Enhanced Chemosensitivity to Irinotecan by RNA Interference-Mediated Down-Regulation of the Nuclear Factor-κB p65 Subunit

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ABSTRACT

In preclinical tumor models, inhibition of nuclear factor-κB (NF-κB) has been associated with increased sensitivity to chemotherapeutic agents such as irinotecan (CPT-11). This is based on the fact that a variety of chemotherapy agents such as CPT-11 activate NF-κB to result in the expression of genes such as *c-IAP1* and *c-IAP2* that might be responsible for the inhibition of chemotherapy-induced apoptosis. In this study, RNA interference [small interfering RNA (siRNA)] was used to down-regulate the NF-κB p65 subunit in the HCT116 colon cancer cell line, and its role, in the presence and absence of CPT-11, was assessed on cell growth and apoptosis. Reduction of endogenous p65 by siRNA treatment significantly impaired CPT-11-mediated NF-κB activation, enhanced apoptosis, and reduced colony formation in soft agar. Furthermore, the *in vivo* administration of p65 siRNA reduced HCT116 tumor formation in xenograft models in the presence but not the absence of CPT-11 administration. These data indicate that the administration of siRNA directed against the p65 subunit of NF-κB can effectively enhance *in vitro* and *in vivo* sensitivity to chemotherapeutic agents.

INTRODUCTION

The nuclear factor-κB (NF-κB) transcription factor is a critical regulator of the expression of genes involved in the immune and inflammatory response as well as in the control of cell growth (1, 2). The NF-κB complex is composed of a variety of homodimers and heterodimers formed by five components including p50, p52, p65 (RelA), RelB, and c-Rel subunit. The best-characterized form of NF-κB consists of the p50 and p65 heterodimer (3, 4). This complex is sequestered in the cytoplasm of most cells, where it binds to a family of inhibitory proteins known as IκB (5, 6). In response to a variety of stimuli such as cytokines, the IκB proteins are phosphorylated on two NH_2-terminal serine residues by the IκB kinases, ubiquitinated, and subsequently degraded by the proteasome (7, 8). Once released from IκB, the NF-κB complex translocates to the nucleus and increases expression of a variety of target genes including inhibitors of apoptosis such as *c-IAP1*, *c-IAP2*, and *Bcl-xL* (9, 10).

Apoptosis induction is an important mechanism in chemotherapy-induced killing of tumors. In contrast, resistance to chemotherapy in a variety of tumors is due to up-regulation of genes that inhibit apoptosis (11). Recent experimental observations have demonstrated that activation of the NF-κB pathway serves as an important mechanism of cell survival in response to chemotherapy treatment by activating a variety of genes such as *c-IAP1*, *c-IAP2*, *TRAF1*, *TRAF2*, and *Bcl-2* homologs that inhibit apoptosis (9, 11). In tumor models, NF-κB is activated in response to radiation and chemotherapy, and inhibition of the NF-κB pathway significantly enhances the apoptotic response to chemotherapy (12, 13). Importantly, systemic use of NF-κB inhibitors such as the proteasome inhibitor PS-341 in conjunction with irinotecan (CPT-11) enhances chemotherapy-induced cell killing by stimulating apoptosis (11, 14).

CPT-11 and its more active metabolite, SN38, are topoisomerase I inhibitors that have efficacy in the treatment of certain neoplasms including colorectal cancer (15). Despite the initial response to therapy, most patients treated with CPT-11 become resistant to this chemotherapy and exhibit tumor progression (16). CPT-11 treatment has been shown to lead to the activation of NF-κB in a variety of human colorectal cancer cell lines (17). Activation of the NF-κB pathway constitutes a potential mechanism of inducible resistance by malignant cells exposed to CPT-11 (9, 11). Thus, reducing NF-κB-mediated activation may help prevent CPT-11-induced resistance to cell killing.

Recently, a gene therapy approach to inhibit the NF-κB pathway using recombinant adenovirus-mediated transfer of a modified nondegraded form of IκBα resulted in significant augmentation of sensitivity to CPT-11 in colon cancer cells (12). An alternative strategy for inhibiting NF-κB activation by CPT-11 in colon cancer cells was demonstrated by the addition of proteasome inhibitors to prevent IκB degradation (14). These findings indicate that NF-κB inhibition might be an effective strategy to enhance sensitivity to CPT-11. Small interfering RNA (siRNA) is a highly specific and efficient tool to evaluate genes that regulate NF-κB-mediated resistance to chemotherapy and also has the potential to be used as a targeted therapeutic agent (18–26). Annealed 21-mer siRNAs can be introduced into

Received 10/7/03; revised 1/16/04; accepted 1/27/04.
Grant support: The Helen and Alden Wagner Cancer Fund and the Nasher Cancer Research Program.
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Note: J. Guo and U. N. Verma contributed equally to this work.
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cells by transfection, bind specifically to the cellular mRNA of interest, and stimulate RNA degradation resulting in a 80–90% specific decrease in the levels of their corresponding protein (27, 28). Previous experiments have shown that siRNA can block specific genes such as β-catenin and survivin in colon cancer cell lines and mouse xenograft models (29, 30).

To evaluate whether inhibiting the p65 component of NF-κB could increase the sensitivity of colon cancer cells to CPT-11, we used siRNA directed against p65 both *in vitro* and *in vivo* using the established colon cancer cell line HCT116. Our studies demonstrate that NF-κB activation induced by CPT-11 is effectively inhibited in both cell culture and xenografts by p65 but not control siRNAs. The p65 siRNA treatment increased sensitivity of colon cancer cells to CPT-11 and reduced tumor growth *in vivo*. Our results demonstrate that inhibition of NF-κB by siRNA can enhance the therapeutic effects of chemotherapy.

MATERIALS AND METHODS

Cell Lines. The colon cancer adenocarcinoma cell lines SW480 (31, 32) and HCT116 (33) were obtained from the American Type Culture Collection (Manassas, VA). These cells were propagated and maintained in McCoy's 5A and Leibovitz L-15 media (Life Technologies, Inc., Rockville, MD), respectively, and supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The HCT116 cells were maintained with 5% CO_2, whereas the SW480 cells were maintained without CO_2.

