

Nuclear Factor κ B Inhibitors Induce Adhesion-dependent Colon Cancer Apoptosis: Implications for Metastasis¹

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ABSTRACT

The transcription factor nuclear factor κ B (NF κ B) is constitutively active in many types of cancer cells and regulates the expression of several antiapoptotic genes. Previous studies demonstrated a role for the inhibition of NF κ B in cancer therapy using a transgenic approach in mice. We found that NF κ B was transiently activated much greater than background constitutive levels during colon cancer cell readhesion, which rendered the readhering colon cancer cells exquisitely susceptible to apoptosis in the presence of soluble NF κ B inhibitors. These compounds greatly reduced colon cancer cell implantation in an *in vivo* seeding model of metastasis. The ability of soluble NF κ B inhibitors to significantly induce apoptosis of readherent colon cancer cells makes them prospective candidates for preventing colon cancer metastasis.

INTRODUCTION

The extracellular matrix is important to epithelial cell proliferation, differentiation, and survival (reviewed in Ref. 1). A complete loss of adhesion causes a rapid and massive induction of apoptosis (termed “anoikis”) of normal epithelial cells, whereas transformed epithelial cells are resistant to anoikis (2). This resistance to anoikis promotes cancer cell invasion and metastasis, a process that involves dynamic changes in cell adhesion. Cell adhesion is transiently absent when cancer cells gain access to the circulatory systems or other body fluid-containing compartments, which occurs commonly in patients with advanced cancers (3, 4). The mechanisms by which cancer cells usurp normal extracellular matrix-derived survival signals are not well known. We have studied a number of proteins known to be important in mediating anchorage-dependent colon cancer cell survival and now present *in vitro* and *in vivo* data showing the role of NF κ B⁴ in this process. The clinical implication of these studies is that soluble NF κ B inhibitors may have a unique role in preventing the spread of colon cancers.

The nuclear transcription factor NF κ B is gaining recognition as an important survival factor and is commonly overexpressed in malignant *versus* normal cells (5–9). Studies have demonstrated recently that inhibition of NF κ B activation resulted in diminished cancer cell proliferation and an increased susceptibility to proapoptotic chemotherapeutic agents (10–12). The exact mechanism(s) of action by which inhibition of NF κ B activation increased the apoptotic activity of chemotherapy agents in these studies is not completely known. Inhibition of NF κ B activity was able to restore the susceptibility of

cancer cells to the proapoptotic effects of TNF- α (10, 13). Other studies have implicated the loss of expression of the antiapoptotic genes, *c-IAP-1*, *c-IAP-2*, *XIAP*, *IEX-1L*, *TRAF-1*, and *TRAF-2*, which are under the transcriptional regulation of NF κ B, as the mechanism of enhanced cancer cell apoptosis resulting from NF κ B inhibition (14–16).

NF κ B is a heterodimeric transcription factor comprised of various protein subunits: p50/p105, p65/RelA, p52/p100, c-Rel, and RelB. NF κ B is localized to the cytoplasm by association with I κ B, which inhibits translocation of NF κ B into the nucleus thus preventing its transcriptional activity (17). Under the influence of cytokines, reactive oxygen species, growth factors, and other stimuli, inhibitor of nuclear factor B kinases phosphorylate I κ B on two critical serine residues (18, 19). This then targets I κ B for ubiquitination and subsequent degradation by the proteasome, allowing free NF κ B to translocate into the nucleus and activate the transcription of various genes possessing κ B consensus DNA binding sites in their promoters (20–22). Many of the genes regulated by NF κ B encode proteins that promote inflammation, including *COX-2* (23–26), and are angiogenesis factors, such as vascular endothelial growth factor (27). In addition, NF κ B mediates the transcription of several antiapoptotic genes: *c-Myc*, *Bcl2*, *p53*, *p21*, *c-FLIP*, *c-IAP-1*, *c-IAP-2*, *XIAP*, *IEX-1L*, *COX-2*, *TRAF-1*, and *TRAF-2* (14–16, 28, 29).

Two NF κ B inhibitors, BAY 11–7082 and BAY 11–7085, shown previously to inhibit I κ B phosphorylation and TNF- α -induced NF κ B activation (30), were tested on the DLD-1, HCT-116, and HT-29 human colon cancer cell lines for their effects on cell tumorigenicity and apoptosis. BAY 11–7082 and BAY 11–7085 induced apoptosis of colon cancer cells in a cell adhesion-dependent fashion: readhesion after transient suspension of the cell lines caused a large and transient activation of NF κ B, which rendered the cells exquisitely sensitive to apoptosis induced by the compounds. Treatment of athymic mice with BAY 11–7085 completely inhibited tumor implantation of the liver after i.p. delivery of HT-29 colon cancer cells. These studies suggest that colon cancer cells use NF κ B as an important survival factor during the establishment of adhesion. This provides an opportunity to use NF κ B inhibitors to prevent colorectal cancer seeding or metastasis.

MATERIALS AND METHODS

Materials. The NF κ B inhibitors, BAY 11–7082 (Biomol), BAY 11–7085 (Biomol), PDTC (ALEXIS), and *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG-132; ALEXIS) were solubilized in DMSO to a final concentration of 1 mM. The antagonistic and chimeric TNF- α monoclonal antibody (cA2) was purchased from the clinical pharmacy at the University of Utah and reconstituted in sterile water at 1 mg/ml. Polyclonal antibodies to *c-IAP-1*, *c-IAP-2*, *TRAF-1*, *TRAF-2*, and I κ B α , and a monoclonal antibody to the NF κ B p65 subunit were obtained from Santa Cruz Biotechnology. Polyclonal antibodies to cleaved PARP were obtained from New England Biolabs. Alkaline phosphatase-conjugated goat-antirabbit antibody was obtained from The Jackson Laboratory. Adenovirus containing the I κ B super-repressor construct was a kind gift from Dr. Richard Rippe at the University of North Carolina (Chapel Hill, NC).

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⁴ The abbreviations used are: NF κ B, nuclear factor κ B; TNF, tumor necrosis factor; I κ B, inhibitor of nuclear factor- κ B; PDTC, pyrrolidinedithiocarbamic acid; PARP, poly (ADP-ribose) polymerase; EMSA, electrophoretic mobility shift assay; COX-2, cyclooxygenase-2; JNK, c-Jun NH₂-terminal kinase.

Cell Culture and Proliferation Assay. DLD-1, HCT-116, Caco-2, and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection and were cultured in DMEM supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. 293 cells stably transfected with *COX-2* (293-COX-2) under the control of a ponasterone sensitive promoter (a generous gift from Dr. Stephen Prescott, University of Utah, Salt Lake City, UT; Ref. 31) were cultured in DMEM supplemented with 10% fetal bovine serum, 400 μg/ml of zeocin, 400 μg/ml of G418, glutamine, penicillin, and streptomycin. For induction of COX-2 protein expression, 293-COX-2 cells were cultured for 48 h in medium supplemented with 1 μg/ml of ponasterone. All of the cells were cultured at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

For the cell proliferation assay, cells were dispersed and plated at 40,000 cells/well in 96-well dishes. At various days in culture, the cells were gently washed twice with 100 μl/well of ice-cold blocking buffer (1% radioimmunoassay grade BSA in PBS) and twice with 100 μl/well of ice-cold PBS. The cells were fixed for 10 min in 100% ice-cold methanol (100 μl/well), then allowed to air-dry. The cells were stained with 100 μl/well of 0.1% crystal violet in H₂O for 10 min, then washed gently four times with ddH₂O and four times with PBS. The plates were then air-dried completely. The stained cells were then solubilized in 1% sodium deoxycholate, and the plates read at 590 nm in a spectrophotometer. The absorption at 590 nm is proportional to the number of attached cells (data not shown).

