Abstract: Numerous dietary and pharmacological agents have been proposed as alternative strategies for treatment and prevention of colorectal cancer. Curcumin, an active ingredient of turmeric, that inhibits growth of malignant neoplasms, has a promising role in the prevention and treatment of colorectal cancer. EGF-R related protein (ERRP), a recently identified pan-erbB inhibitor, is a potential therapeutic agent for colorectal cancer. Here we examine whether curcumin together with ERRP will cause a greater inhibition of growth of colon cancer cells than either agent alone and the mechanisms of this inhibition. Human colon cancer HCT-116 or HT-29 cells were incubated with increasing doses of curcumin (up to 10 µM) or ERRP (up to 5 µg/ml), or a combination of both for 48 h. We observed that the cell growth inhibition and stimulation of apoptosis in response to the combinatorial treatment was significantly greater than that caused by either agent alone. These changes were associated with decreased activation (tyrosine phosphorylation) of EGFR, ErbB-2, ErbB-3, and/or IGF-1R. Whereas curcumin inhibited constitutive activation of both EGFR and IGF-1R, ERRP decreased activation of EGFR, ErbB-2, and ErbB-3 but had no effect on IGF-1R. Further, the combination therapy caused a greater attenuation of downstream effectors such as NF-κB, Akt and BAD activation, and down-regulation of procaspase-3 than that noted with either agent alone. The superior effects of the combinatorial treatment could partly be attributed to inhibition of constitutive activation of EGFRs and IGF-1R signaling pathways.

Introduction

Colorectal cancer is one of the leading causes of cancer deaths in both men and women in the United States with about 140,000 newly diagnosed cases per year. Despite recent advances in medicine, mortality remains unacceptably high. Therefore, there has been a surge of activity to develop alternative therapeutic strategies. Advances in molecular pathogenesis of cancer have aided in formulating strategies to develop targeted therapies. However, it is becoming increasingly clear that many solid tumors, including those in the colon, show up-regulation or constitutive activation of multiple signaling pathways, specifically EGFR and/or its family member(s) (referred to as EGFRs) and also IGF-1R, that promote growth, angiogenesis, and metastasis (1). Therefore, inhibition of these pathways with tolerable doses of multiple noncytotoxic agents represents a logical therapeutic approach for colorectal cancer.

Members of the receptor tyrosine kinase family that include EGFR, ErbB-2/HER-2, ErbB-3/HER-3, and ErbB-4/HER-4 (also referred to as EGFRs), are frequently implicated in experimental models of epithelial cell neoplasia as well as in human cancers (2,3). The vast number of patients with tumors expressing EGFRs provides a logical setting for the use of pan erbB signal transduction inhibitors. Indeed, a number of drugs that target either EGFR or HER-2 have been developed, but with limited success. This may partly be the result of the fact that most solid tumors, including those in the colon, express more than one EGFR, and coexpression of multiple EGFRs leads to an enhanced transforming potential and worsened prognosis (4–6). Therefore, inhibitors targeting multiple members of the EGFR family are likely to provide a therapeutic benefit to a broad range of patient population.

EGFR Related Protein (ERRP) was isolated recently and characterized as a pan-erbB inhibitor (7–13). Results from our laboratory also suggest that ERRP could be a potential therapeutic agent for colorectal and perhaps other epithelial cancers as well (8–13). The basis for this postulation comes from the observations that ERRP inhibits growth and attenuates basal- as well as ligand-induced activation of EGFR and HER-2 in different colon and other epithelial cancer cells that express varying levels of EGFRs (7,8). Comparison with cetuximab (MoAb to EGFR) or trastuzumab (MoAb to HER-2) has revealed that whereas cetuximab or trastuzumab inhibits the growth of colon and other epithelial cancer cells that express high levels of EGFR or HER-2, respectively, ERRP is effective on all epithelial cancer irrespective of the levels of EGFRs (8). We have also demonstrated that ERRP inhibits growth of xenografts of colon or pancreatic cancer...
cells in SCID mice without any sign of toxicity. However, it is becoming increasingly clear that the development and progression of many malignancies, including colorectal cancer, are associated with up-regulation or constitutive activation of multiple signaling pathways (14). This raises the possibility of whether combining the ERRP treatment with another non-toxic agent would provide a better therapeutic outcome.

