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## Bromoethylindole (BEI-9) redirects NF- $\kappa$ B signaling induced by camptothecin and TNF $\alpha$ to promote cell death in colon cancer cells

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### Abstract

Chemotherapeutic regimens containing camptothecin (CPT), 5-fluorouracil, and oxaliplatin are used to treat advanced colorectal cancer. We previously reported that an indole derivative, 3-(2-bromoethyl)indole (BEI-9), inhibited the proliferation of colon cancer cells and suppressed NF- $\kappa$ B activation. Here, we show that a combination of BEI-9 with either CPT or tumor necrosis factor alpha (TNF $\alpha$ ) enhances cell death. Using colorectal cancer cells, we examined the activation of NF- $\kappa$ B by drugs, the potential of BEI-9 for inhibiting drug-induced NF- $\kappa$ B activation, and the enhancement of cell death by combination treatments. Cells were treated with the chemotherapeutic drugs alone or in combination with BEI-9. NF- $\kappa$ B activation, cell cycle profiles, DNA-damage response, markers of cell death signaling and targets of NF- $\kappa$ B were evaluated to determine the effects of single and co-treatments. The combination of BEI-9 with CPT or TNF $\alpha$  inhibited NF- $\kappa$ B activation and reduced the expression of NF- $\kappa$ B-responsive genes, Bcl-xL and COX2. Compared to CPT or BEI-9 alone, sequential treatment of the cells with CPT and BEI-9 significantly enhanced caspase activation and cell death. Co-treatment with TNF $\alpha$  and BEI-9 also caused more cytotoxicity than TNF $\alpha$  or BEI-9 alone. Combined BEI-9 and TNF $\alpha$  enhanced cell death through caspase activation and cleavage of the switch-protein, RIP kinase. BEI-9 reduced the expression of COX2 both alone and in combination with CPT or TNF. We postulate that BEI-9 enhances the effects of these drugs on cancer cells by turning off or redirecting NF- $\kappa$ B signaling. Therefore, the combination of BEI-9 with drugs that activate NF- $\kappa$ B needs to be evaluated for clinical applications.

### Keywords

Bromoethylindole; Apoptosis; NF- $\kappa$ B; Camptothecin; Colorectal cancer

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## Introduction

Standard therapy for colorectal cancer (CRC) involves surgery, chemotherapy, or a combination of the two based on the stage of the disease. Surgical removal of affected tissue is performed for stages I–III, either with or without the administration of chemotherapy or radiation. For the most advanced disease, stage IV, surgery is often impractical due to extensive involvement of lymph nodes and other organs. Chemotherapy for CRC includes targeted (EGFR or VEGF inhibitors) and broad-acting cytotoxic drugs (5-fluorouracil [5-FU], oxaliplatin, leucovorin, irinotecan, capecitabine) as single agents or in combination. Management of stage IV disease invariably includes chemotherapy.

Although the combination of cytotoxic and targeted drugs has improved the survival of CRC patients (1, 2), the efficacy for both targeted agents and immunotherapy against CRC lags behind that for other types of cancer, such as melanoma and lung cancer. Such low efficacy may be explained, in part, by compensatory and alternative pathways in some CRCs or by lack of knowledge of patient subgroups that may benefit. An additional challenge relates to the unknown mechanisms for resistance (2). Until these mechanisms are identified and targeted, broad-acting cytotoxic drugs will remain the mainstay of CRC therapy.

Camptothecin (CPT), an inhibitor of topoisomerase I (TOPO1), interferes with cell cycle progression by inhibiting DNA replication. CPT-related drugs (i.e., irinotecan, irrisa, and topothecan), often in combination with other drugs or biologics, constitute the therapeutic regimen for stage IV CRC. For example, irinotecan is administered as part of the FOLFIRI (FOLinic acid, Fluorouracil, IRInotecan) and FOLFOXIRI (FOLinic acid, Fluorouracil, OXaliolatin, IRInotecan) regimens or even as a single agent ([https://www.cancer.gov/types/colorectal/hp/colon-treatment-pdq#section/\\_145](https://www.cancer.gov/types/colorectal/hp/colon-treatment-pdq#section/_145), <http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-by-stage-colon>).

We and others have previously reported that treatment of CRC-derived cell lines with CPT leads to activation of NF- $\kappa$ B signaling (3, 4). The mechanism for NF- $\kappa$ B activation is suggested to be signaling in response to DNA damage through ATM/ATR followed by IKK activation (5). We also showed that the downstream targets of NF- $\kappa$ B signaling activated by CPT are different from those activated by TNF $\alpha$  (3), illustrating a divergence of effectors of signaling initiated by cell surface receptors and those initiated by DNA damage. TNF $\alpha$  increased the expression of the anti-apoptotic *cIAP2* (*BIRC3*) gene, and CPT up-regulated expression of the cytokines CXCL1 and CXCL8, which signal through CXCR1 and CXCR2.

In addition to chemotherapy, radiation therapy activates NF- $\kappa$ B (6–8). However, the role of NF- $\kappa$ B activation subsequent to either radiation or chemotherapy remains incompletely understood. NF- $\kappa$ B activation may be a pathophysiological response to tissue damage during therapy, but the benefits of such activation may be overshadowed by the downstream expression of inflammatory cytokines, which in turn may drive the oncogenic process. Moreover, infiltration of damaged tumor tissue by inflammatory cells and macrophages may mitigate efficacy of the therapy. The most desirable outcome would be the recruitment and

activation of effector cytotoxic cells that destroy the surviving, perhaps damaged, cancer cells. However, selective modulation of the molecules that activate effector T-cells without enhancing cancer cell survival has not yet been achieved.

In a recent study on selected indole-based compounds, we identified 3-(2-bromoethyl)indole (BEI-9 or BEI) as a potent inhibitor of NF- $\kappa$ B signaling and cell proliferation (9). In the present work, we used BEI-9 to shut down NF- $\kappa$ B signaling activated by either CPT or TNF $\alpha$ . In colon cancer cells treated with either of the drugs, inhibition of NF- $\kappa$ B signaling resulted in enhanced cell death by apoptosis, marked by cleavage of RIP kinase. Switching NF- $\kappa$ B signaling from survival of cancer cells to apoptosis by use of a small molecule has implications for cancer therapy and for inflammatory conditions in which continued activation of NF- $\kappa$ B may lead to survival and escape of neoplastic cells or to exacerbated inflammation.

