xL knockdown produced a quantitative supra-additive enhancement of the global activity of SN38 in assays of total surviving adherent cell numbers.

In summary, our data allow construction of a model for the determinants of response to SN38 in HCT116 colorectal cancer cells. In HCT116-wt cells a sustained p53- and p21/WAF1-dependent SN38-induced senescent-like growth arrest protects cells from a p53-independent apoptotic pathway, whereas Bcl-xL induction suppresses a p53- and Bax-dependent apoptotic pathway. Thus, ablation of the p21/WAF1-mediated sustained growth arrest or antisense-mediated Bcl-xL knockdown can both, independently and additively, enhance the apoptotic response to SN38.

The capacity of antiapoptotic Bcl-2 family members to switch the chemotherapeutic response from apoptotic cell death to drug-induced senescence has been demonstrated recently in vivo, after cyclophosphamide treatment of Bcl-2 overexpressing lymphoma in a transgenic murine model, and drug-induced senescence was associated with late relapse after therapy (1). Our results demonstrate that this chemotherapy resistance mechanism is not restricted to lymphoma or to cyclophosphamide treatment only, but is a more general phenomenon. In addition, we have identified a potential therapeutic approach to circumventing such resistance. Disruption of Bcl-xL function, if achieved in tumors in vivo, using antisense technology or using small molecule inhibitors currently under development (57, 58), might provide a means of enhancing the chemotherapeutic effect, especially in those cancer patients whose tumors retain wild-type p53 and Bax function. Therefore, the combination of irinotecan and Bcl-xL antisense merits testing in models of colorectal cancer in vivo.

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