NFκB Activation Assays. The NFκB EMSA was performed as described previously (32).

Activation of NFκB was also determined using the Trans-a.m. assay (Active Motif, Carlsbad, CA, per the manufacturer's instructions; Ref. 33). Briefly, cell monolayers in 35-mm dishes were washed with ice-cold PBS and removed by incubating in trypsin-PBS or scraping. The cells were centrifuged for 10 min at 1000 rpm at 4°C and resuspended for 10 min in 100 μl of 4°C lysis buffer. Lysates (5 μg of total protein) were incubated in 96-well dishes containing immobilized oligonucleotides containing the NFκB consensus DNA binding site (5'-GGGACTTCC-3') for 1 h at room temperature. The wells were then washed three times, and 100 μl of p65 subunit monoclonal antibody (only binds to p65-containing NFκB heterodimers that are bound to DNA) supplied with the kit was diluted 1:1000 and added to each well for 1 h at room temperature. The wells were washed three times, and 100 μl of horseradish peroxidase-conjugated secondary antibody diluted 1:1000 was added to each well for 1 h at room temperature without agitation. The wells were washed four times, and 100 μl of developing solution was added to each well for 10 min at room temperature. One-hundred μl of stop solution was added to each well after which the absorbance was determined on a plate spectrophotometer at 450 nm. Specificity of binding was determined using 200-fold excess wild-type and mutated NFκB oligonucleotides.

Apoptosis Assay. Single cell suspensions were plated at 100,000 cells/well in 24-well plates in medium containing DMSO, BAY 11-7082, BAY 11-7085, PDTG, or MG-132 for 8–24 h in an incubator. Alternatively, DMSO, BAY 11-7082, BAY 11-7085, or MG-132 was added to cells that were confluent and adherent for several days. The nonadherent cells were aspirated off with the medium and spun for 3 min at 2,000 rpm in 15-ml polypropylene tubes. The adherent cells were washed twice with PBS, dispersed in trypsin, and spun down for 3 min at 2,000 rpm in 15-ml polypropylene tubes. The nonadherent and adherent cells were resuspended in 1× binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂] to a final concentration of 10⁶ cells/ml. One-hundred μl of the cell solution were transferred to a 5-ml culture tube and incubated with 5 μl of FITC-conjugated annexin V (PharMingen) and 10 μl of propidium iodide (from a 50 μg/ml stock solution made in PBS) in the dark at 25°C for 15 min. Four-hundred μl of binding buffer was added to each tube, and the cells were analyzed by flow cytometry immediately. The percentage of apoptosis was determined as the percentage of annexin V-positive, propidium iodide-negative cells of the propidium iodide-negative cells counted.

DLD-1 and HT-29 cells that were approximately 60–70% confluent were transduced with Ad-IκB super-repressor by adding 1–50 μl of purified virus to the cells. After 3 days, the expression of the IκB super-repressor was ascertained by immunoblot. In a parallel experiment, the transduced cells were resuspended and allowed to readhere in BAY 11-7085 for 8 h. The percentage of surviving (nonapoptotic) cells was determined using crystal violet staining (see cell proliferation assay, above).

Immunodetection. Immunoblotting of cellular proteins was performed as described previously (34). The primary antibodies were diluted at 1:1000–1:2000 in blocking buffer (1% BSA in Tris-buffered saline).

Immunofluorescence staining of cells was performed as described previously (34). Primary antibody was used at a 1:50 dilution, and detection antibody (Cy3-conjugated) was used at a dilution of 1:1000.

Athymic Mouse Colon Cancer Xenograft Studies. HT-29 and HCT-116 cells were harvested in 0.25% trypsin-PBS-EDTA, washed once each in medium and PBS, and resuspended in PBS at 1 million cells/200 μl. One-million HT-29 cells were injected either s.c. in the backs or i.p. in 5 week-old female *nu/nu* athymic mice (Charles River Labs). For the mice with s.c. tumors, the tumors were allowed to establish themselves for 10 days after which the mice were randomized to BAY 11-7085 or DMSO. For the mice with i.p. tumors, the mice received BAY 11-7085 (5 mg/kg) or an equal volume of DMSO (~200 μl) 24 h before the HCT-116 or HT-29 cells were injected i.p. All of the mice received BAY 11-7085 (5 mg/kg) or an equal volume of DMSO (~200 μl) twice weekly for 21–32 days. s.c. tumor sizes were determined by measuring the length and width with calipers. These studies were approved by the University of Utah Institutional Review Board and Institutional Animal Care and Use Committee, and performed in the University of Utah Animal Resource Center. Mice were euthanized when they experienced a >10% loss in body weight or if they appeared ill. Postmortem examinations included sectioning of kidney, lung, and liver that were stained with H&E followed by examination for tissue toxicity/damage by an experienced mouse pathologist (E. J. E.) who was blinded to therapy.

RESULTS

NFκB Inhibitor-induced Apoptosis Is Associated with a Loss of Cell Adhesion. An NFκB EMSA was performed to determine the inhibitory effects of BAY 11-7082 and BAY 11-7085 on colon cancer cells. The potency of BAY 11-7085 was greater than BAY 11-7082 in HT-29 cells (Fig. 1A). In addition, HT-29 colon cancer cells demonstrated constitutive activation of NFκB. DLD-1 and HCT-116 cells demonstrated constitutive activation of NFκB by EMSA as well (data not shown).

When HT-29 colon cancer cells were treated with the NFκB inhibitors, BAY 11-7085 or MG-132 for 24 h, the percentage of apoptotic cells (annexin V-positive and propidium iodide-negative) was relatively greater for MG-132 than BAY 11-7085 (Fig. 1B), and was proportional to the number of floating cells (data not shown). We confirmed this in HT-29 cells that were treated with increasing concentrations of BAY 11-7085 for 24 h. After separating the HT-29 cells that remained adherent and those that became nonadherent after BAY 11-7085 treatment, the percentage of apoptotic cells was determined in each group. Whereas the majority of nonadherent cells were apoptotic, only a minority of the adherent cells was apoptotic (Fig. 1C). Note that the percentage of nonadherent apoptotic cells decreased after treatment with 50 and 100 μM BAY 11-7085 compared with lower concentrations of BAY 11-7085, because many of the cells were positive for both annexin V and propidium iodide indicating that they had completed apoptosis and were now necrotic (data not shown). Shown another way, HT-29 cells were treated with 20 μM MG-132 for 8 h, after which the adherent and nonadherent cells were collected separately, and then lysed and immunoblotted for cleaved PARP, a product of caspase cleavage. Whereas there was only a small increase in cleaved PARP in the cells that remained adherent, there was a large increase in cleaved PARP in the nonadherent cells (Fig. 1D). Previous studies showed that the loss of colon cancer cell adhesion was associated with the induction of apoptosis by IFN and TNF-α (35). Taken together with our data, the possibility that an inhibition of colon cancer cell adhesion might be related to the apoptotic effects of the NFκB inhibitors tested was raised.