RNA Oligonucleotides. Double-stranded siRNAs with two thymidine residues (dTdT) at the 3′ end of the sequence extending between amino acid residues 347 and 353 were designed for the NF-κB p65 subunit (sense, 5′-GCCCUAUCCCUUUACGUCA-3′; Ref. 34). SiRNAs with two thymidine residues (dTdT) at the 3′ end of the sequence extending between amino acid residues 60 and 66 along with their corresponding antisense oligonucleotides were designed for human T-cell lymphotrophic virus type I Tax gene (sense, 5′-GAUGGACGCGUUAUCGGCU-3′; Ref. 27; Dharmacon Research Inc., Lafayette, CO). These RNAs were dissolved in 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA as 200 μM solutions. Double-stranded siRNAs were generated by mixing the corresponding pair of sense and antisense RNA oligonucleotides at a concentration of 20 μM in annealing buffer [30 mM HEPES-KOH (pH 7.9), 100 mM potassium acetate, and 2 mM magnesium acetate]. The reaction mixture was heated to 95°C for 2 min, allowed to come to room temperature over 90 min, and then aliquoted and stored at –20°C.

Transfection of RNA Oligonucleotides. Approximately 1×10^6 cells were plated per 10-cm plate in media containing 10% fetal bovine serum to give 30–50% confluence. Transfection of the RNA oligonucleotides was performed using Oligofectamine (Invitrogen, Carlsbad, CA) to result in a final RNA concentration of 50 nM. The cells were harvested at different time points and lysed in PD buffer [40 mM Tris-HCl (pH 8.0), 500 mM sodium chloride, 0.1% NP40, 6 mM EDTA, 6 mM EGTA, 10% glycerol, 10 mM sodium fluoride, and 1 mM sodium orthovanadate] for Western blot analysis.

Western Blot Analysis. Cells were prepared in PD buffer, and Western blot analysis was performed as described previously (35). The antibodies and dilutions used included anti-p65 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-IAP1 and Bcl-2 (1:500; Transduction Laboratories, San Diego, CA), anti-actin (1:1000; Sigma Chemical Co.), and anti-c-IAP2 (1:500; Santa Cruz Biotechnology). After extensive washing, the membranes were incubated with antimouse or antirabbit IgG-horseradish peroxidase conjugate antibody (Amersham Biosciences, Piscataway, NJ) at a 1:1000 dilution for 1 h at room temperature and developed using enhanced chemiluminescence (Amersham Biosciences).

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay. Forty-eight hours after transfection with siRNA to p65 or Tax, 5000 HCT116 or SW480 cells were seeded into a 96-well plate. Twenty-four h later, CPT-11 (Pharmacia and Upjohn Co., Kalamazoo, MI) was added at varying concentrations in triplicate. After 24 or 48 h, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml in PBS) were added to each well, the plate was incubated for 4 h at 37°C, and 100 μl of 0.04 N HCL in isopropanol were added. Within an hour, the absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of surviving cells at each concentration relative to the nontreated group was plotted.

Gel Retardation and Supershift Assays. Whole cell extracts from HeLa cells were prepared in PD buffer, and ^{32}P-labeled NF-κB or SP1 oligonucleotides (Promega, Madison, WI) were used for gel retardation assay. The binding reactions containing 60,000 cpm of the radiolabeled probe, 10 μg of cell extracts, 500 ng of poly(deoxyinosinic-deoxycytidylic acid) (Amersham Biosciences), 10 μg of BSA, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1% NP40, 5% (v/v) glycerol, and 5 mM DTT in a final volume of 20 μl were incubated at room temperature for 30 min and subjected to electrophoresis on a 5% (w/v) polyacrylamide gel in Tris-EDTA buffer. For supershift assays, 1 μg of rabbit polyclonal antibody directed against p65 or p50 (Santa Cruz Biotechnology) was added to the binding reactions and incubated for 1 h on ice before gel electrophoresis. The gels were dried and exposed to X-ray film.

Effect of siRNA on NF-κB Reporter Constructs. Cells were transfected with a NF-κB luciferase (1 μg) and a Rous sarcoma virus-β-galactosidase (0.3 μg) reporter using GeneJuice (Novagen, Madison, WI) 24 h after either p65 or Tax siRNA transfection (31). After an additional 24 h, the cells were treated with 10 μM CPT-11 for either 24 or 48 h, lysed in a luciferase analysis buffer (Promega), and analyzed for luciferase and β-galactosidase activity using specific assays (Promega).

Caspase-3 Assays. Induction of apoptosis was assessed by detecting caspase activity in cell lysates 48 h after siRNA transfection. The activity of DEVD (Asp-Glu-Val-Asp)-specific proteases was measured using an EnzCheck Caspase-3 Assay Kit (Molecular Probes, Eugene, OR) as suggested by the manufacturer. Forty-eight h after siRNA transfection, the cells were harvested, and 1×10^6 cells were frozen at −80°C until the time of assay. The frozen cells were resuspended in 50 μl of lysis buffer and incubated on ice for 30 min, and cellular debris was pelleted. Lysates (50 μl) were transferred to 96-well plates and incubated at room temperature for 50 min with substrate (Z-

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DEVD-AMC) in 2× reaction buffer at a final concentration of 100 μM. Cell lysate alone with the caspase inhibitor Ac-DEVD-CHO at a final concentration of 100 μM was also included. Fluorescence was measured by a fluorometer at an excitation wavelength of 350 nm and a detection wavelength of 450 nm.

Soft Agar Colony Assays. Twenty-four h after siRNA transfection, the cells were mixed with tissue culture media containing 0.6% agar to result in a final agar concentration of 0.4%. One ml of this cell suspension was immediately plated in 6-well plates coated with 0.6% agar (1 ml/well) in tissue culture media. The colonies were counted in triplicate 15 days after plating, and the number of colonies per 10^5 cells was calculated.

Murine Xenograft Model. Institutional guidelines and an Animal Research Committee-approved protocol were followed for mouse studies. For these studies, 4–6-week-old female nude$^{nu/nu}$ mice were obtained from Charles River (Wilmington, MA) and housed in clean, specific pathogen-free rooms in groups of five and in cages containing microisolator tops. HCT116 cells were transfected with siRNA to p65 (experimental group) or Tax (control group). Twenty-four h after transfection, the cells were harvested and washed twice in ice-cold serum-free McCoy 5A media, and the number of viable cells was counted by trypan blue exclusion. The cells were resuspended in the same medium, and 2.5×10^6 cells in a volume of 0.25 ml were injected s.c. into the right flank of each mouse. One day after tumor cell implantation, five mice in the experimental group and the control group were treated with 0.25 ml (4 mg/ml) of CPT-11 by i.p. injection (Pharmacia and Upjohn Co.) twice a week for 2 weeks. The tumors were measured in three axes from day 7 onward, and the tumor volume was calculated from these measurements.

In another experiment, 2.5×10^6 untreated HCT116 cells were injected into nude mice s.c. These mice were randomly divided into four groups of eight mice each. Eight mice received p65 siRNA, eight mice received Tax siRNA, eight mice received CPT-11 and p65 siRNA, and eight mice received CPT-11 and Tax siRNA. Each mouse was given 250 pmol of siRNA diluted into 0.25 ml of PBS twice a week by tail vein injection. The mice randomized to the CPT-11 groups received 0.25 ml (2 mg/ml) of CPT-11 by i.p. injection twice a week. Serial measurements of tumor size were initiated once tumors were palpable. Tumors were allowed to grow until they reached a maximum size of 2 cm^3 or ulcerated or for 80 days.