Curcumin (diferuloylmethane), the major active ingredient of turmeric (Curcuma longa) with no discernable toxicity, has been shown to inhibit the growth of transformed cells (15,16) and colon carcinogenesis at the initiation, promotion, and progression stages of colorectal cancer in carcinogenic-induced rodent models (17–19). Curcumin has also been shown to prevent the development of adenomas in the intestinal tract of Min-/+ mice, a model of human familial adenomatous polyposis (20). In a Phase I clinical trial, curcumin has been shown to be effective in inhibiting the growth of tumors (21). Recent studies have linked the growth inhibitory effects of curcumin to inactivation of NF-κB (22).

The present investigation was undertaken to test the hypothesis that curcumin together with ERRP will produce a greater inhibition of growth of colon cancer cells in vitro than either agent alone. Furthermore, because members of the receptor tyrosine kinase family, particularly EGFRs and IGF-1R, are known to be involved in the development and progression of carcinogenesis, we also examined whether the inhibition of the growth of colon cancer cells in response to curcumin and/or ERRP could be attributed to attenuation of signaling by these receptors.

Materials and Methods

Chemicals

Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic/antimycotic were obtained from Gibco BRL (Bethesda, MD). Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant ERRP was immuno-affinity purified as detailed previously (9). Anti-caspase-3 and anti-Akt (Ser 473) as well as anti-phospho ErbB-2 (Tyr 1173) and anti-phospho-ErbB-3 (Tyr 1289) antibodies were obtained from Cell Signaling (Beverley, MA). Antibodies to phospho-EGFR (Tyr 1173) were obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies to α-tubulin were purchased from Oncogene (San Diego, CA).

Cell Lines and Cell Cultures

Human colon cancer HCT-116 and HT-29 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in tissue culture flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO2. Medium was supplemented with 10% FBS and 1% antibiotic/antimycotic. Medium was changed three times a week and cells were passaged using trypsin/EDTA.

Growth Inhibition Assay

Inhibition of cell growth in response to curcumin and/or recombinant ERRP was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (7,9). Briefly, cells were dispersed by trypsin-EDTA treatment and 2.5 × 10^4 cells/ml, resuspended in DMEM containing 10% of FBS and seeded into 96-well culture plates with six replicates. After 24 h of plating, the medium was replaced with that contained 2.5% of FBS, and the incubation was continued for another 48 h in absence (control) or presence of curcumin or ERRP or a combination of both as described in the figure captions. At the end of the 48 h incubation period, the reaction was terminated by adding 20 μl of 5 mg/ml stock of MTT to each well. The reaction was allowed to proceed for 3–4 h at 37°C. The culture medium was then removed. The formazan crystals were then dissolved by adding 0.1 ml of dimethyl sulfoxide (DMSO). The intensity of the color developed, which is the reflection of the number of live cells, and was measured at a wavelength of 570 nm. All values were compared to the corresponding controls. All assays were performed with six replicates.

Assessment of Apoptosis

Approximately 1 × 10^5 cells/well were plated in DMEM/10% FBS. After 24 h of plating the medium was changed to contain 2.5% FBS to minimize the contribution of serum-derived growth factors and subsequently treated the same way as described previously for growth inhibition study. At the end of the incubation period, the cells were lysed, and the levels of apoptosis were determined using the Cell Death Detection ELISA PLUS kit from Roche Diagnostics GmbH (Penzberg, Germany), which measures the cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes). The levels of apoptosis were further validated by measuring the relative abundance of activated form of caspase 3 by immunocytochemistry as described subsequently.

Immunocytochemistry

HCT-116 cells were grown in lab tek chamber slides (NUNC INC). Cells were treated with curcumin, ERRP, or a combination of both for 48 h. Supernatant was discarded and the cells were fixed in 10% neutral buffered formalin solution. Immunostaining was performed according to the standard procedure described in the ABC kit from vector Laboratories (Burlingame, CA).