Many adherent cells undergo apoptosis after a loss of cell adhesion; a process termed anoikis (2). We hypothesized that NFκB inhibitors

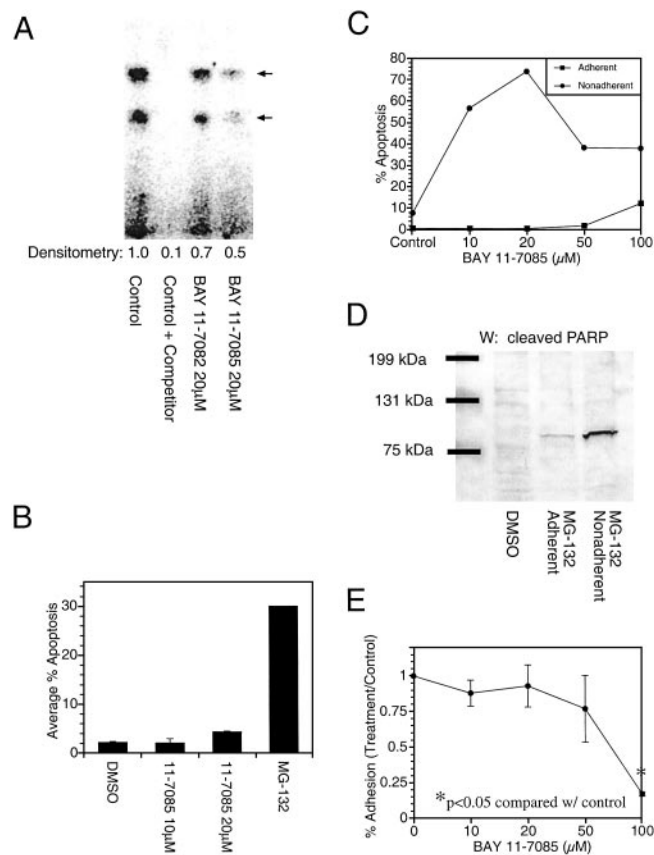


Fig. 1. Effect of NFκB inhibitors on apoptosis of colon cancer cells. **A**, adherent HT-29 cells were treated with BAY 11-7082 or BAY 11-7085 for 3 h after which an NFκB EMSA was performed (see "Materials and Methods"). Each lane contains an equal total protein concentration of nuclear lysate. The control cells were treated with DMSO alone. The *top arrow* shows the p65/p50 heterodimer and the *bottom arrow* shows the p50/p50 homodimer. The *numbers below the lanes* represent densitometry data (both bands) normalized to the control. **B**, adherent HT-29 cells were treated with DMSO alone (*control*), 10–20 μM BAY 11-7085, or 20 μM MG-132 for 24 h. The data points represent the average percentage of annexin V-positive, propidium iodide-negative cells (of the total cells counted) from triplicate experiments; *bars*, ± SE. **C**, adherent HT-29 cells were treated with BAY 11-7085 at various concentrations for 24 h after which the nonadherent and adherent cells were collected separately. The results shown are a graphic representation of individual flow cytometry results expressed as the percentage of propidium iodide-negative cells that were annexin V-positive. **D**, adherent HT-29 cells were treated with 20 μM MG-132 for 8 h after which the cells were collected. An immunoblot of the lysates for cleaved PARP was performed. Each lane represents equal total protein concentrations. **E**, confluent monolayers of HT-29 cells were treated with various concentrations of BAY 11-7085 for 3 h after which they were dispersed with trypsin-PBS. The cells were allowed to adhere to plastic dishes for 1 h after which they were stained with crystal violet. The cells were solubilized in deoxycholate, and the absorbance was detected in a spectrophotometer at 590 nm. The adherent cells were expressed as a fraction of the controls. Each data point represents the average of triplicate experiments; *bars*, ± SE.

might cause a loss of cell adhesion resulting in anoikis. To test this, HT-29 cells treated with various concentrations of BAY 11-7085 for 3 h were assayed for changes in cell adhesion. Cell adhesion was significantly inhibited only at the higher doses (≥ 50 μM) of BAY 11-7085 demonstrating a minor role for NFκB in promoting colon cancer cell adhesion (Fig. 1E). Furthermore, only a minority (17%) of HT-29 cells was apoptotic after culture in suspension for 24 h on dishes coated with poly-HEMA, a substrate that completely inhibited HT-29 cell adhesion (data not shown). The modest inhibition of cell adhesion and the overall resistance of HT-29 cells to anoikis suggested that changes in cell adhesion could not alone account for the apoptotic activity of the NFκB inhibitors.

Changes in Colon Cancer Cell Adhesion Increase the Apoptotic Effect of NFκB Inhibitors. We hypothesized that an inhibition of cell adhesion was insufficient but perhaps necessary for efficient

colon cancer cell apoptosis caused by the NFκB inhibitors. To test this, HT-29 cells were transiently suspended in trypsin-PBS for ~15 min before replating them in the presence of various concentrations of BAY 11-7085 for 8 h. Various concentrations of BAY 11-7085 were also added to adherent HT-29 cell monolayers for 8 h for comparison.