RESULTS

CPT-11-Mediated Growth Inhibition of SW480 and HCT116 Cell Lines. First, we addressed the response of two colon cancer cell lines, SW480 and HCT116, to cell killing by CPT-11. The sensitivity of these cell lines was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Both cell lines were exposed to CPT-11 for 24 h at concentrations of 0, 1, 5, 10, 15, and 20 μM. The SW480 cell line was more sensitive to CPT-11 than the HCT116 cell line. In SW480 cells, CPT-11 resulted in an IC_{50} of 13 μM, whereas HCT116 cells did not reach an IC_{50} at the concentrations of CPT-11 tested (Fig. 1). Results similar to those for CPT-11 were seen with SN38, the active metabolite of CPT-11, but at concentrations 100-fold less, as described previously (data not shown; Refs. 36 and 37). Given the relative resistance of the HCT116 cells to CPT-11 killing, this cell line was selected for subsequent experiments to determine whether inhibition of NF-κB activation by CPT-11 would enhance cell death.

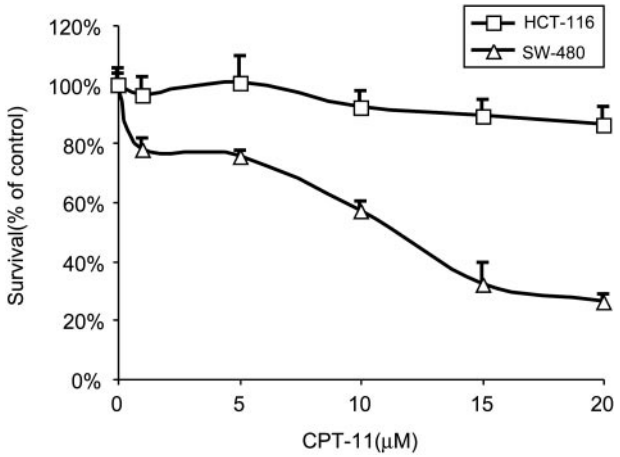

Fig. 1 Growth inhibition by irinotecan (CPT-11) in SW480 and HCT116 cells. HCT116 and SW480 cells were plated on 96-well plates and treated with 0, 1, 5, 10, 15, and 20 μM CPT-11 for 24 h. Cell survival was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the cell number was plotted as a percentage of the control (cells not exposed to CPT-11). Each value represents the mean ± SD of triplicate readings. Similar results were observed in three other independent experiments.

Prolonged p65 Protein Suppression and Enhanced Chemosensitivity to CPT-11 in HCT116 Cells by p65 siRNA. HCT116 cells were transfected with 21-mer sense and antisense RNA oligonucleotides (siRNAs) directed against a portion of the NF-κB p65 subunit or siRNAs directed against the human T-cell leukemia virus Tax gene, and Oligofectamine-transfected or mock-transfected cells were used as controls. Cells were harvested at 2, 4, 8, and 10 days after transfection, and extracts were prepared and analyzed by Western blot for p65 and actin. The levels of p65 protein were decreased 2 days after siRNA transfection and remained decreased for 8 days (Fig. 2*A*). These results suggested that siRNA transfection leads to specific and prolonged suppression of p65 protein expression.

Next we addressed whether p65 siRNA would enhance chemosensitivity to CPT-11 in HCT116 cells. Forty-eight h after transfection with p65 siRNA, Tax siRNA, or Oligofectamine or mock-transfection, HCT116 cells were treated with 0, 1, 5, 10, 15, or 20 μM CPT-11 for 48 h. Cells exposed to p65 siRNA in the presence of CPT-11 showed increased sensitivity to CPT-11 (IC_{50} = 11 μM) as compared with cells treated with either Tax siRNA, Oligofectamine alone, or control transfection (Fig. 2*B*). These results indicate that p65 siRNA-mediated decreases in the NF-κB subunit resulted in enhanced cell killing by CPT-11.

NF-κB Activation Induced by CPT-11 Is Inhibited by p65 siRNA. To address whether the NF-κB pathway was activated in HCT116 cells in response to CPT-11 treatment and whether siRNA directed against p65 could prevent this process, siRNA directed against either p65 or Tax was used with CPT-11, and NF-κB activity was assayed by gel retardation experiments. At 72 h after transfection of these siRNAs, HCT116 cells

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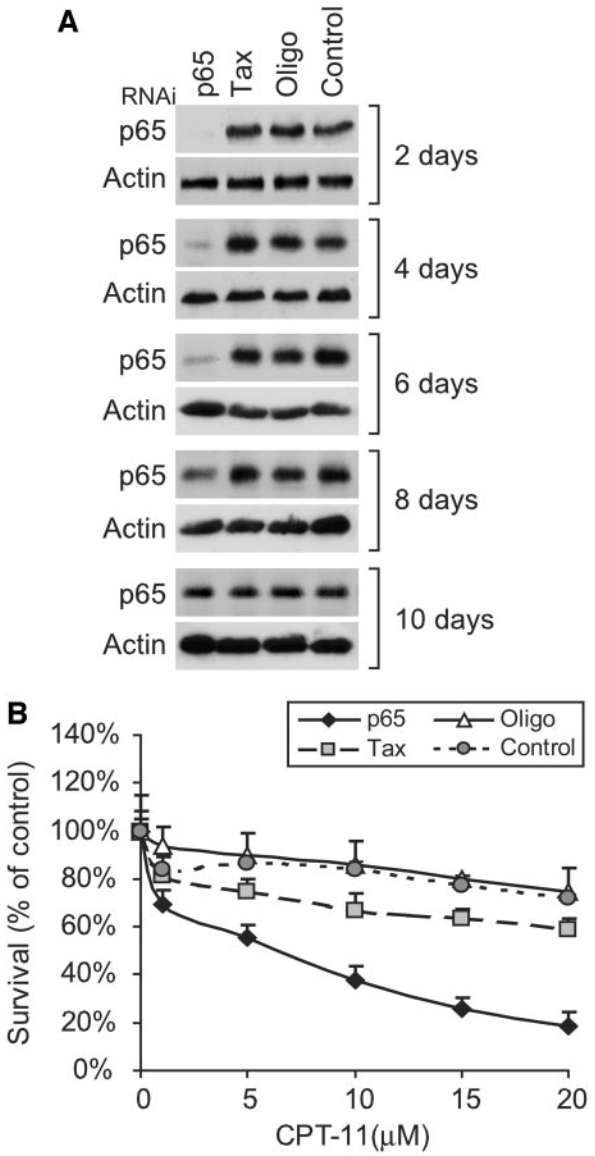