Western Blot Analysis

Western blot analysis was performed essentially according to our standard protocol (7). Briefly, the cells were
solubilized in lysis buffer (7). Following clarification at 10,000 g for 15 min, the supernatant was used for Western blot analysis. In all analyses, protein concentration, determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), was standardized among the samples. Aliquots of cell lysates containing 50 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were transferred electrophoretically onto supported nitrocellulose membranes (Osmonics, Gloucester, MA). Membranes were incubated for 1 h at room temperature with blocking buffer, TBS-T (20 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Tween-20) and 5% nonfat dry milk with gentle agitation. After washing the membranes with TBS-T, they were incubated overnight at 4°C in TBS-T buffer containing 5% milk and with one of the following antibodies (1:1000 dilution): phospho-EGFR (Tyr^{1173}), phospho-ErbB-2/HER-2 (Tyr^{1248}), phospho-ErbB-3/HER-3 (Tyr^{1289}) or phosphorylated Akt (Ser^{473}), PARP, or caspase-3. The membranes were washed three times with TBS-T, and subsequently incubated with appropriate secondary antibodies (1:5000 dilution) in TBS-T containing 5% milk for 2 h at room temperature with gentle agitation. The membranes were washed again with TBS-T, and the protein bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham). The membranes containing the electrophoresed proteins were exposed to X-Omat film, and the signals were quantitated by densitometry using ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA). Membranes were stripped (2 × for 15 min at 55°C) in stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl pH 6.7. The membranes were then reprobed for the levels of total (non-phosphorylated) EGFR, Akt, or α-tubulin using corresponding antibodies. α-Tubulin was used as loading control. All Western blots were performed at least three times for each experiment.

**Gel Mobility Shift Assays**

Nuclear protein extracts were prepared from untreated and treated HCT-116 colon cancer cells according to procedures described previously (12). The oligonucleotides having NF-κB consensus sequences were purchased from Stratagene (La Jolla, CA). The oligonucleotides were annealed and labeled using T<sub>4</sub> polynucleotide kinase and [γ<sup>32</sup>P]ATP, followed by their purification on G-50 sephadex spin column. DNA-protein binding was measured by using gel electrophoretic mobility shift assays essentially as described before (12). Briefly, 10 μg of the nuclear protein extracts were incubated on ice for 15 min with 2 μg of polydeoxyinosinic-deoxyctydilic acid, 5 μg of bovine serum albumin and 1X binding buffer [10mM HEPES (pH 7.5), 1 mM β-mercaptoethanol, 10% glycerol (vol/vol), and 50 mM KCl]. After the addition of 32P-labelled probe DNAs (150 pg of probe/reaction), the mixture was incubated for another 15 min on ice. Excess unlabeled specific and nonspecific competitor DNAs were added 15 min before adding the labeled probe. The specific competitor DNA consisted of above unlabeled, annealed oligos for NF κB sequences, whereas the non-specific competitor DNA consisted of 48 bp size, XhoI plus XmaI cut, gel purified p21WAF1/CIP1 3′UTR fragment of plasmid pBSK-WAF clone 42.C.12 (12). The binding reactions were electrophoresed onto a 5% non-denaturing polyacrylamide gel (acylamide: bisacylamide, 30:0.8) containing 5% glycerol at 150 V in 1X low TAE buffer [1mM EDTA, 3.3 mM sodium acetate, and 6.7 mM Tris (pH 7.5)]. The gels were dried at 60°C, and DNA-protein binding was visualized by autoradiography.

**Statistical Analysis**

Unless otherwise stated, data are expressed as mean ± SEM. Where applicable, the results were analyzed using analysis of variance followed by Fischer’s protected least significant differences or Scheffe’s test. A P value of < 0.05 was designated as the level of significance.