## Materials and Methods

### Cell culture

Parental SW480 and SW620 cells were purchased from the American Type Culture Collection (ATCC). Both cell lines were grown in McCoy's 5A medium containing 10% fetal bovine serum (FBS) and 50  $\mu$ g/ml ciprofloxacin. SW480 and SW620 cells expressing NF- $\kappa$ B-reporter luciferase were generated and used as described previously (23). Other cell lines, also purchased from ATCC, were maintained in DMEM containing FBS and ciprofloxacin. Cells were incubated in a humidified incubator under 5% CO<sub>2</sub> at 37°C. Reporter cells were used for luciferase assays; parental cells were used for all other biochemical and phenotype assays.

### Chemicals

Dimethylsulfoxide (DMSO, used as solvent for drugs), ciprofloxacin, and McCoy's 5A medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). BEI-9 was purchased from Sigma Aldrich and dissolved in DMSO. Drugs used for treatment of cells were obtained from Sigma Aldrich (5-FU, oxaliplatin, TNF $\alpha$ ) or LC laboratories (erlotinib, phleomycin, CPT).

### MTS Assay

To assess the viability of treated cells, we used the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay (Promega) according to manufacturer's instructions. For the assays, 10<sup>4</sup> cells/well were seeded in 96-well plates, then treated with the indicated drugs for the indicated number of hr, after which the assay was performed. Normalization of the data was accomplished by using readings from vehicle-treated cells. To express the results, viability indices, which represent relative percentages compared to the controls, were used. Variations were recorded as standard deviations from the mean values.

## Microscopy

Phase-contrast images of cells were taken with an Olympus IX71 inverted microscope attached to a digital camera equipped with CellSens® Image Capture software (Olympus America, Inc., Center Valley, PA, USA). Images were stored in TIFF format, and, afterward, were cropped and resized in Microsoft Power Point.

## Cell cycle analysis

The cell cycle was analyzed by flow cytometry. Cells were harvested by use of 0.25% trypsin-EDTA (Invitrogen Corp., Carlsbad, CA, USA) and then centrifuged for 3–5 min at 1200 rpm (about 800 *g*). The supernatant was removed, and cells were suspended in 300  $\mu$ l of phosphate-buffered saline (PBS) without calcium and magnesium. Cells were fixed with 700  $\mu$ l of cold 100% ethanol, stored at  $-20^{\circ}\text{C}$ , and analyzed within two days. The fixed samples were centrifuged at 1600 rpm for 5 min, the supernatant was removed, and FACS staining solution (320 mg/ml RNase A and 0.4 mg/ml propidium iodide [PI]) in PBS without calcium and magnesium was added for staining. Stained cells were passed through filters (70- $\mu\text{m}$  pore size) and analyzed on a FACScalibur® Flow Cytometer (BD, San CA USA). Data processing and preparation of histograms were accomplished with FlowJo software (Cytek® Biosciences, Fremont, CA).

## NF- $\kappa$ B response reporter luciferase assays

SW480 or SW620 NF- $\kappa$ B luciferase reporter cells were seeded in 96-well plates and treated with the test drugs for 18–24 hr. To perform the luciferase assay, the medium was removed by aspiration, and 50  $\mu$ l of 1X luciferin substrate solution prepared in PBS was added to each well. Luminometer readings were at  $37^{\circ}\text{C}$ . Plates were read immediately after adding the substrate solution and after 5 and 10 min. Readings at the time point when the peak luminescence values were recorded were used to obtain the relative luciferase units (RLUs) or fold activation. RLUs were normalized against readings of control wells and recorded as relative NF- $\kappa$ B reporter activity.

## Immunoblotting

For immunoblotting experiments, cells were treated in 6-cm culture dishes and lysed in RIPA buffer to which a cocktail of protease and phosphatase inhibitors was added. Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad) according to the instructions. Samples containing equivalent protein concentrations were mixed with Laemmli's buffer and boiled for 5 min. Proteins were transferred to polyvinylidene difluoride membranes, and membranes were blocked with 5% non-fat dry milk prepared in PBS containing 0.1% Tween-20. After 30 min to 1 hr of blocking, the membranes were incubated overnight with primary antibodies. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in 2.5% non-fat dry milk containing blocking buffer. Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific) was used to visualize the proteins. X-Ray films were used for the detection of chemiluminescent bands, except when the signals were strong, in which case C-DiGiT Scanner (LI-COR®, Lincoln, NE) was used to scan the signals. Primary antibodies, at 1:1,000 dilutions, were used to target TOPO1 (Upstate

Biotechnology, Lake Placid, NY, USA), p53, and Bcl-xL (Millipore, Darmstadt, Germany). Rabbit anti-GAPDH antibody was purchased from OriGene Technologies® (Rockville, MD). Peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were purchased from Millipore (Temecula, CA) and used at 1:3,000 dilutions.

### **Annexin-V apoptosis assay**

This assay was conducted with Annexin V-FITC apoptosis detection kits (#APOAF-50TST, Sigma-Aldrich, St Louis MI), according to the manufacturer's instructions. Cells were incubated with treatment compounds for 16–18 hr. Annexin V readings (FL1 channel) and PI (FL3 channel) data were collected and analyzed with a FACSCalibur flow cytometer and FlowJo® analysis software.

### **FITC VAD FMK and caspase 3/7 activation assay**

Fluorescent caspase activation assays for pan-caspase were performed with casPACE FITC-VAD-FMK *in situ* marker (Promega, Madison, WI), and caspase 3/7 activation was assayed with CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen, Carlsbad, CA). Cells were treated with compounds or vehicle control (DMSO) for 24–28 hr before caspase assays. After incubation with the detection reagents and washing to remove excess reagents, images were taken with an inverted fluorescent microscope (Olympus IX71) and analyzed by CellSense® image capture software as described above.