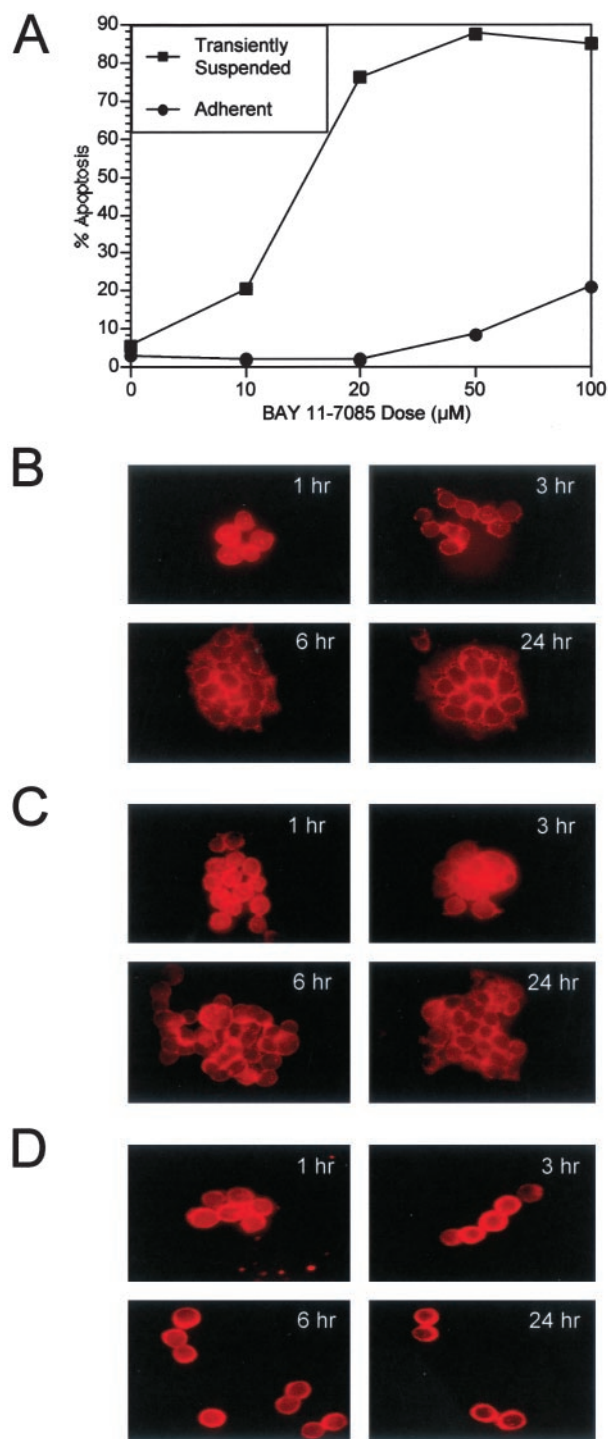


Fig. 2. Relationship of cell adhesion to NFκB inhibitor-induced apoptosis. **A**, HT-29 cells that were either adherent or transiently suspended with trypsin-EDTA were treated at time zero with DMSO or 10–100 μM BAY 11-7085. The percentage of apoptotic cells was determined using the annexin V flow cytometry assay and graphed *versus* the dose of BAY 11-7085. **B**, DLD-1, **C**, HCT-116, and **D**, HT-29 cells were transiently suspended in trypsin-EDTA and allowed to readhere to glass coverslips for 1–24 h. The cells were fixed and immunostained with an NFκB p65 subunit monoclonal antibody. Note the strong nuclear and cytoplasmic staining at 1 h *versus* the markedly reduced nuclear staining by 24 h. All cells shown are adjacent but not layered.

Transient suspension of HT-29 cells greatly increased their susceptibility to the apoptotic effects of BAY-11-7085 at all of the concentrations tested (Fig. 2A). On the other hand, there was a relatively small increase in apoptosis of adherent HT-29 cells and only at the highest doses of BAY 11-7085 used (Fig. 2A). Similar results were obtained with BAY 11-7082 at the same doses and under similar conditions (data not shown).

One potential explanation for these results was that the transient suspension of colon cancer cells caused an activation of NFκB, which in turn inhibited apoptosis. To test this, DLD-1, HCT-116, and HT-29 cells were transiently suspended in trypsin-PBS and then quickly allowed to readhere. Nuclear localization of the p65 subunit of NFκB was seen in colon cancer cells 1 h after replating (Fig. 2, B-D). Thereafter, nuclear p65 localization decreased from 3-24 h after replating. By 24 h, the vast majority of the p65 staining was cytoplasmic indicating a marked decrease in NFκB activation. These results demonstrated that transient suspension of colon cancer cells resulted in activation of NFκB.

Colon Cancer Cell Readhesion Increases NFκB Activation.

Activation of NFκB-DNA binding induced by transient suspension of colon cancer cells was measured. Adherent monolayers of DLD-1, HCT-116, and HT-29 cells were scraped off plates or transiently suspended with trypsin, followed by nuclear isolation and lysis. The nuclear lysates were added to 96-well plates containing immobilized DNA oligonucleotides corresponding to the NFκB consensus-binding sequence. The levels of NFκB in the nuclear lysates were measured using a monoclonal antibody capable of recognizing the NFκB p65 subunit only when bound to DNA. The assay showed that there was relatively little NFκB activation in any of the samples compared with the positive control (HeLa cells treated with TNF-α; Fig. 3A). In contrast, readhesion of transiently suspended HT-29 cells caused a large activation of NFκB (Fig. 3B). On the basis of the p65 localization results for HT-29 cells (Fig. 2B), the duration of NFκB activation appeared to last up to 6 h. Thus it was cell readhesion after transient suspension that caused the large and transient increase in NFκB activation.

The same assay was used to determine whether NFκB inhibitors could diminish readhesion-induced activation of NFκB. HT-29 cells were transiently suspended with trypsin and allowed to readhere for 3 h in the presence of increasing concentrations of BAY 11-7085. The strong activation of NFκB caused by HT-29 cell readhesion was greatly inhibited by treatment of the cells with increasing concentrations of BAY 11-7085 (Fig. 3B).

To determine whether readhesion-induced activation of NFκB would render other colon cancer cells more susceptible to BAY 11-7085-induced apoptosis, four colon cancer cell lines were transiently suspended and allowed to readhere in the presence of BAY 11-7085. BAY 11-7085 caused marked increases in apoptosis of readherent DLD-1 and HT-29 cells, a moderate increase for Caco-2 cells, but much less for HCT-116 cells (Fig. 3C).

Two other soluble NFκB inhibitors, MG-132 and PDTC, were used to test their activity in the induction of apoptosis of colon cancer cells during readhesion. Compared with BAY 11-7085, MG-132 more potently induced apoptosis of HT-29 colon cancer cells during readhesion (Fig. 3D). PDTC caused apoptosis of HT-29 cells during readhesion, but with less potency than MG-132 or BAY 11-7085 (Fig. 3D).

The specific involvement of NFκB in the apoptotic mechanism of BAY 11-7085 was examined specifically. To accomplish this, DLD-1 and HT-29 cells were transduced with an adenovirus containing the IκB super-repressor construct to specifically inhibit NFκB. The IκB super-repressor was created previously by mutating two key serine residues within the IκB gene resulting in an encoded IκB protein that