Fig. 2 Enhanced chemosensitivity to irinotecan (CPT-11) in HCT116 cells by p65 small interfering RNA (siRNA). *A*, HCT116 cells were transfected with 21-mer annealed p65 siRNA (50 nM), Tax siRNA (50 nM), or Oligofectamine alone or mock-transfected (control). Forty-eight h after transfection, cells were treated with 0, 1, 5, 10, 15, and 20 μM CPT-11 for 48 h. Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the cell number was plotted as a percentage of the control (cells not exposed to CPT-11). Each value represents the mean ± SD of triplicate readings. Similar results were observed in three independent experiments. The IC_{50} was defined as the drug concentration needed to produce a 50% reduction of growth relative to the control. *B*, HCT116 cells were transfected with 21-mer annealed RNA oligonucleotides (50 nM) directed against p65 or Tax or mock-transfected. Extracts of the cells were prepared at 2, 4, 6, 8, and 10 days after transfection and analyzed for p65 and actin expression by Western blot analysis.

were treated with 10 μM CPT-11 and harvested at 0, 1 or 6 h after treatment. The effect of p65 siRNA on the level of NF-κB activation induced by CPT-11 was determined by gel retardation and supershift assays. There was little NF-κB binding of the p50/p65 heterodimer in cell extracts prepared from the p65 siRNA-transfected cells as compared with that seen in Tax and control cells (Fig. 3*A*, *top panel*). Supershifted p65 and p50 bands were demonstrated in control extracts treated with CPT-11 (Fig. 3*A*, *top panel*, *Lanes 11* and *12*). In contrast, there was equivalent binding of SP1 in these extracts (Fig. 3*A*, *second panel*). Quantification of gel retardation experiments from three independent experiments is shown (Fig. 3*A*, *graph*). Western blot analysis with antibodies directed against p65 indicated that the p65 but not the Tax siRNA specifically blocked p65 protein expression (Fig. 3*A*, *third panel*), whereas there was no change in the actin expression (Fig. 3*A*, *fourth panel*). These results demonstrated that inhibition of p65 expression markedly decreases CPT-11-induced NF-κB activation.

Next, a NF-κB luciferase reporter construct was transfected into HCT116 cells pretreated with either p65 or Tax siRNA, and luciferase activity was determined after CPT-11 treatment for either 0, 6, 12, or 24 h. SiRNA directed against p65 reduced NF-κB activation approximately 4–5-fold as compared with siRNA directed against Tax in cells treated with 10 μM CPT-11 for either 12 or 24 h (Fig. 3*B*). These results indicate that p65 siRNA could reduce NF-κB induction in HCT116 cells.

p65 siRNA Increases CPT-11-Mediated Apoptosis by Decreasing c-IAP1 and c-IAP2 Expression. To determine whether p65 siRNA alters the ability of CPT-11 to induce apoptosis in HCT116 cells, the activity of caspase-3 and related DEVD-specific proteases was determined. The assay involved the fluorescent measurement of the proteolytic cleavage of Z-DEVD-AMC to the fluorescent molecule AMC in both the absence and presence of the caspase-3 inhibitor Ac-DEVD-CHO. The results of this analysis demonstrated that the transfection of p65 siRNA increased the ability of CPT-11 to activate caspases as compared with transfection of Tax siRNA or control (Fig. 4*A*). Western blot analysis was performed to determine whether the levels of the antiapoptotic proteins c-IAP1, c-IAP2, and Bcl-2 were altered in the presence of p65 siRNA and CPT-11. Cells exposed to CPT-11 for 0, 24, or 48 h in the presence of p65 siRNA had a significant reduction in the levels of c-IAP1 and c-IAP2 but not Bcl-2 as compared with controls (Fig. 4*B*). This set of experiments indicates that p65 siRNA-mediated increases in CPT-11-induced apoptosis occur by down-regulation of the NF-κB target genes, c-IAP1 and c-IAP2, but not on other inhibitors of apoptosis that are not regulated by NF-κB. These results are consistent with the role of the NF-κB-regulated inhibition on increasing CPT-11-mediated cell killing.

p65 siRNA and CPT-11 Reduce HCT116 Colony Formation. Next, we determined whether p65 siRNA alters colony formation of HCT116 cells in soft agar assays in the presence of CPT-11. Cells were transfected with siRNA directed against either p65 or Tax, and at 24 h after transfection, the cells were placed into media with soft agar. Colony formation was assayed 15 days after plating. Exposure of the cells to 1 μM CPT-11 inhibited colony formation 9-fold in the presence of p65 siRNA as compared with that seen with Tax siRNA (Fig. 5). Colony formation was inhibited by 25-fold in the presence of 5 μM CPT-11 and p65 siRNA, as compared with that seen with 5 μM CPT-11 and Tax siRNA. There was less than a 2-fold decrease in HCT116 colony formation between p65 and Tax siRNA transfection in the absence of CPT-11 (Fig. 5). These

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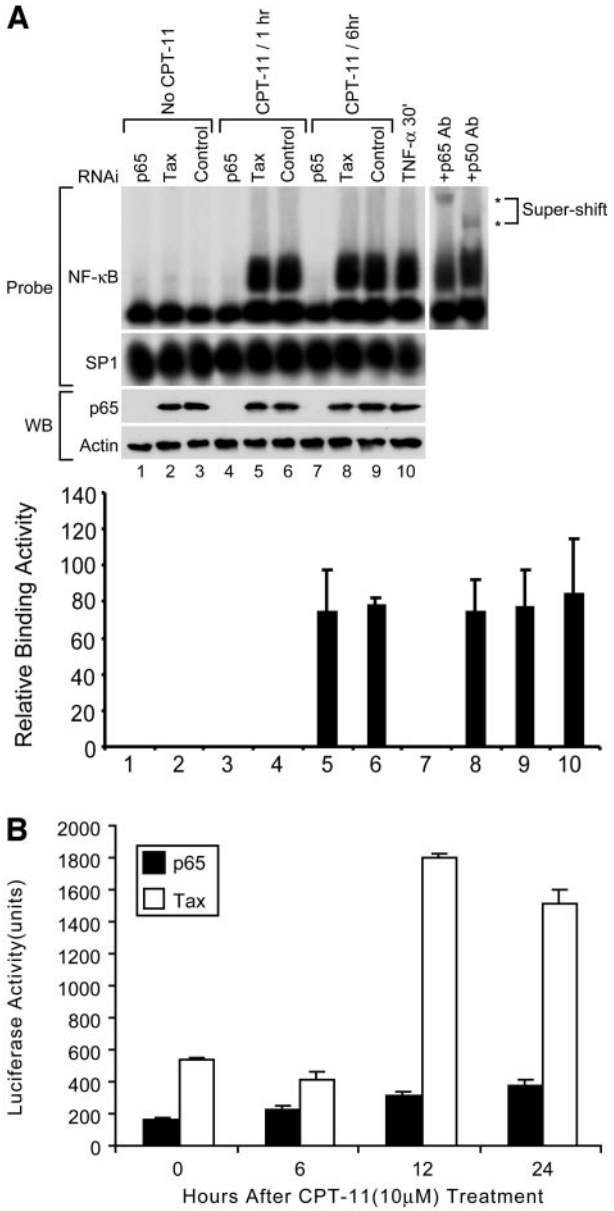