## **Results**

### **CPT and TNF $\alpha$ increase NF- $\kappa$ B activity in metastasis-derived SW620 cells**

In a previous report, we showed that SW480 cells, derived from a primary colon carcinoma, respond to CPT by activating NF- $\kappa$ B signaling. Since CPT-based drugs are clinically used primarily to treat advanced and recurrent CRCs, we tested the reaction of SW620 cells, which were derived from a lymph node metastasis of the same patient from whom SW480 cells were obtained. NF- $\kappa$ B reporter SW620 cells were derived as described earlier (3). SW480 and SW620 cells showed a similar pattern of NF- $\kappa$ B activity upon treatment with various drugs (Fig. 1A–B). The dose-response of SW620 cells treated with 0–10  $\mu$ M CPT was determined (Fig. 1C). Exposure of the cells to CPT at concentrations between 0.6 and 2.5  $\mu$ M (peak at 1.25  $\mu$ M) resulted in higher NF- $\kappa$ B activity relative to lower and higher concentrations.

### **BEI-9 suppresses NF- $\kappa$ B activation caused by CPT and induces cell death upon sequential treatment**

We next determined if the combination of BEI-9 with CPT alters NF- $\kappa$ B-activation. Co-treatment of BEI-9 and an NF- $\kappa$ B-inducing concentration of CPT was evaluated on NF- $\kappa$ B reporter SW620 cells. The simultaneous combination of 5  $\mu$ M BEI-9 and 1  $\mu$ M CPT resulted in complete suppression of the elevated reporter activity (Fig. 2A). However, microscopic examination of these cells indicated that cell death did not account for the loss of NF- $\kappa$ B activation (not shown). Therefore, we determined if sequential treatment of the cells with CPT and BEI-9 induced cell death in addition to inhibiting NF- $\kappa$ B signaling. To this end, parental SW620 cells were treated with sequential combinations of CPT followed by BEI-9

and analyzed by flow cytometry. Marked cell death, as measured by the proportion of sub-G1 population, was induced when treatment with a 1  $\mu$ M concentration of CPT for 24 hr was followed by a 24-hr treatment with BEI-9 (Fig. 2B) or when varied CPT concentrations for 24 hr were followed by a 2.5- $\mu$ M concentration of BEI-9 (Fig. 2C). Of note, single-agent treatments by either CPT or BEI-9 induced minimal cell death, showing a potential synergistic effect of these sequential treatments. To corroborate the induction of cell death by sequential combinations of the two agents, the Annexin-V/PI procedure was used to assess the induction of apoptotic cell death. This method is used to analyze the early events of apoptosis characterized by alterations in cell membrane phosphatidyl serine orientations. In these experiments, cells were exposed to CPT for 24 hr and then to BEI-9 for 12 hr. Treatment of CPT-exposed cells induced a concentration-dependent increase in Annexin-V and Annexin/PI double-positive cells (Fig. 2D), showing an increase in the proportion of apoptotic cells that lose membrane integrity and transition to cell death. Again, treatment with either of the compounds alone failed to result in appreciable cell death, further demonstrating a synergistic effect of the combination.

To investigate the time-dependent increase in cell death by CPT - BEI-9 combination, we exposed SW620 cells to CPT (1  $\mu$ M) for 6, 10, or 24 hr and then treated them with BEI-9 (3  $\mu$ M) for additional 24 hr. Cell cycle profiles were analyzed by flow cytometry to assess the development of sub-G1 population. As shown in Fig. 2E, sequential combination enhanced sub-G1 population in a time-dependent manner.

### **Cell death by the sequential combination of CPT and BEI-9 proceeds through loss of Bcl-xL and caspase activation**

The expression of the anti-apoptotic protein, Bcl-xL, in SW620 cells treated with a combination of 1  $\mu$ M CPT and 1–3  $\mu$ M BEI-9 was examined. Similar to the effect of caspase activation, the expression of Bcl-xL was reduced in cells exposed to the combination (Fig. 3A). Single agents were not effective in eliminating the anti-apoptotic protein, corroborating the results showing a synergistic effect. To characterize cell death induced by the combination, caspase activation in treated cells was evaluated by use of the fluorescent pan-caspase activation assay (FITC-VAD-FMK). In this method, a fluorescent caspase substrate binds to activated caspases in cells and is detected by fluorescent microscopy. Therefore, the proportion of fluorescent cells in a treated population is indicative of the degree of caspase activity. SW620 cells were treated with the individual compounds or the combination scheme of 1  $\mu$ M CPT and 5  $\mu$ M BEI, and caspase-positive fluorescent cells were counted as proportions of the total population. The proportion of caspase-positive cells was higher, 3-fold or greater, than treatment with either of the single agents (Fig. 3B). Thus, since *Bcl-xL* is an NF- $\kappa$ B-responsive gene, inhibition of upstream signaling, caspase activation, and elimination of Bcl-xL together accelerate cell death induced by the combination.

### **BEI-9 inhibits NF- $\kappa$ B activation by TNF $\alpha$ and induces cell death**

We next ascertained if BEI-9 inhibited NF- $\kappa$ B signaling activated by TNF $\alpha$  and if combination treatment also induced cell death. Similar to experiments with CPT, we determined if single or combination treatments had differential effects on parental and reporter SW620 cells. However, unlike the CPT and BEI-9 sequential experiments, cells



were treated simultaneously with BEI-9 and TNF $\alpha$  for 24 hr, and the effects of treatment were measured by flow cytometry, reporter activity, or viability assays. BEI-9 was a weak inducer of apoptosis (as measured by the sub-G1 population), even when the cells were exposed to concentrations as high as 50  $\mu$ M (Fig. 4A–C). Indeed, there was no significant difference among the proportions of cell death induced by varied concentrations of BEI-9. When NF- $\kappa$ B reporter SW620 cells were treated with TNF $\alpha$  alone, activation of NF- $\kappa$ B reporter activity was evident. However, treatment with BEI-9 alone or BEI-9 in combination with TNF $\alpha$  eliminated the signaling (Fig. 4D). To determine if the reduction in NF- $\kappa$ B signaling was due to cell death, a cell viability assay was performed on the same cells immediately after the reporter assay was completed. Cells markedly lost viability only when they were exposed to the combination of BEI-9 and TNF $\alpha$  (Fig. 4E). Although treatment with BEI-9 alone had a 100-fold reduced reporter activity, only the combination of BEI-9 with TNF $\alpha$  resulted in cell death. The cell death outcome was visible microscopically as abundant apoptotic cells with membrane blebs (supplementary Fig. 1A) and increased numbers of cells in the sub-G1 phase of the cell cycle (Fig. 4F).