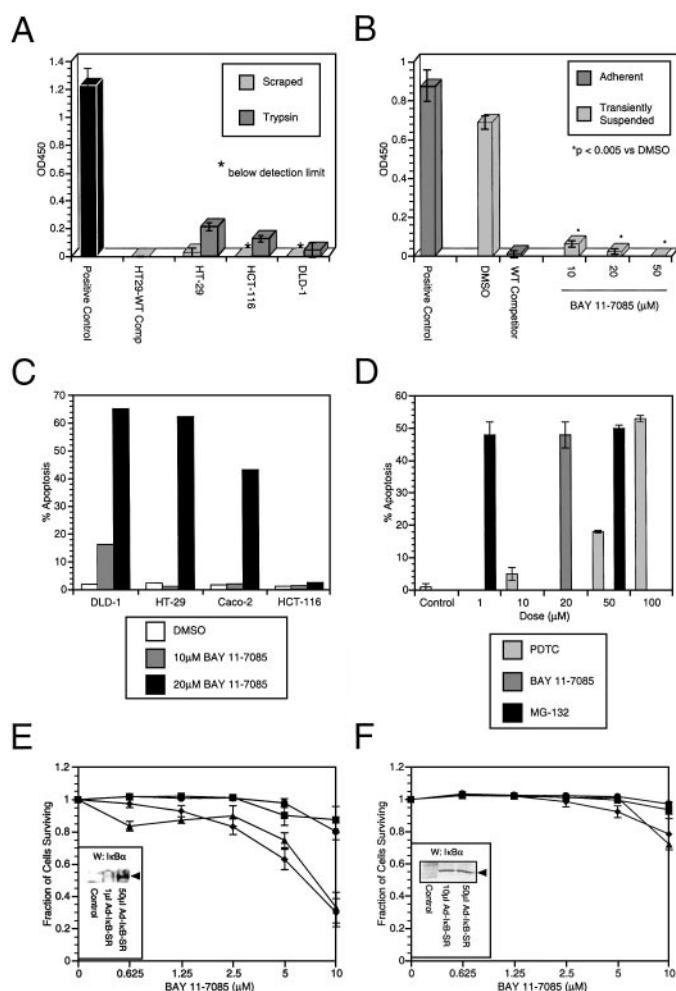


Fig. 3. Effect of cell adhesion on NFκB activation and susceptibility to NFκB inhibitors. **A**, NFκB binding activity assay (TransAM) of DLD-1, HCT-116, and HT-29 cells that were transiently suspended by scraping or with trypsin-EDTA. The transiently suspended cells were lysed and allowed to bind to DNA oligonucleotides, containing the NFκB consensus binding site, that were immobilized to 96-well plates. NFκB protein bound to the oligonucleotides was detected by an antibody to the p65 subunit that only recognized p65 that is bound to DNA. The positive control was HeLa cells that were stimulated with TNF-α. A control was performed using excess free NFκB oligonucleotides (Competitor). **B**, HT-29 cells were transiently suspended and allowed to readhere in the presence or absence of BAY 11-7085 for 3 h after which they were lysed. NFκB binding activity was determined by the TransAM assay. **C**, transiently suspended DLD-1, HT-29, Caco-2, and HCT-116 cells were allowed to readhere in the presence of DMSO (control) or BAY 11-7085 for 8 h after which the percentage of apoptotic cells was determined by the flow cytometric annexin V assay. The data shown represent typical results of three separate experiments. **D**, HT-29 cells were transiently suspended and allowed to readhere in the presence or absence of various concentrations of the NFκB inhibitors, PDTC, BAY 11-7085, and MG-132 for 4 h. The percentage of apoptotic cells was determined using the annexin V assay. The data points represent the averages of three experiments; bars, ± SE. **E**, DLD-1 and **F**, HT-29 cells were transduced with an adenovirus containing the IκB super-repressor construct. After 3 days, the cells were transiently suspended and allowed to readhere in the presence of subapoptotic concentrations of BAY 11-7085 or DMSO (vehicle) for 8 h. The data are expressed as fractions of cells surviving compared with the controls (no adenovirus) and were quantitatively determined by staining the remaining adherent cells with crystal violet (see "Materials and Methods"). Each data point is the average of triplicate experiments; bars, ± SE. The insets show the relative levels of IκB super-repressor protein expressed in DLD-1 (**E**) and HT-29 (**F**) cells. Each lane of the immunoblots represents 50 μg of total protein. Legend for graphs: ■ = 0, ● = 1, ▲ = 10, ◆ = 50 μl of adenovirus-IκB-super repressor per 60-mm dish of cells.

is incapable of being targeted for ubiquitination and, hence, degradation by the proteasome (36). The transduced DLD-1 and HT-29 cells were then transiently suspended and allowed to readhere in concentrations of BAY 11-7085 below the *in vitro* apoptotic threshold (>20 μM) for these cell lines. Expression of the IκB super-repressor in DLD-1 and HT-29 cells significantly lowered the apoptotic threshold

of BAY 11-7085 compared with the controls (Fig. 3, *D* and *E*). The effect of the I κ B super-repressor was much greater for DLD-1 than HT-29 cells because the former expressed higher levels of I κ B super-repressor protein than the latter cells (see insets in Fig. 3, *D* and *E*). Furthermore, the I κ B super-repressor protein was functional in both cell lines (data not shown). These data showed that inhibition of NF κ B was indeed important to the apoptotic effect of BAY 11-7085 during colon cancer cell readhesion.

Effects of NF κ B Inhibitors on Adherent Colon Cancer Cells *in Vivo*. Because BAY 11-7085 did not cause much increase in apoptosis of adherent colon cancer cells, we hypothesized that the drug would not have a significant effect on adherent colon cancer cells *in vivo*. To test this, BAY 11-7085 was tested on human colon cancer xenografts in athymic mice, in which the colon cancer cells would be in an adherent state.

HT-29 colon cancer cell lines were used to generate s.c. tumors in athymic female mice. The mice were then randomly assigned to receive vehicle (DMSO) alone or BAY 11-7085 at 5 mg/kg i.p. twice weekly for 28 days. HT-29 xenografts showed a statistically significant but modest overall reduction in tumor volumes in the BAY 11-7085 treatment group compared with the control group (Fig. 4A). In addition, the colon cancer xenografts were resected and the percentage of apoptotic cells was determined using the terminal deoxyuridine end-labeling method. Only a very small percentage (<5%) of apoptotic cells were found in the xenografts, and there was no significant difference in the percentage of apoptotic cells between tumors from mice treated with BAY 11-7085 or vehicle alone (data not shown). The modest antitumorigenic activity of BAY 11-7085

may have been because of the antiproliferative effects of BAY 11-7085 seen *in vitro* (Fig. 4B).

Intra-Abdominal Colon Cancer Seeding Model. During colorectal cancer surgery, seeding of the peritoneal cavity by tumor cells with tumor cell implantation of the peritoneal surfaces can occur (4, 37). Seeding results from the transient suspension of cancer cells, through a loss of adhesion either naturally or because of surgical displacement, followed by readhesion to other tissues. Because BAY 11-7085 induced apoptosis of readherent HT-29 cells *in vitro*, the ability of the drug to prevent readhesion or seeding of intra-abdominal tissues with colon cancer cells was tested in an *in vivo* model. Athymic mice were injected i.p. with either vehicle alone (DMSO) or BAY 11-7085 (5 mg/kg). Twenty-four h later, transiently suspended HT-29 and HCT-116 colon cancer cell lines were injected into the abdominal cavities of the mice. The mice were then injected (i.p.) twice weekly with vehicle or BAY 11-7085 (5 mg/kg) for a total of 21 days.

There was no macroscopic evidence of tumoral implantation of the intra-abdominal parietal or visceral peritoneal surfaces 7 days after the introduction of colon cancer cells (data not shown). After 21 days, the mice injected with HCT-116 cells showed numerous (>100) tumor implants involving the abdominal parietal and visceral peritoneum, and liver regardless of whether they had received vehicle or BAY 11-7085 (Fig. 5, A-C). However, of the 6 mice injected intra-abdominally with HT-29 cells and treated with BAY 11-7085, only 2 mice demonstrated parietal peritoneal tumor implants (two implants/mouse) and none showed any evidence of hepatic implants (Table 1). Of the 6 mice that had been injected with HT-29 cells and treated with vehicle, 5 developed tumor implants of the visceral and parietal peritoneum (Fig. 5, *D* and *E*), and all 6 developed tumor implants of the liver (Fig. 5, *F-I*; Table 1).