Fig. 3 Irinotecan (CPT-11)-induced activation of nuclear factor-κB (NF-κB) pathway is reduced by p65 small interfering RNA (siRNA) treatment. *A,* HCT116 cells were transfected with 21-mer annealed p65 siRNA (50 nM) or Tax siRNA (50 nM) or mock-transfected (control). Seventy-two h after transfection, the cells were either not treated (*Lanes 1–3*) or treated with CPT-11 (10 μM) for 1 (*Lanes 4–6*) or 6 h (*Lanes 7–9*) or treated with tumor necrosis factor α (5 ng/ml) for 30 min (*Lane 10*). Whole cell extracts were prepared from these cells and used in gel retardation assays with labeled oligonucleotide probes corresponding to NF-κB or SP1. Supershift analysis using p65 or p50 antibody was performed to confirm the presence of p65 and p50 in the NF-κB complex (*Lanes 11* and *12*). Western blot analysis was performed on the same extracts using antibodies directed against p65 or actin. Quantitation of three different gel retardation assays performed under similar experimental conditions is also shown (*graph*). *B,* cells were transfected with a NF-κB luciferase reporter (1 μg) and a Rous sarcoma virus-β-galactosidase reporter (0.3 μg) at 24 h after transfection of either p65 or Tax siRNA. Twenty-four h later, the cells were treated with 10 μM CPT-11 for 0, 6, 12, and 24 h; lysed in luciferase analysis buffer; and analyzed for luciferase and β-galactosidase activity.

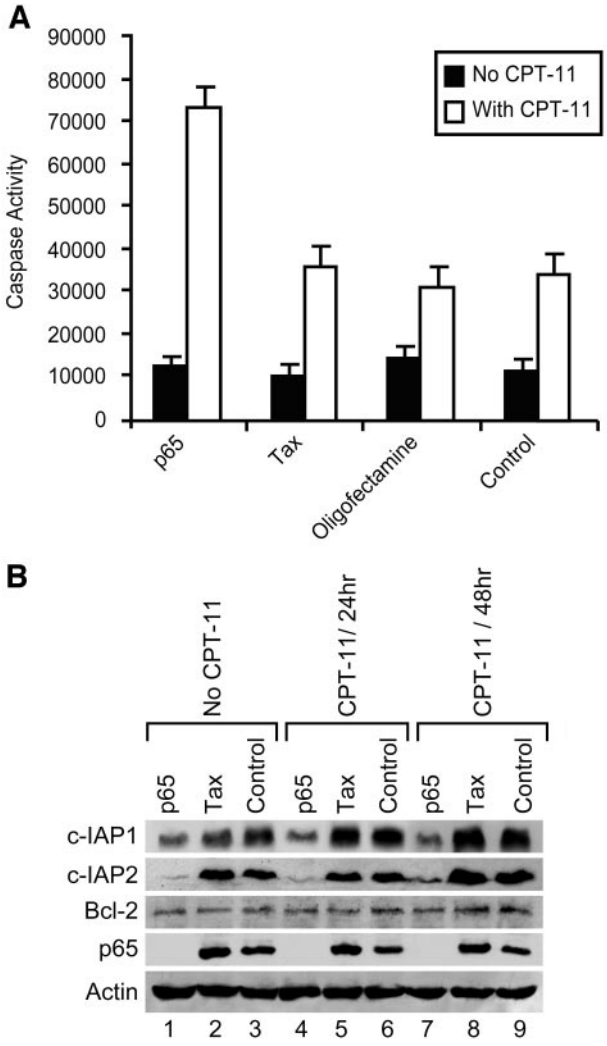

Fig. 4 Enhancement of irinotecan (CPT-11)-induced apoptosis by p65 small interfering RNA (siRNA). *A,* 48 h after transfection of p65 and Tax siRNAs (cells transfected with Oligofectamine alone and mock-transfected cells were used as controls), HCT116 cells were treated with 10 μM CPT-11 or left untreated. Caspase-3 activities were determined 48 h after treatment with CPT-11 as described in "Materials and Methods." *B,* HCT116 cells were exposed to 10 μM CPT-11 for 0, 24, or 48 h after transfection with either p65 or Tax siRNAs or mock-transfection. Western blot analysis was performed using antibodies directed against c-IAP1, c-IAP2, Bcl-2, p65, and actin.

results indicate that reduction of p65 protein synergizes with CPT-11 to inhibit colony formation in soft agar. In contrast, p65 siRNA alone had no significant effect on the ability of HCT116 cells to form colonies (Fig. 5).

siRNA Directed against p65 Reduces *in Vivo* Tumor Growth. Finally, we addressed whether the transient exposure of HCT116 cells to p65 siRNA in cell culture altered the ability of these cells to proliferate after injection into nude mice. In addition, we asked whether the i.v. administration of p65 siRNA and i.p. injections of CPT-11 could alter the growth of HCT116 xenografts in nude mice. Thus, we could address the potential *in*

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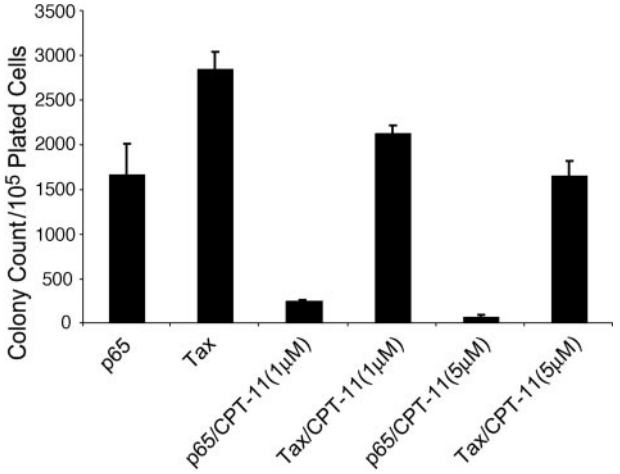

Fig. 5 Effects of p65 small interfering RNA (siRNA) and irinotecan (CPT-11) on colony formation of HCT116 cells. HCT116 cells were plated in media containing either CPT-11 (10 μM) and soft agar or soft agar alone at 24 h after transfection with p65 or Tax siRNA. The colonies were counted in three different wells at 15 days after transfection, and the averages were plotted. The reduction in the number of colonies in the p65 siRNA-transfected, CPT-11-treated group was statistically significant ($P < 0.01$) compared with that seen with the Tax siRNA-transfected, CPT-11-treated group.

vivo effects of siRNA on enhancing CPT-11-mediated cell killing of HCT116 cells.