Annexin-V/PI staining flow cytometry was then performed for SW620 cells treated with TNF $\alpha$  and BEI-9 to determine if the phenotype of the cells treated with the combination was the result of apoptosis. These results (Fig. 4G–H) showed that the combination resulted in an increased proportion of Annexin-V-positive cells that transitioned to PI-positivity at higher concentrations of BEI-9 in combination with TNF $\alpha$ . Although these observations suggest cell-death through transitioning from Annexin-V positivity to Annexin-PI double positivity, outright induction of PI-positivity (necrotic death) can not be excluded.

Since the membrane blebs, increased sub-G1 population, and increased Annexin-V positivity were all suggestive of apoptotic cell death, we checked for the activation of caspase 3/7 in SW620 cells treated with single agents (CPT, BEI-9, TNF $\alpha$ ) or with combinations of CPT and BEI-9 or TNF $\alpha$  and BEI-9. Increased numbers of caspase-positive cells were found only when TNF $\alpha$  was combined with BEI-9 (supplementary Fig. 1B). Simultaneous treatment of cells with CPT and BEI-9 did not result in caspase activation. Thus, the CPT-BEI-9 combination was cytotoxic only when the compounds were added sequentially.

### **PARP cleavage, elimination of Bcl-xL and COX2, and pathway-switch as mechanisms of apoptotic cell death induced by combination treatment**

To understand the mechanisms underlying cell death induced by the combination of BEI-9 with either CPT or TNF $\alpha$ , the expression of proteins involved in NF- $\kappa$ B signaling and cell death were examined. Immunoblotting was performed for detection of PARP, p53, Bcl-xL, and TOPO1. Additionally, levels of the NF- $\kappa$ B-signaling regulator protein, RIPK1, and the NF- $\kappa$ B target and mediator of inflammatory signaling, COX2, were determined. CPT and BEI-9 were sequentially added to cells, whereas TNF $\alpha$  and BEI-9 were simultaneously added. Results from these analyses showed that PARP was partially cleaved by the combination of CPT and BEI-9, but completely cleaved by the TNF-BEI-9 combination (Fig. 5A). In contrast, expression of Bcl-xL was reduced by the CPT-BEI-9 combination (see also Fig. 3B) but was only moderately reduced by TNF $\alpha$  plus BEI-9. TP53 expression,

although moderately increased by CPT, did not change by the other treatments. CPT treatment also reduced the expression of TOPO1, the primary target of topoisomerase inhibitors. The expression of TOPO1 was also reduced in cells treated with the combination of TNF $\alpha$  and BEI-9. Since TNF $\alpha$  and BEI-9, as single agents, did not affect the isomerase western blot band (Fig. 5A, lanes 3, 6 and 7), the reason for such a reduction is unknown.

Signaling pathways downstream of TNF-receptor engagement involve multiple regulators that determine the outcome of TNF $\alpha$  signaling. TNF signaling could lead to cytokine secretion, cell survival and proliferation, apoptosis, or necroptosis (reviewed in (10)). Since TNF-activated apoptosis proceeds through a mechanism that involves RIP1K cleavage, we tested if this event occurs when BEI-9 is combined with either CPT or TNF $\alpha$ . The combination of TNF $\alpha$  with BEI-9 induced cleavage of RIP1 kinase (Fig. 5B, upper panel), suggesting the switching of TNF-mediated signaling towards apoptosis. Although combination of CPT with BEI-9 reduced the level of full-length RIP1 kinase, there was no visible cleaved product in that lane (Fig. 5B, 4<sup>th</sup> lane). Although the effect of the BEI-9-combination on the TNF pathway is evident, it is not possible to exclude the involvement of RIP1K in apoptosis induced by CPT-BEI-9.

The expression of COX2 was reduced by both the CPT-BEI-9 and TNF $\alpha$ -BEI-9 combinations. TNF $\alpha$  treatment resulted in increased COX2 levels, and CPT moderately reduced it (Fig. 5B, middle panel). BEI-9 as a single agent also reduced the expression of COX2, suggesting inhibition of expression of NF- $\kappa$ B target genes. These results show the involvement of caspase-mediated cleavage of intracellular targets in the process of cell death induced by the combination of BEI-9 with either CPT or TNF $\alpha$ . The primary mechanism of action of BEI-9 also appears to involve inhibition of the NF- $\kappa$ B pathway and switching of NF- $\kappa$ B signaling towards apoptosis through RIP1K cleavage.

### **Effect of the BEI-9-TNF $\alpha$ combination on various colon cancer cell lines**

To demonstrate that the apoptotic effect of the combination of BEI-9 with CPT or TNF $\alpha$  is not specific to SW480 or SW620 cells, HCT116, RKO, and Colo205 cells were exposed to TNF $\alpha$  (25 or 50 ng/mL) and to BEI-9 (10  $\mu$ M) either singly or in combination. HCT116 and Colo205 cells were as sensitive to the combination of the two agents as were SW480 and SW620 cells. RKO cells were sensitive to 10  $\mu$ M BEI-9, but the combination of TNF $\alpha$  with BEI did not result in enhanced cell death as in the other cells (supplemental Figures 2–3).

### **Discussion and Conclusions**

In this study, we demonstrated that BEI-9 acts as a sensitizer to the effects of CPT and TNF $\alpha$  through inhibition of NF- $\kappa$ B signaling and through elimination of intermediate molecules, such as Bcl-xL, that mediate survival. Moreover, the results show that combination treatment of colon cancer cells with CPT or TNF $\alpha$  and BEI-9 induces apoptosis marked by caspase activation. The mechanism of cell death by the combination of TNF $\alpha$  and BEI-9 appears to proceed through a signal switch from survival to apoptosis, characterized by cleavage of the RIP kinase protein, an indicator of the switch.