Mechanism of NF κ B Inhibitor-induced Apoptosis. Attention was focused on potential mechanisms of apoptosis resulting from NF κ B inhibition. Inhibition of NF κ B increased the susceptibility of cancer cells to TNF- α -induced apoptosis in previous studies (10, 13). Furthermore, TNF- α is expressed by a number of colon cancer cell lines including HT-29 cells (38) and can activate NF κ B in HT-29 cells (39). This suggested that BAY 11-7082 and BAY 11-7085 might be sensitizing colon cancer cells to TNF- α -induced apoptosis. cA2 is a humanized mouse chimeric antibody, which inhibits TNF- α binding to TNF- α receptors (40). To demonstrate the activity of cA2 on colon cancer cells, cell proliferation of DLD-1, HCT-116, and HT-29 colon cancer cells was measured during treatment with various concentrations of cA2. At concentrations of 10 and 50 μ g/ml, cA2 inhibited cell proliferation of all three of the colon cancer cell lines, which implicated TNF- α as an autocrine growth factor (Fig. 6A). Pretreatment of HT-29 cells with 50 μ g/ml of cA2 for 48 h did not diminish BAY 11-7085-induced apoptosis during readhesion (Fig. 6B). This suggested that endogenous TNF- α was not necessary for BAY 11-7085-induced apoptosis.

An important difference between the cell lines selected in these studies is that HCT-116 cells do not express COX-2, whereas DLD-1, Caco-2, and HT-29 cells do (41-43). COX-2 is commonly overexpressed in colorectal tumors, and inhibition of its activity in colorectal cancer cells causes apoptosis (41-43). The four colon cancer cell lines that were tested with BAY 11-7085 expressed differing levels of COX-2 protein (Fig. 6C). There was a correlation between the susceptibility of the colon cancer cell lines to BAY 11-7085-induced apoptosis and the relative levels of COX-2 expression (compare Fig. 3C and Fig. 6C).

To determine whether COX-2 may mediate BAY 11-7085-induced apoptosis, 293 kidney epithelial cells stably transfected with a ponasterone-inducible COX-2 construct (293-COX-2) were studied. Treatment of 293-COX-2 cells with 1 μ g/ml of ponasterone for 48 h

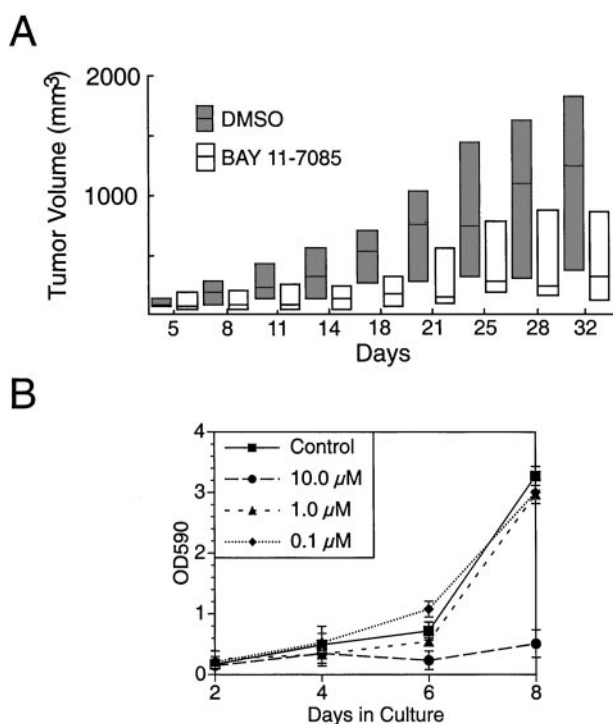


Fig. 4. Effect of BAY 11-7085 on colon cancer xenografts. *A*, athymic mice received s.c. injections of HT-29 cells, followed by 5 mg/ml of BAY 11-7085 (white bars) or DMSO (gray bars) i.p. twice weekly. The bars represent the 25-75% ranges of the tumor volumes and the horizontal lines in the bars are the medians. The Kruskal-Wallis statistical test was used to compare the significance of the changes in median tumor volumes before and after treatment with BAY 11-7085 versus DMSO and yielded a *P* of 0.005. *B*, effect of BAY 11-7085 on HT-29 colon cancer cell proliferation *in vitro*. Adherent HT-29 cells were treated with various concentrations of BAY 11-7085 for 8 days. The number of cells was quantified using a crystal violet staining method (see "Materials and Methods"); bars, \pm SE.

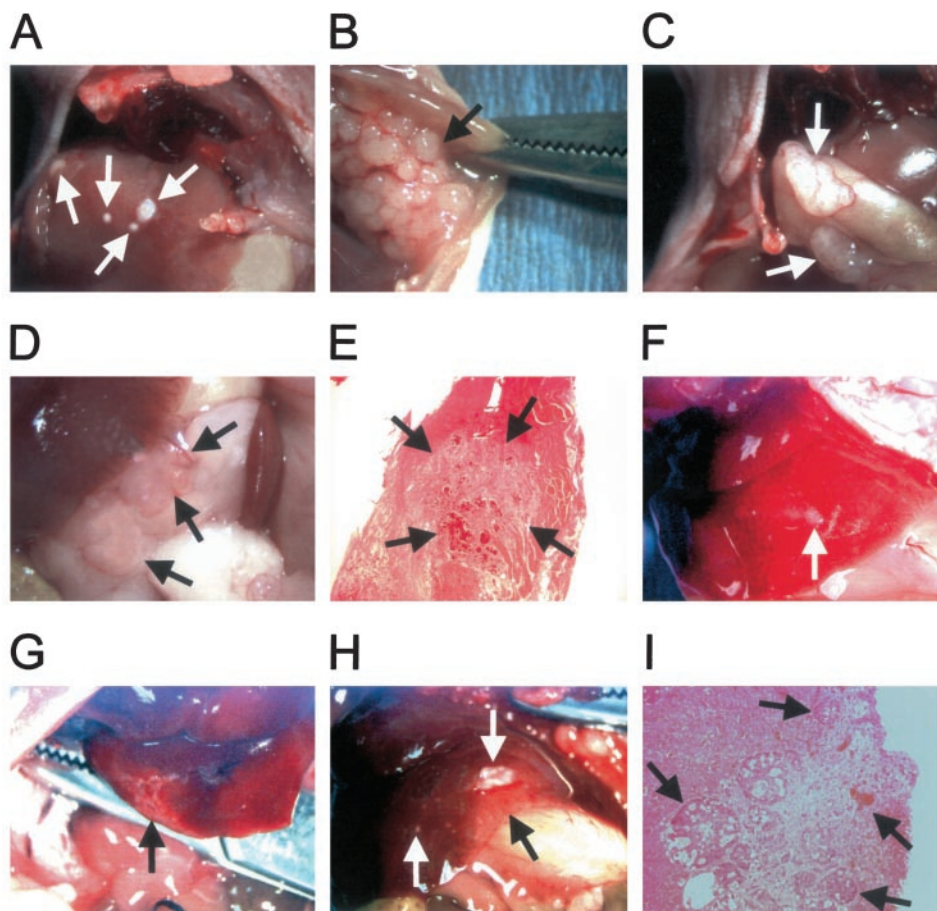


Fig. 5. Intraabdominal seeding model. *A*, liver, *B*, abdominal wall, and *C*, intestinal tumor implants (white and black arrows) in three athymic mice 21 days after receiving intra-abdominal injections of HCT-116 cells. All of these mice received 5 mg/kg of BAY 11-7085 twice weekly for 21 days. *D-I* are from athymic mice 21 days after receiving i.p. injections of HT-29 cells. The mice received i.p. DMSO 24 h before injection of cancer cells and then twice weekly for a total of 21 days. *D*, HT-29 colon cancer tumor implants (black arrows) of the visceral peritoneum overlying the small intestine. *E*, photomicrograph of HT-29 cell tumor implant of the parietal peritoneum (black arrows). *F-H*, tumor implants of the liver (white and black arrows). *I*, photomicrograph showing tumor invasion through the liver capsule. This section was obtained from the tumor implant from the mouse in *F*.