In the first series of experiments, HCT116 cells were first transfected with siRNA directed against either p65 or Tax for 24 h. Mice were divided into four cohorts (5 mice/cohort), and 2.5×10^6 of the siRNA-transfected HCT116 cells were injected to each mouse. Mice were treated with i.p. CPT-11 at a dose of 0.25 ml (4 mg/ml) starting 1 day after the injection of the tumor cells and continued twice weekly for 2 weeks. Twelve days after injection of siRNA-transfected HCT116 cells, the size of the tumors was measured in three dimensions twice a week for 5 weeks, and the tumor volume was calculated from these measurements. As seen in Fig. 6*A*, there was no difference in the size of the HCT116 tumors transfected with p65 siRNA as compared with cells transfected with Tax siRNA in the absence of CPT-11 (Fig. 6*A*). In contrast, p65 siRNA combined with CPT-11 treatment led to a dramatic reduction of tumor growth as compared with that seen with Tax siRNA and CPT-11 (Fig. 6*B*). These results were statistically significant with a P of <0.001 and <0.05, respectively (Student's t test). Western blot analysis of the HCT116 cells at 24 h after transfection of the siRNAs showed more than a 3-fold reduction in p65 protein levels as compared with Tax protein. Quantification of the gel is shown as the ratio of p65 or Tax to actin (Fig. 6*C*).

To evaluate whether the systemic administration of p65 siRNA could enhance sensitivity to CPT-11, we randomized mice ($n = 8$ mice/group) with established s.c. HCT116 tumor xenografts to four treatment groups. The mice received either (*a*) p65 siRNA (250 pmol/mouse) by tail vein injection, (*b*) Tax siRNA (250 pmol/mouse) by tail vein injection, (*c*) p65 siRNA by tail vein injection with CPT-11 by i.p. injection, or (*d*) Tax siRNA by tail vein injection with CPT-11 by i.p. injection. The CPT-11 dose used in this study was of 0.25 ml (2 mg/ml) per mouse. The siRNAs were delivered i.v. twice a week starting 9 days after HCT116 cells had been injected, and the CPT-11 was injected i.p. twice weekly starting 1 day after siRNA injection. Tumor size in the mice injected with p65 siRNA or Tax siRNA alone was analyzed over a 30-day time period. There was no statistically significant difference in tumor volume in mice when

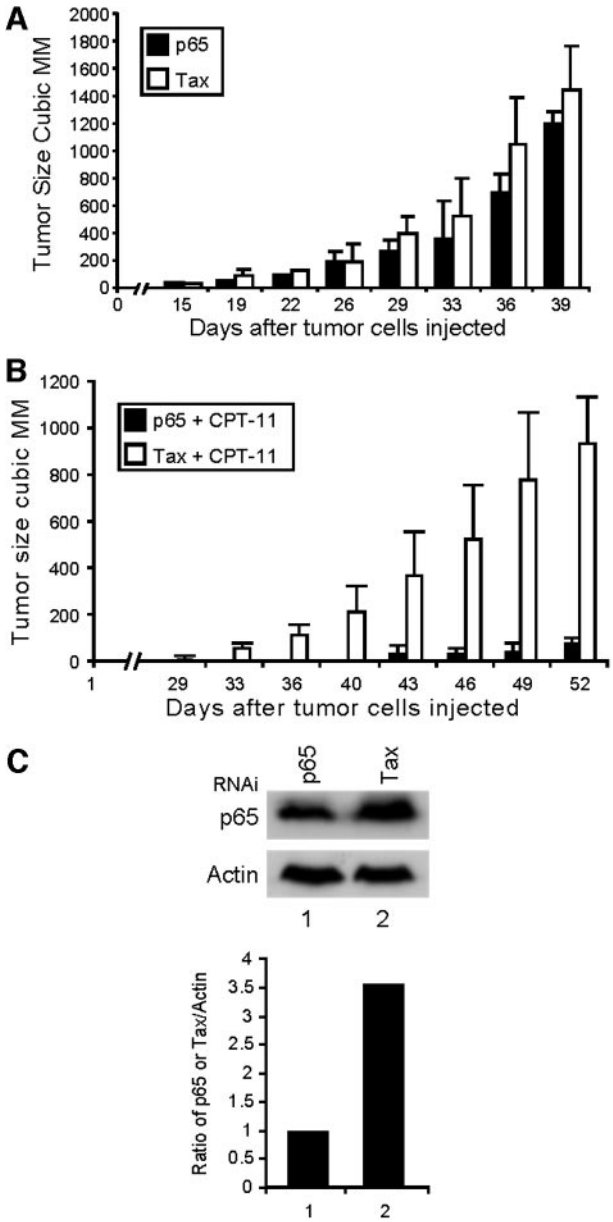

Fig. 6 Effects of p65 small interfering RNA (siRNA) and irinotecan on HCT116 tumor growth in nude mice. *A*, cohorts of five nude mice received s.c. injection with 2.5×10^6 HCT116 cells transfected with either p65 siRNA or Tax siRNA, and tumors were measured at day 15 twice weekly for 3 weeks. *B*, 1 day after the tumor cells were injected, the mice received i.p. injection of irinotecan twice a week for 2 weeks, and tumor size (in mm^3) was obtained from days 29 to 52. *C*, HCT116 cells were transfected with p65 or Tax siRNA (50 nM), and 24 h after transfection, extracts were prepared and analyzed by Western blot.

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treated with p65 siRNA or Tax siRNA alone (Fig. 7*A*). The tumor growth in mice treated with these siRNAs followed by CPT-11 was measured up to day 80. There was a significant reduction of tumor growth in the p65 siRNA + CPT-11-treated group as compared with the Tax siRNA + CPT-11-treated group (Fig. 7*B*). At day 80, three of these siRNA- and CPT-11-treated mice were sacrificed, their tumors were dissected, and Western blot analysis was performed with antibodies directed against p65 and actin. Quantification of the Western blot is shown as the ratio of p65 or Tax to actin (Fig. 7*C*). These results indicated that p65 siRNA decreased the p65:actin ratio as compared with the Tax:actin ratio for each sample analyzed. Thus, siRNA appeared quite effective in reducing tumor growth when administered by tail vein injection in combination with CPT-11.