Although CPT and TNF $\alpha$  activate the NF- $\kappa$ B pathway, our previous study and data from other reports suggest that there are different mechanisms through which the two agents activate NF- $\kappa$ B signaling (3, 11). CPT, a TOPO1 inhibitor, has been proposed to activate NF- $\kappa$ B signaling through the ATM/ATR-mediated DNA damage-response pathway that leads to the activation of NF-Kappa-B essential modulator (NEMO) and hence to translocation of the p65 subunit (4, 5, 12), resulting in transcriptional activation of NF- $\kappa$ B target genes. In contrast, TNF $\alpha$  activates NF- $\kappa$ B signaling through surface receptors (TNFR1 or TNFR2), which, upon engagement, assemble the signaling complex consisting of TRAF2, inhibitors of apoptosis (IAPs), caspases, and RIP kinase (13, 14). Unperturbed downstream signaling from the receptor complex in non-immune cells proceeds through IKKs and other signaling intermediates, leading to the transcription of NF- $\kappa$ B genes (14, 15). The downstream targets of TNF $\alpha$ - or CPT-activated NF- $\kappa$ B are apparently different.

As determined by studies with cancer cell lines, CPT and TNF $\alpha$  do not appear to be potent or rapid inducers of cell death. The anticancer activities of CPT are dependent on the inhibition of TOPO1, which leads to cell cycle arrest followed by cell death. Moreover, the molecular actions of CPT may be dose-dependent, from inhibition of DNA unwinding and reversal of fork progression to induction of double-strand breaks (16). For NF- $\kappa$ B activation in SW480 and SW620 cells, peak activation by CPT occurred at concentrations between 0.5  $\mu$ M and 2  $\mu$ M, tapering down at other concentrations. The functional consequence of such concentration-dependent activation in response to CPT treatment of colon cancer cells remains unknown. Our previous data indicate that, when NF- $\kappa$ B is activated by CPT, it results in a cytokine response, as shown by increased expression of CXCL8 and CXCL1 cytokines and CXCR2, the receptor for the two cytokines (3). This is in contrast to TNF $\alpha$ , which induces expression of the anti-apoptotic *cIAP2* (*BIRC3*) gene, in addition to that of CXCL8. Studies with experimental animals are needed to address the importance of these differential cytokine-receptor responses. However, given the functions of the CXCR2 axis (17–21), it is plausible to hypothesize that induction of NF- $\kappa$ B followed by increased CXCR2-mediated signaling may favor the survival of cells exposed to chemotherapy.

Unlike cytokines such as Fas and TRAIL, which rapidly activate cell death, TNF $\alpha$  is not a typical activator of death in epithelial cells (13, 22, 23). Indeed, NF- $\kappa$ B activation and promotion of survival may be the default pathway upon engagement of TNF $\alpha$ . Apoptosis ensues when TNF $\alpha$ -induced NF- $\kappa$ B survival signaling is blocked by loss of signaling intermediates or reduced expression of regulators through negative-feedback mechanisms (24).

A sensitizing effect of CPT on NF- $\kappa$ B inhibition has been reported (11, 25, 26). The potent induction of apoptosis by the combination of BEI-9 with either CPT or TNF $\alpha$  suggests that BEI-9 acts by inhibiting NF- $\kappa$ B signaling downstream of the drug or cytokine primary targets. Loss of the anti-apoptotic protein, Bcl-xL, was prominent when CPT was combined with BEI-9, indicating that, in this case, the induction of apoptosis is achieved through elimination of proteins of the anti-apoptotic BCL-family. Of note, simultaneous treatment of colon cancer cells with CPT or pre-treatment of cells with BEI-9 did not induce cell death, showing a temporal difference in the actions of these two compounds. We hypothesize that CPT activates NF- $\kappa$ B through a slow process, sensitizing the cells to BEI-9 only after a

period of treatment. Combined with CPT, BEI-9 induces apoptosis through a mechanism that involves the elimination of Bcl-xL.

In contrast, with the combination of BEI-9 and TNF $\alpha$ , cleavage of RIP-kinase and therefore inhibition of the default NF- $\kappa$ B survival signaling pathway apparently promotes cell death. Since TNF $\alpha$  initiates downstream signaling within minutes, the effects of co-treatment of cells with BEI-9 and TNF $\alpha$  did not require a sequential treatment with the two drugs. Of note, BEI-9 as a single agent inhibited the expression of COX2, a pro-inflammatory protein activated by NF- $\kappa$ B, suggesting potent inhibition of basal NF- $\kappa$ B signaling in these cells by BEI-9. Combined with either CPT or TNF $\alpha$ , BEI-9 further reduced the expression of COX2. This finding is relevant, because COX2 is the target for anti-inflammatory drugs such as NSAIDs (27, 28), which indicates that BEI-9 is an inhibitor of inflammatory signaling. It is not clear whether the inhibition of COX2 expression by BEI-9 would contribute to enhanced cell death or if it is only a marker for the inhibition of NF- $\kappa$ B signaling.

It remains to be determined if inhibition of the NF- $\kappa$ B pathway after radiation or chemotherapy is beneficial to clinical outcomes (29–33). For effective immunotherapy, active inflammatory signaling could be needed to attract immune cells, and a sustained inflammatory state could be deleterious. Since NF- $\kappa$ B may activate a diverse array of genes involved in both inflammation and survival, it is essential to delineate NF- $\kappa$ B targets that determine the survival of treated cancer cells versus those that recruit immune cells. Carefully timed sequential targeting strategies that first activate inflammation followed by dampening of the signaling may be optimal.

In sum, we have shown that, to activate cell death, BEI-9 enhances the elimination of NF- $\kappa$ B-responsive pro-survival proteins, Bcl-xL and RIP kinase. The potential for compounds such as BEI-9 to switch NF- $\kappa$ B signaling from survival to apoptosis and to chemo-sensitize cancer cells needs to be investigated in pre-clinical models.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