led to a large induction of COX-2 protein, whereas the uninduced cells showed no measurable COX-2 protein expression by Western blot (Fig. 6C). BAY 11-7085 did not cause an increase in apoptosis of uninduced 293-COX-2 cells, but caused a marked increase in apoptosis of ponasterone-treated cells (Fig. 6D). This data suggested that COX-2 expression was important to the mechanism of BAY 11-7085-induced apoptosis, and could potentially explain the susceptibility of DLD-1, Caco-2, and HT-29 cells, and the resistance of HCT-116 cells to BAY 11-7085-induced apoptosis. Whereas BAY 11-7085 did not cause a decrease in COX-2 protein expression by Western blot in the colon cancer cells (data not shown), it is presently unknown as to whether BAY 11-7082 or BAY 11-7085 can directly inhibit COX-2 activity.

NFκB regulates the expression of a number of antiapoptotic genes, including *c-IAP-1*, *c-IAP-2*, *TRAF-1*, and *TRAF-2* (14-16). One potential mechanism for the apoptotic effects of NFκB inhibitors would be to cause the reduced expression of one or more of these antiapoptotic gene products. HT-29 cells did not express detectable levels of *c-IAP-1* by Western blots (data not shown). Treatment of HT-29 cells with BAY 11-7085 resulted in a decrease in the protein expression of *c-IAP-2*, *TRAF-1*, and *TRAF-2* proteins (Fig. 7).

DISCUSSION

Cancer cells derived from tumors arising from various types of tissues demonstrated constitutive activation of NFκB (5-9). Similarly, we found a low level of constitutive NFκB activation in adherent colon cancer cells. However, readhesion of transiently suspended colon cancer cells caused a marked and transient induction of NFκB activation, which could be inhibited almost completely by treatment

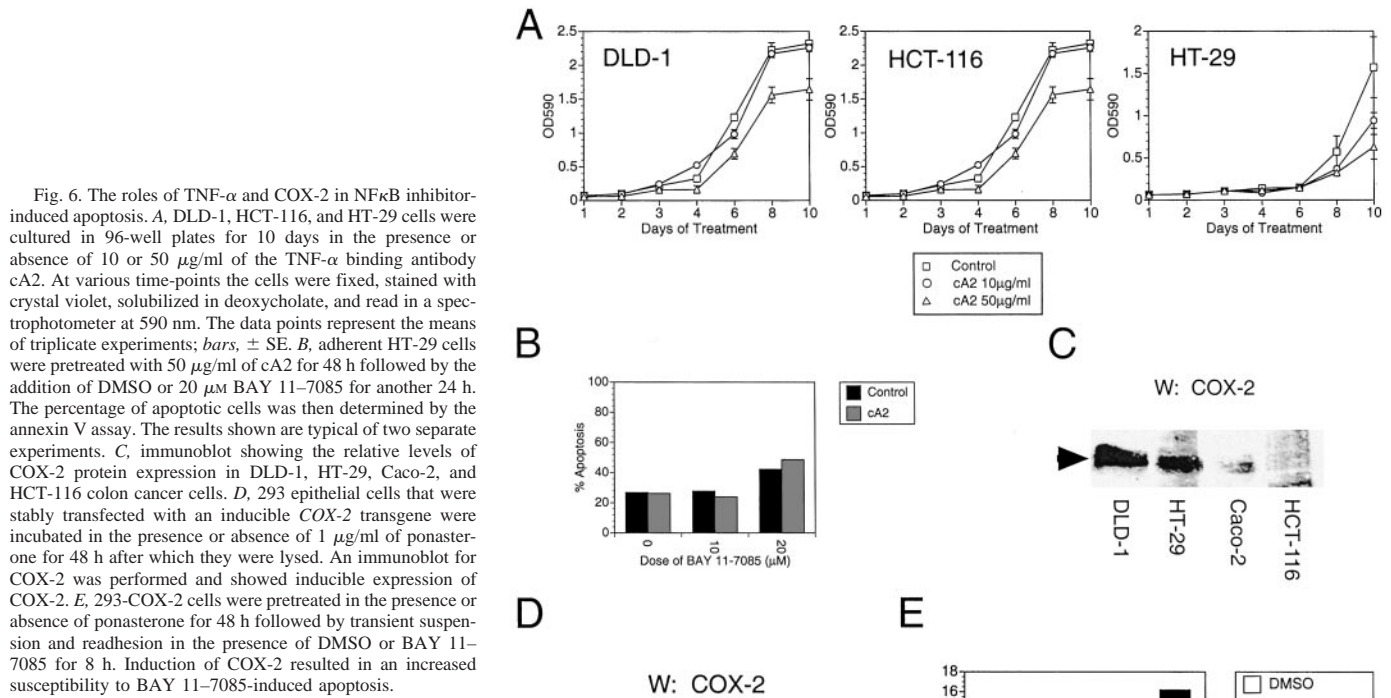
with BAY 11-7085. This is consistent with the studies of Pierce *et al.* (30) who showed that BAY 11-7082 and BAY 11-7085 inhibited TNF-α-induced NFκB activation. The transient induction of NFκB during cell readhesion rendered DLD-1 and HT-29 colon cancer cells exquisitely susceptible to apoptosis induced by either BAY 11-7082 or BAY 11-7085, as well as the NFκB inhibitors PDTC and MG-132.

DLD-1 and HT-29 cells were transduced with an adenovirus containing the IκB super-repressor construct, which encodes a non-degradable IκB protein that directly inhibits NFκB, to study the role of NFκB in the apoptotic mechanism of BAY 11-7085 (36). BAY 11-7085 displayed a fairly uniform minimum dose (~20 μM) for causing apoptosis of readhering colon cancer cells *in vitro*. Expression of the IκB super-repressor protein in these cells significantly increased the apoptotic effect of BAY 11-7085 at concentrations below the apoptotic threshold. This demonstrated the direct role of NFκB in the apoptotic mechanism of BAY 11-7085.