DISCUSSION

Many anticancer agents that induce apoptosis, such as CPT-11, also activate the NF-κB pathway (11, 38, 39). When activated, NF-κB increases the expression of genes that promote cell survival and block apoptosis (9, 11). Inducible chemotherapy resistance to CPT-11 has been shown to be reversed by inhibiting NF-κB (12, 39). Several methods of inhibiting NF-κB activation such as antisense oligonucleotides, proteasome inhibitors, and siRNA are currently under investigation (23–26, 40, 41).

In the current study, the functional consequences of p65 siRNA-mediated decreases in NF-κB activation by CPT-11 in an established colon cancer cell line were determined using assays of caspase-dependent apoptosis, soft agar formation, and *in vitro* and *in vivo* assays of tumor growth. This analysis demonstrates that transfection of tumor cells with siRNA directed against the p65 NF-κB subunit significantly decreases inducible NF-κB activation in response to treatment with CPT-11. These studies also demonstrated that the enhanced response to chemotherapy was associated with increased levels of apoptosis. Furthermore, inhibition of NF-κB activation in association with CPT-11 treatment significantly suppresses colony formation in soft agar and tumor growth in nude mice. As a result, there was enhanced chemosensitivity to CPT-11 both *in vitro* and *in vivo*.

NF-κB activation has been shown to regulate apoptosis in several different experimental systems (9, 10). The mechanism responsible for the antiapoptotic effects of NF-κB activation after exposure to chemotherapy have not been fully elucidated, although induction of inhibitors of apoptosis is an important mechanism that can prevent tumor cell killing by chemotherapy. Thus, it is likely that downstream gene targets that are NF-κB-regulated are involved in this inducible survival mechanism (42). In this study, we examined c-IAP1, c-IAP2, and Bcl-2 protein expression in p65 siRNA-transfected HCT116 cells in the presence and absence of CPT-11. Down-regulation of the NF-κB-inducible genes *c-IAP1* and *c-IAP2*, but not *Bcl-2*, was demonstrated, suggesting that the decreased expression of these proteins by inhibiting the NF-κB pathway may play an important role in enhancing apoptosis induced by CPT-11.

To extend our *in vitro* studies, we first established tumors by s.c. injection of HCT116 cells into nude mice transfected with either p65 or Tax siRNAs. There was a 95% reduction in

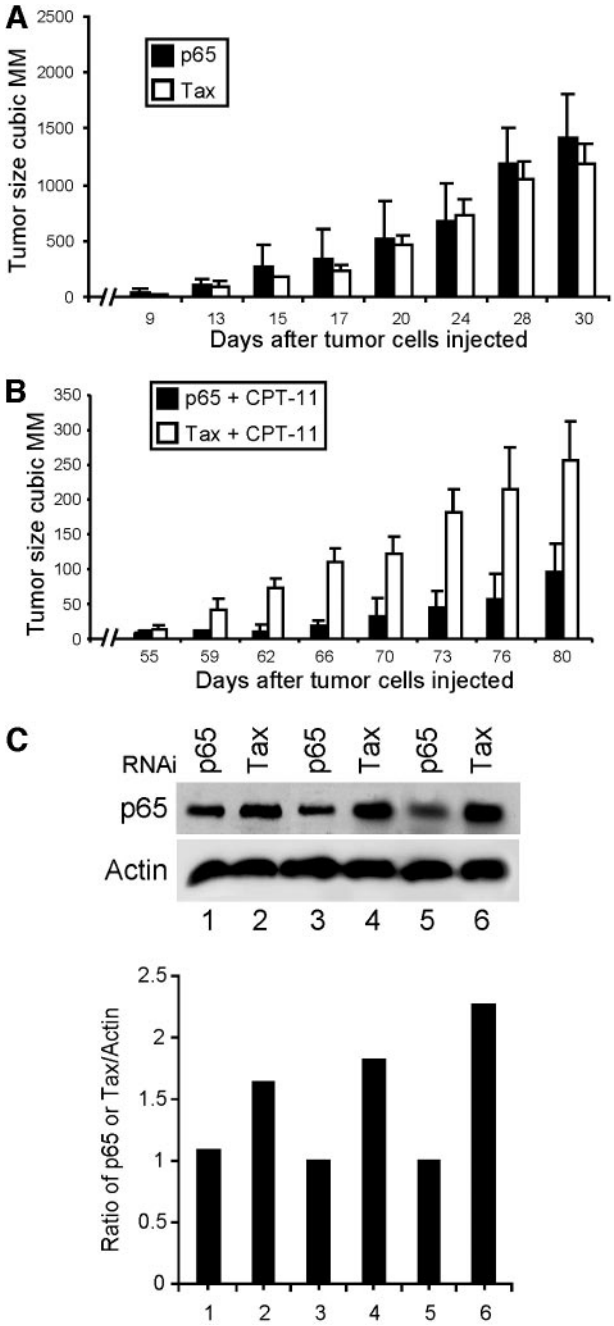

Fig. 7 Effects of i.v. p65 small interfering RNA (siRNA) and irinotecan on HCT116 tumor growth in nude mice. *A*, cohorts of five nude mice received s.c. injection with 2.5×10^6 cells, and the tumors were allowed to grow for 9 days. Starting on day 10, the mice were treated twice a week by tail vein injection with 250 pmol of siRNA directed against p65 or Tax. The tumor size was recorded in three dimensions and measured twice a week for 3 weeks. *B*, a cohort of mice received 0.25 ml (4 mg/ml) of i.p. irinotecan twice week in addition to tail vein injection of p65 or Tax siRNAs. The tumor growth was measured in three dimensions twice a week for 80 days. *C*, tumors dissected from three mice in each group from *B* were minced in PD buffer and left on ice for 40 min followed by a Western blot analysis with antibodies directed against p65 and actin. The results were analyzed as the ratio of p65 or Tax to actin.

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tumor growth in the nude mice implants with HCT116 cells treated with i.p. injections of CPT-11 and transfected with p65 siRNA as compared with the Tax siRNA group of mice. There was no statistically significant difference in the growth of the tumors in the absence of CPT-11. Because we have shown previously (30) that siRNA can work by the i.v. route in xenograft models, we next treated these mice with CPT-11 and i.v. siRNA directed against either p65 or Tax. Systemic therapy with p65 siRNA did not limit tumor growth as compared with Tax siRNA. However, when combined with CPT-11, p65 siRNA significantly delayed tumor growth with reductions in tumor volume of 75% as compared with that seen with Tax siRNA. These xenograft studies demonstrated antitumor effects of i.v. p65 siRNA administration in combination with CPT-11 in an *in vivo* model of human colon cancer.