**Results**

The primary objective of this investigation was to determine whether a combinational treatment of curcumin and ERRP would produce a greater inhibition of growth of colon cancer cells than either agent alone. Indeed, when colon cancer HCT-116 or HT-29 cells were incubated for 48 h with increasing doses of curcumin and ERRP, we observed that for each combinational treatment there was a greater induction of apoptosis or inhibition of cell growth than that caused by either agent alone, when compared with the controls (Figs. 1 and 2). This was evident when a suboptimal dose of ERRP (2.5 μg/ml) and curcumin (5 μM) was utilized. Together they produced 40% enhanced apoptosis when compared with 15–20% induction with either agent alone indicating an additive effect (Fig. 1). On the other hand, the additive effect was not observed when higher doses of curcumin and ERRP were utilized. Whereas 10 μM curcumin caused approximately 30% increase in apoptosis of HCT-116 cells, ERRP at a dose of 5 μg/ml produced a 70% induction in apoptosis, and in combination they stimulated apoptosis by about 85%, when compared with the untreated controls (Fig. 1A). The growth of HCT-116 cells, as determined by MTT assay, was also similarly affected by this treatment regimen (Fig. 1B). Similar changes in cell growth and apoptosis were also observed in HT-29 colon cancer cells in response to either curcumin or ERRP, each alone or in combination (Fig. 2). Taken together, the results suggest that the inhibition of cell growth and induction of apoptosis by the combinational treatment of curcumin and ERRP is not cell type specific, since similar effects were noted for both cell lines. All subsequent experiments were performed on HCT-116 cells using curcumin and ERRP doses of 5 μM and 2.5 μg/ml, respectively.

Earlier we reported that ERRP inhibits cell growth by attenuating the signal transduction pathways induced by EGFR.
and its family members, particularly ErbB-2 and ErbB-3 (8,9). Curcumin has also been shown to inhibit EGFR signaling (23). In addition to EGFR and/or its family members, receptors of IGFs, particularly IGF-1R, have been implicated in regulating growth of normal and neoplastic cells (24–27). Because the combinatorial treatment produced a greater effect on cell growth and apoptosis than that elicited by either agent alone, we measured the constitutive levels of the activated (tyrosine phosphorylation) form of EGFR and IGF-1R following treatments with curcumin, ERRP, or a combination of both. Interestingly, ERRP inhibited the constitutive levels of tyrosine phosphorylated form of EGFR, while curcumin by itself attenuated tyrosine phosphorylation of both EGFR and IGF-1R (Figs. 3A and 3B). Moreover, the magnitude of reduction of EGFR and IGF-1R in response to the combinatorial treatment was found to be considerably greater than that noted by either agent alone, when compared with the controls (Fig. 3A and 3B). This was accompanied by a concomitant reduction in the constitutive levels of EGFR (Fig. 3A) suggesting that decreased activation (phosphorylation) of EGFR in response to the combinatorial treatment could partly be the result of down-regulation of the receptor. Similar to EGFR, the combinatorial treatment of curcumin and ERRP also markedly inhibited constitutive activation of ErbB-2 and ErbB-3 in HCT-116 cells, when compared with the controls (Fig. 3C). However, the current dose of neither curcumin nor ERRP produced any significant change in constitutive activation of either ErbB-2 or ErbB-3, over the control (Fig. 3C).

Activation of EGFR and/or IGF-1R leads to stimulation of a number of downstream signaling pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK) kinase