These are the first studies to demonstrate that colon cancer cells are highly dependent on inducible NFκB activation for survival during the establishment of cell adhesion. These data suggested a potential therapeutic role for NFκB inhibitors in preventing colon cancer seeding *in vivo*. At the time patients with abdominal malignancies undergo resection of their tumors, seeding of the peritoneal cavity and the

Table 1 Peritoneal and liver metastases following i.p. injections of HT-29 cells into athymic mice

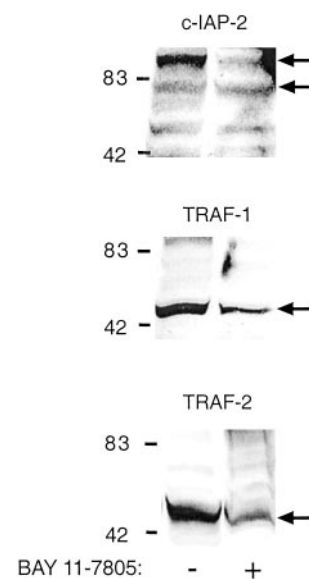
Treatment	Peritoneal metastases	Liver metastases
DMSO (n = 6)	5/6	6/6
BAY 11-7085 (n = 6)	2/6	0/6



circulatory system occurs (4, 37). This is particularly worrisome, because iatrogenic seeding can negate the curative intent of the surgery. The results obtained with the athymic mouse model, whereby seeding was simulated by i.p. injection of colon cancer cells, demonstrated that peritoneal tumor implantation of HT-29 colon cancer cells could actually be prevented with BAY 11-7085 administration. BAY 11-7085 completely prevented tumor implantation of the liver by HT-29 colon cancer cells, whereas 100% of control mice developed multiple tumor implants of their livers. HT-29 cells probably did not metastasize to the liver hematogenously, because histological sections of the livers of control mice showed only superficial invasion of cancer cell implants through the liver capsule, whereas tumor cells in the deep interior regions of the livers were absent. Consistent with the *in vitro* studies, BAY 11-7085 did not significantly decrease tumor implantation by HCT-116 colon cancer cells. Not only were there superficial tumor implants of the liver, but deeper sections showed islands of tumor cells suggesting that hematogenous metastases may have occurred.

Unlike DLD-1, HT-29, and Caco-2 colon cancer cells, HCT-116 cells were consistently resistant to the apoptotic and antitumorigenic effects of BAY 11-7082 or BAY 11-7085. The colon cancer cell lines used in this study were selected on the basis of key molecular genetic differences: HCT-116 colon cancer cells demonstrate microsatellite instability because of mutations in both *hMLH1* alleles, whereas DLD-1, HT-29, and Caco-2 cells are microsatellite stable (44-46). Previous studies showed that COX-2 overexpression is not typically found in colorectal tumors with high levels of microsatellite instability (47). Consistent with this observation, DLD-1, HT-29, and Caco-2 cells express COX-2, whereas HCT-116 cells do not (43, 48, 49). COX-2 is a major target for nonsteroidal anti-inflammatory drugs and is commonly overexpressed in colon tumors but not in normal colonic

epithelium (50, 51). Whereas NF κ B is important for COX-2 gene expression in normal cells (23-26), the mechanism for its overexpression in colon tumors is unknown (52, 53). The efficacy of BAY 11-7085 to cause apoptosis of colon cancer cells correlated with the relative levels of COX-2 expression in the various colon cancer cell lines. Results obtained in our study using 293 kidney epithelial cells



transfected with an inducible *COX-2* transgene demonstrated a potential role for COX-2 protein overexpression in the apoptotic response to BAY 11-7085. However, in other studies, COX-2 overexpression inhibited colon cancer cell apoptosis, increased cell adhesion, and increased resistance to butyrate-induced apoptosis in rat intestinal epithelial cells (54). A possible explanation for this paradox is that BAY 11-7082 and BAY 11-7085 may inhibit COX-2 activity. However, we found that sulindac sulfide, a COX-2 inhibitor known to induce colon cancer cell apoptosis (50, 51, 55), caused much less apoptosis of readherent HT-29 cells than similar doses of BAY 11-7082 or BAY 11-7085 (data not shown). This suggests that BAY 11-7082 and BAY 11-7085 do not cause colon cancer cell apoptosis by inhibiting COX-2 alone. The fact that HCT-116 cells lack COX-2 expression and are resistant to BAY 11-7085-induced apoptosis suggests that they use different survival mechanisms than DLD-1, HT-29, and Caco-2 cells.

To determine how the NFκB inhibitors caused apoptosis of colon cancer cells, NFκB-mediated survival pathways that may be used during colon cancer cell readhesion were examined. Recent studies in cancer cells demonstrated that NFκB inhibition resulted in an increased susceptibility of the cells to TNF-α-induced apoptosis (10, 13). Our results did not suggest this as a mechanism. We found that the anti-TNF-α monoclonal antibody had an antiproliferative effect on the colon cancer cell lines used, implicating TNF-α as an autocrine growth factor in colon cancer cells, which is a novel finding.

We examined whether BAY 11-7085 reduced the expression of survival genes under the regulation of NFκB and shown to play a role in cancer cell survival. BAY 11-7085 did diminish the expression of c-IAP-2, TRAF-1, and TRAF-2, which have been shown previously to be involved in mediating cancer cell survival (14-16). We did not examine BAY 11-7085-induced changes in other known survival genes under the regulation of NFκB, such as *XIAP* or *IEX-1L*, so there may be other genes important to cell survival that were targeted by BAY 11-7082 or BAY 11-7085 as well in the colon cancer cells studied. The fact that NFκB mediates the expression of multiple survival genes makes it an important and rational target for cancer chemotherapy.

A caveat to the interpretation of our results is that BAY 11-7082, BAY 11-7085, PDTC, and MG-132, whereas known to inhibit NFκB activity, are not completely specific for NFκB. MG-132 has a general inhibitory effect on proteasomal protein degradation; thus, its effects do not exclude the role of cellular proteins other than NFκB, of which the activity and/or expression are regulated by the proteasome (56). BAY 11-7082 and BAY 11-7085 also activate JNK/stress-activated protein kinase and p38/Hog (30). JNK/stress-activated protein kinase and p38/Hog are members of the mitogen-activated protein kinase family, and are known to be involved in the induction of apoptosis (57). Whereas we observed constitutive phosphorylation of JNK in HT-29 cells by immunoblot,⁵ there was no change in the level of JNK phosphorylation after treatment of HT-29 cells with proapoptotic doses of BAY 11-7085 (data not shown). No increased phosphorylation of p38 was seen in the HT-29 cells after treatment with BAY 11-7085 either (data not shown). Nevertheless, the ability of BAY 11-7085 to inhibit both tumorigenicity and seeding of colon cancer cells *in vivo* demonstrate the strong potential of this agent as a novel chemotherapeutic drug.

Most studies on the inhibition of NFκB activity *in vivo* have used a gene therapy approach through the introduction of a nondegradable IκB mutant, which prevents nuclear translocation of NFκB. The advantage of soluble inhibitors is that their delivery would be easier

and more efficient than gene transfer *in vivo*. Whereas the chemical structures of BAY 11-7082 and BAY 11-7085 suggest that they are potential Michael acceptors, no significant renal, hepatic, or pulmonary tissue toxicity was seen with large doses of these compounds in the mice in our studies. Indeed, the mice appeared to tolerate these compounds well, and a previous study using up to 20 mg/kg/day of these agents in rats for 21 days did not reveal obvious toxicity (30). Soluble NFκB inhibitors may be useful adjuncts to colon cancer chemotherapy regimens, and as neoadjuvant therapy before surgical resection in preventing seeding and metastasis.

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