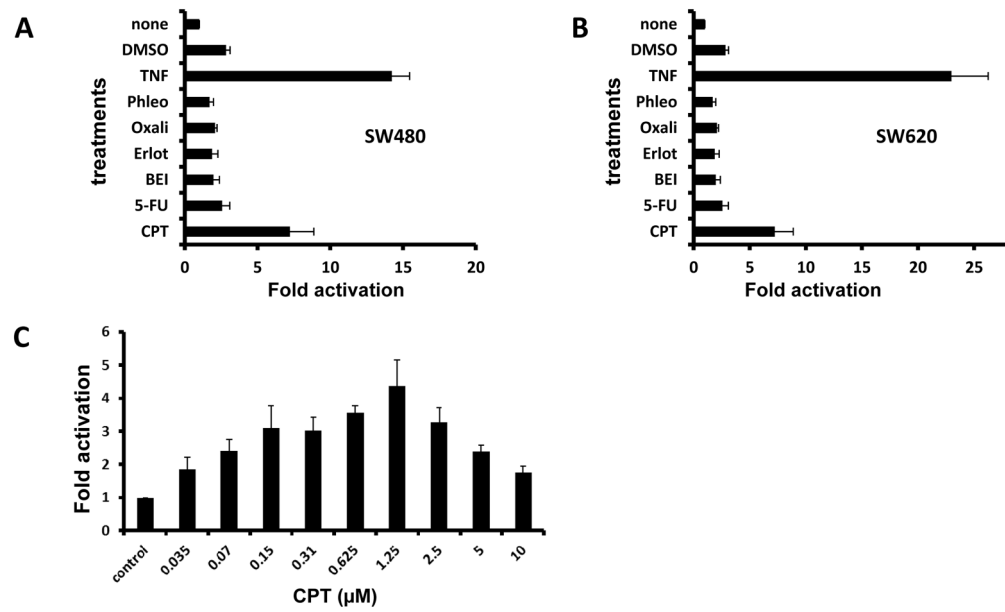
This study was supported by grants from the National Institutes of Health through National Institute of General Medical Sciences NIGMS grant #SC3109314, National Institute of Minority Health and Health Disparities NIMHD grant #U54MD008149, and RCMI Core Facility Grant #G12MD007585 to Tuskegee University. The reported content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH.

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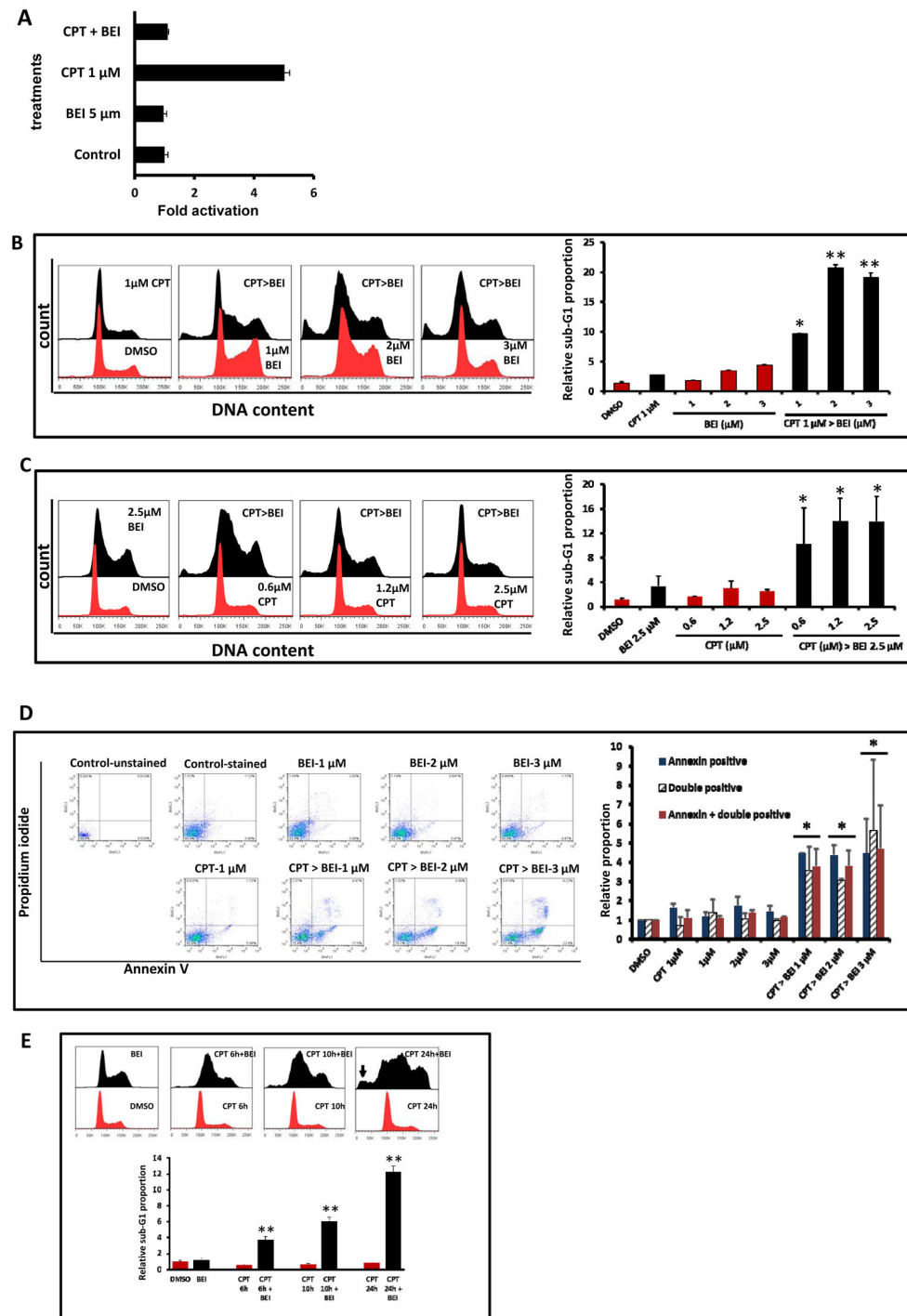
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**Fig. 1.**