As compared with other methods such as proteasome inhibitors that affect multiple components of the cell, siRNA has the advantage of decreasing the level of specific components of the NF-κB pathway. Furthermore, inhibition of the NF-κB pathway by p65 siRNA in the absence of CPT-11 did not alter tumor growth, suggesting that it would potentially only inhibit cell growth after administration of chemotherapy or other agents that induce its expression. Additional studies will determine whether p65 siRNA will increase the therapeutic index of chemotherapy-induced cell death in cancer cells as compared with normal cells. In summary, the studies reported here demonstrate that siRNA provides a simple, reproducible, and highly efficient means to demonstrate the role of the NF-κB pathway in enhancing the chemosensitivity and increased apoptosis of colon cancer cells both in cell culture and in xenografts. Our *in vivo* data suggest that siRNA may have therapeutic potential for inhibiting the expression of genes that enhance the growth of tumors.

ACKNOWLEDGMENTS

We thank Noelle Williams for helpful comments during the study, Shane Scoggin for technical assistance, Alex Herrera for preparation of the figures, and Robert Nunnan and Sonol Reitmeyer for the preparation of chemotherapy.

REFERENCES

1. Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–83.

2. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.

3. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141–79.

4. Thanos D, Maniatis T. Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell 1995;83:1091–100.

5. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell 2002;109(Suppl):S81–96.

6. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev 1995;9:2723–35.

7. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature (Lond) 1997;388:548–54.

8. Mercurio F, Zhu H, Murray BW, et al. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science (Wash DC) 1997;278:860–6.

9. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science (Wash DC) 1998;281:1680–3.

10. Chen X, Kandasamy K, Srivastava RK. Differential roles of RelA (p65) and c-Rel subunits of nuclear factor kappa B in tumor necrosis factor-related apoptosis-inducing ligand signaling. Cancer Res 2003;63:1059–66.

11. Baldwin AS Jr. Series introduction: the transcription factor NF-kappaB and human disease. J Clin Investig 2001;107:3–6.

12. Wang CY, Cusack JC Jr, Liu R, Baldwin AS Jr. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat Med 1999;5:412–7.

13. Jones DR, Broad RM, Madrid LV, Baldwin AS Jr, Mayo MW. Inhibition of NF-kappaB sensitizes non-small cell lung cancer cells to chemotherapy-induced apoptosis. Ann Thorac Surg 2000;70:930–6; discussion 936–7.

14. Cusack JC Jr, Liu R, Houston M, et al. Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-kappaB inhibition. Cancer Res 2001;61:3535–40.

15. Wasserman E, Sutherland W, Cvitkovic E. Irinotecan plus oxaliplatin: a promising combination for advanced colorectal cancer. Clin Colorectal Cancer 2001;1:149–53.

16. Calvo E, Cortes J, Rodriguez J, et al. Irinotecan, oxaliplatin, and 5-fluorouracil/leucovorin combination chemotherapy in advanced colorectal carcinoma: a Phase II study. Clin Colorectal Cancer 2002;2:104–10.

17. Xu Y, Villalona-Calero MA. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. Ann Oncol 2002;13:1841–51.

18. Bass BL. Double-stranded RNA as a template for gene silencing. Cell 2000;101:235–8.

19. Sharp PA. RNA interference—2001. Genes Dev 2001;15:485–90.

20. Lu PY, Xie FY, Woodle MC. siRNA-mediated antitumorigenesis for drug target validation and therapeutics. Curr Opin Mol Ther 2003;5:225–34.

21. Kurreck J. Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 2003;270:1628–44.

22. Scherr M, Morgan MA, Eder M. Gene silencing mediated by small interfering RNAs in mammalian cells. Curr Med Chem 2003;10:245–56.

23. Trompouki E, Hatzivassiliou E, Tsichritzis T, et al. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature (Lond) 2003;424:793–6.

24. Brummelkamp TR, Nijman SM, Dirac AM, Bernards R. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature (Lond) 2003;424:797–801.

25. Kovalenko A, Chable-Bessia C, Cantarella G, et al. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature (Lond) 2003;424:801–5.

26. Laderach D, Compagno D, Danos O, Vainchenker W, Galy A. RNA interference shows critical requirement for NF-kappaB p50 in the production of IL-12 by human dendritic cells. J Immunol 2003;171:1750–7.

27. Elbashir SM, Harborth J, Lendeckel W, et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature (Lond) 2001;411:494–8.

28. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001;15:188–200.

29. Williams NS, Gaynor RB, Scoggin S, et al. Identification and validation of genes involved in the pathogenesis of colorectal cancer

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using cDNA microarrays and RNA interference. Clin Cancer Res 2003; 9:931–46.

30. Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. Clin Cancer Res 2003;9:1291–300.

31. Morin PJ, Sparks AB, Korinek V, et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science (Wash DC) 1997;275:1787–90.

32. Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc Natl Acad Sci USA 1995;92: 3046–50.

33. Ilyas M, Tomlinson IP, Rowan A, Pignatelli M, Bodmer WF. Beta-catenin mutations in cell lines established from human colorectal cancers. Proc Natl Acad Sci USA 1997;94:10330–4.

34. Takaesu G, Surabhi RM, Park KJ, et al. TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. J Mol Biol 2003;326:105–15.

35. Kwak YT, Guo J, Prajapati S, et al. Methylation of SPT5 regulates its interaction with RNA polymerase II and transcriptional elongation properties. Mol Cell 2003;11:1055–66.

36. Ma MK, McLeod HL. Lessons learned from the irinotecan metabolic pathway. Curr Med Chem 2003;10:41–9.

37. Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res 1991;51:4187–91.

38. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science (Wash DC) 1996;274:787–9.

39. Cusack JC Jr, Liu R, Baldwin AS Jr. Inducible chemoresistance to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11) in colorectal cancer cells and a xenograft model is overcome by inhibition of nuclear factor-kappaB activation. Cancer Res 2000;60: 2323–30.

40. Stahel RA, Zangemeister-Wittke U. Antisense oligonucleotides for cancer therapy-an overview. Lung Cancer 2003;41(Suppl 1): S81–8.

41. Shah SA, Potter MW, McDade TP, et al. 26S proteasome inhibition induces apoptosis and limits growth of human pancreatic cancer. J Cell Biochem 2001;82:110–22.

42. Shangary S, Johnson DE. Recent advances in the development of anticancer agents targeting cell death inhibitors in the Bcl-2 protein family. Leukemia (Baltimore) 2003;17:1470–81.

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Enhanced Chemosensitivity to Irinotecan by RNA Interference-Mediated Down-Regulation of the Nuclear Factor-κB p65 Subunit

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Clin Cancer Res 2004;10:3333-3341.

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