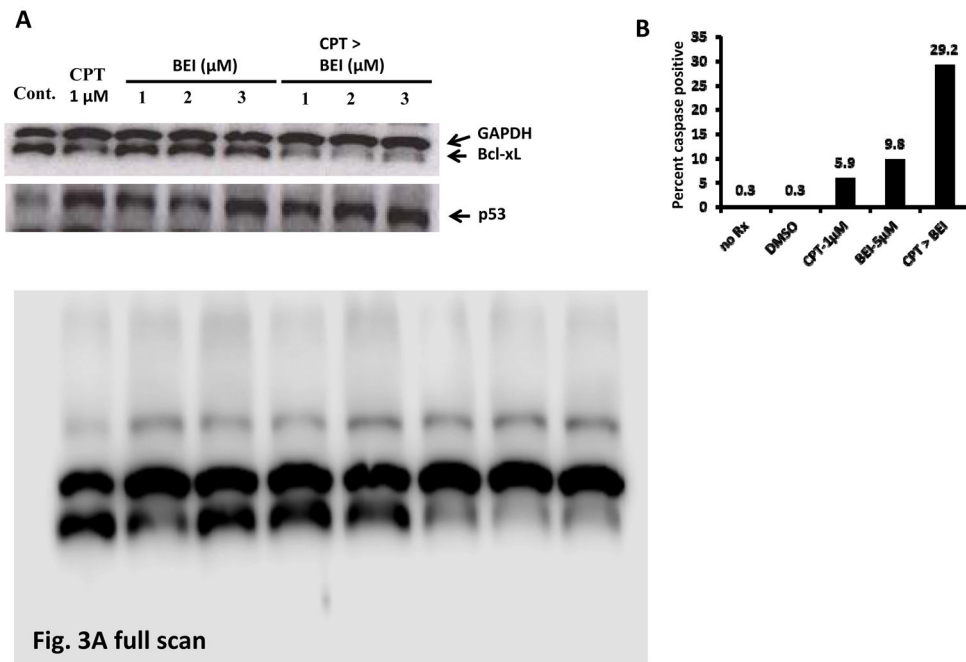
CPT and TNF $\alpha$  increase NF- $\kappa$ B activity in SW480 and SW620 cells. SW480 (A) or SW620 (B) reporter cells were treated as shown on the vertical axis, and NF- $\kappa$ B reporter activity was measured after 24 hr. Fold activation of the reporter activity relative to the untreated cells (none) is shown on the horizontal axis. C. The response of SW620 reporter cells to varying concentrations of the CPT is shown. The Y-axis shows the luminescence units registered for the treatment conditions.



**Fig. 2.** BEI prevents the NF- $\kappa$ B activation caused by CPT and enhances cell death. **A.** SW620 reporter cells were treated as shown with CPT, BEI, or simultaneously with CPT and BEI. NF- $\kappa$ B reporter activity was measured at 24 hr after treatment. **B–C.** Sequential treatment of SW620 cells with CPT for 24 hr followed by BEI (CPT>BEI) for another 24 hr increases the sub-G1 cell population in the cell cycle. In panel B, a fixed concentration of CPT (1  $\mu$ M)

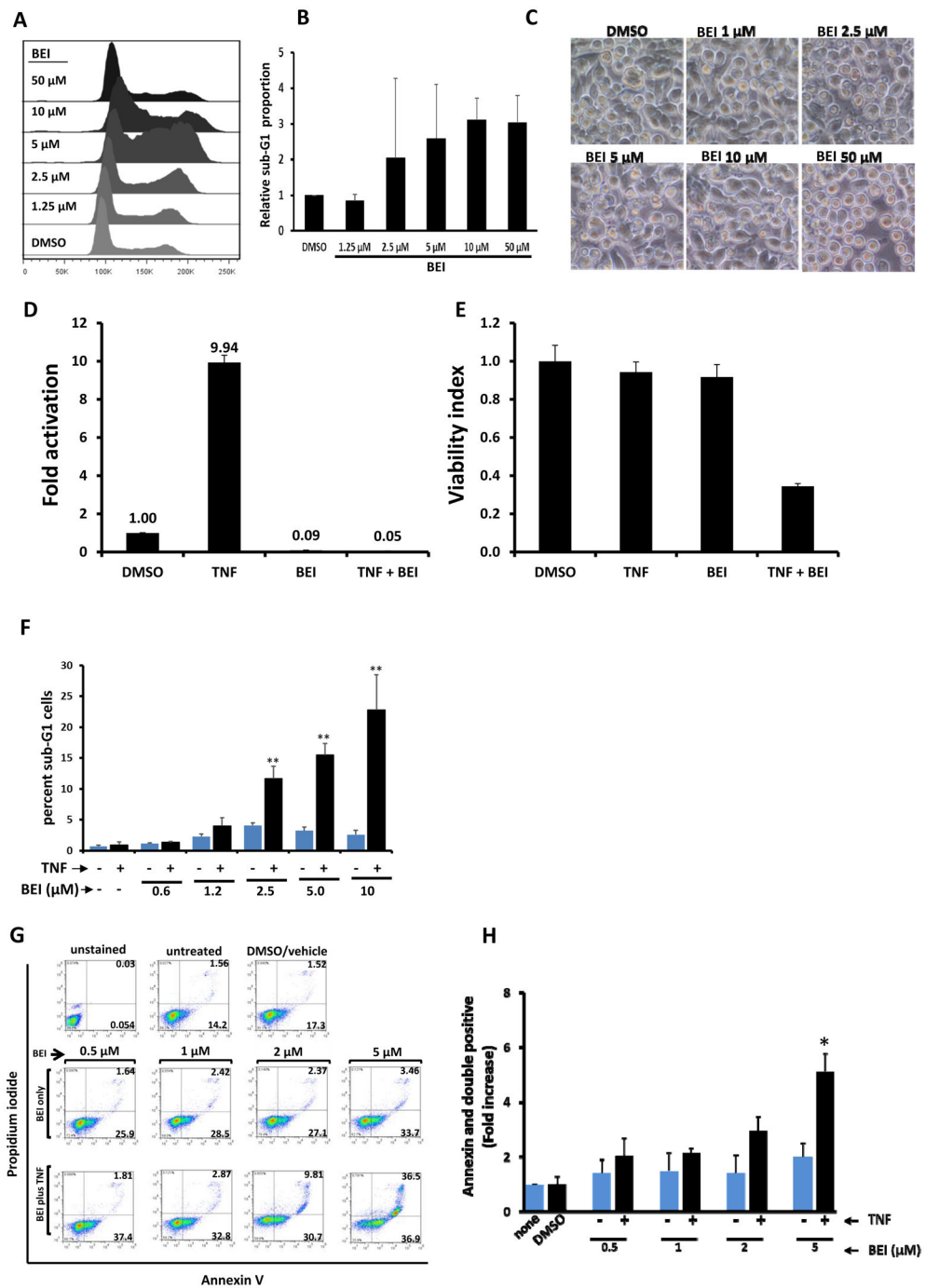


was combined with BEI (1, 3, or 5  $\mu\text{M}$ ); in panel C, a fixed concentration of BEI (2.5  $\mu\text{M}$ ) was combined with CPT (0.625, 1.25, or 2.5  $\mu\text{M}$ ). Bar graphs on the right side of the cell cycle histogram panels show the percentage of the sub-G1 population after each treatment. **D.** Dot plots showing Annexin V/PI staining of SW620 cells treated with BEI (1, 2, or 3  $\mu\text{M}$ ) or CPT (1  $\mu\text{M}$ ) or sequentially with CPT followed by BEI (CPT>BEI). The bar graph on the right shows the quantitation of Annexin-V-positive and Annexin V/PI-double-positive populations derived from the dot plots. **E.** SW620 cells were treated with DMSO (vehicle) or BEI (3  $\mu\text{M}$ ) for 24 hr (first panel) or with CPT for 6, 10, or 24 hr followed by 3  $\mu\text{M}$  BEI for 24 hr (panels 2–4). Cell cycle profiles were analyzed by flow cytometry. Arrow indicates sub-G1 population in CPT>BEI treated cells. Bar graphs show the relative sub-G1 populations in those different treatment groups, summarized from experiment in triplicates. \*\* = *T-test,  $p < 0.01$  (vs. CPT alone)*.



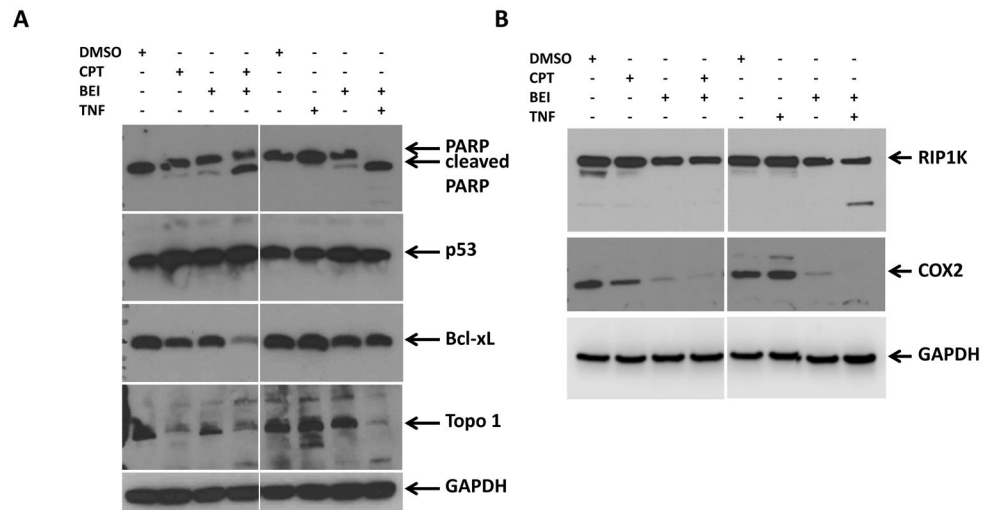
**Fig. 3.**

The CPT-BEI combination induces loss of Bcl-xL and caspase activation. **A.** SW620 cells were treated with CPT (1  $\mu$ M), BEI (1–3  $\mu$ M), or sequentially with CPT followed by BEI (CPT>BEI). The expressions of the NF- $\kappa$ B responsive gene, Bcl-xL, and of p53 were determined by immunoblotting. The results show that p53 levels were increased by the treatments and that Bcl-xL was down-regulated. **B.** SW620 cells seeded in 6-well plates were treated as shown on the X-axis. A FITC-VAD-FMK fluorescent caspase activation assay was performed, and the percentage of green fluorescent cells was determined from a total of >100 cells counted per well. no Rx = untreated control cells.



**Fig. 4.** In combination with TNF $\alpha$ , BEI antagonizes TNF $\alpha$ -induced NF- $\kappa$ B activity and induces cell death. **A–C.** BEI alone is a weak inducer of cell death in SW620 cells. Panel **A** shows the cell cycle profiles of SW620 cells treated with the indicated concentrations of BEI or with the vehicle, DMSO. Panel **B** shows the percentage of sub-G1 population in the cell cycle histograms, and panel **C** shows microphotographs of the treated and control cells. **D–E.** Combined with TNF $\alpha$ , BEI reduces cell viability. Reporter cells were treated with the

vehicle (DMSO), TNF $\alpha$  (25 ng/ml), BEI or a combination of TNF $\alpha$  and BEI. Following completion of the reporter assay (panel **D**), cell viability was determined by MTS assays. Panel **E** presents the results of the cell viability assay, showing loss of viability only in the TNF $\alpha$ +BEI treated cells. **F**. percent sub-G1 population of SW620 cells treated with TNF $\alpha$ , BEI (1, 2, 5, or 10  $\mu$ M), or in combinations as shown. \*\* = *T-test, p<0.01 (vs. BEI alone)*. **G**. Annexin-V/PI staining of SW620 cells treated for 16–24 hr with vehicle, BEI (0.5 – 5  $\mu$ M), or in combination as shown (representative of two independent experiments). A combination of TNF $\alpha$  with 5  $\mu$ M BEI shows the highest proportion of Annexin-V or Annexin-V/PI double-positive cells. **H**. Bar graph showing average and standard deviation of data from two independent experiments standardized to controls. \* = *T-test, p<0.05 (vs. BEI alone)*.



**Fig. 5.** PARP and RIP1K cleavage, and Bcl-xL and COX2 downregulation characterize the results of a combination of BEI with CPT or TNF $\alpha$ . **A.** SW620 cells were treated as shown with single agents or combinations. Immunoblotting results show that dual treatment induces PARP cleavage (an indicator of apoptosis) and a reduction of expression of the NF-kB response gene, Bcl-xL. Target engagements are noticeable by an increase in p53 levels and a decrease in TOPO1 levels. **B.** The expressions of additional NF-kB pathway target genes, RIP and COX2, were assessed by immunoblotting. Although RIP cleavage was specific for the TNF $\alpha$ -BEI combination, the expression of COX2 was essentially eliminated by BEI alone and by the combination of BEI and CPT or TNF $\alpha$ .