---

**Figure 1.** Effects of curcumin or EGF-R related protein (ERRP) alone or in combination on A: apoptosis and B: cell growth of colon cancer HCT-116 cells. The cells were incubated for 48 h in the absence (control) or presence of indicated concentrations of curcumin and/or ERRP. Values represent mean ± SEM of 5–6 observations. *, P < 0.01, compared to the controls; +, P < 0.05, compared ERRP alone.
(MEK)/MAPK and phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathways that regulate growth related processes (28,29). However, although induction of MEK/MAPK signaling is shown to augment the proliferative potential, stimulation of PI3-K/Akt pathway has been linked to reduction in apoptosis leading to increase in cell survival (30,31). Therefore, to determine whether curcumin and/or ERRP-induced apoptosis of colon cancer cells could be attributed to attenuation of Akt signaling, we examined the levels of phosphorylated forms of Akt and Bad. It is generally accepted that the proapoptotic properties of Bad is enhanced with reduction in phosphorylation, whereas phosphorylation renders Bad inactive through sequestration by 14–3–3 family of proteins. As shown in Fig. 4, exposure to 5 µM curcumin produced only marginal reduction in the levels of phosphorylated forms of Akt and Bad in HCT-116 cells. ERRP (2.5 µg/ml), on the other hand, caused a significant reduction in the levels of phosphorylated forms of Akt and Bad, compared with the untreated controls. These changes were accompanied by parallel alterations in the levels of pro-caspase-3 (Fig. 4). To further substantiate our observation of stimulation of apoptosis by curcumin and/or ERRP in HCT-116 cells, we examined the relative abundance of cleaved (active) caspase 3 by immunocytochemistry. Although curcumin or ERRP caused a reduction in the number of cells, when compared to the controls, the intensity of staining as well as the number of activated caspase-3 immunoreactive cells within the high powered field was found to be considerably higher in cells exposed to the combinatorial therapy than those treated with either agent alone (Fig. 5).
Figure 3. Western-blot showing changes in the levels of total and tyrosine phosphorylated form of A: EGFR, ErbB-2, and ErbB-3; B: tyrosine phosphorylated form of IGF-1R; and C: ErbB-2 and ErbB-3 in HCT-116 cells in response to curcumim (5 µM), EGF-R related protein (ERRP; 5 µg/ml), or a combination of both. pEGFR, tyrosine phosphorylated EGFR; pIGF-1R, tyrosine phosphorylated IGF-1R; Y^{1248} ErbB-2, tyrosine phosphorylated ErbB-2; Y^{1289} ErbB-3, tyrosine phosphorylated ErbB-3.

Figure 4. Western-blot showing changes in the levels of phosphorylated forms of Akt (p-Akt) and BAD (pBAD) as well as the levels of procaspase-3 in HCT-116 cells response to curcumim (5 µM), EGF-R related protein (ERRP; 5 µg/ml), or a combination of both. α-Tubulin respresents loading control.
Akt mediated stimulation of cell survival is transduced, in part, by activation of NF-κB (28,30). However, both curcumin and ERRP have been shown to inactivate NF-κB in certain epithelial cancer cells (12,13, 22,32). To determine, therefore, whether attenuation of Akt activation by these agents will lead to reduction in NF-κB activity, we examined binding of NF-κB to its consensus sequence by EMSA. Initially, the specificity of NF-κB binding to its consensus sequence probe was confirmed by supershift assay using anti-p65 antibody (Fig. 6A). In addition, a competition experiment was performed in the 100-fold molar excess of unlabeled probe that revealed a significant reduction in binding of NF-κB (Fig. 6A). Together these observations confirm the validity and specificity of NF-κB binding to its consensus sequence. We then utilized nuclear proteins derived from HCT-116 cells that were treated with curcumin and/or ERRP. We observed that treatments with either curcumin or ERRP alone resulted in a 20–30% reduction in the DNA binding activity of NF-κB. The magnitude of this reduction, however, was greatly elevated (60–70%) with nuclear extracts from cells treated with a combination of curcumin and ERRP (Fig. 6A).

To further determine the regulatory mechanisms for curcumin and/or ERRP mediated inhibition of DNA binding activity of NF-κB in HCT-116 cells, we examined the levels of activated (phosphorylated) form of IKKα, an enzyme that phosphorylates the NF-κB inhibitory proteins, IκBs (33,34). The phosphorylated IκBs are degraded leading to translocation NF-κB to the nucleus for transcriptional control. We found that curcumin or ERRP alone caused a small reduction (10–20%) in the levels of phosphorylated IKKα, compared to the controls. On the other hand, the combination of ERRP and curcumin caused about 50% reduction in IKKα phosphorylation (Fig. 6B).

Discussion

Members of the receptor tyrosine kinase family are frequently implicated in experimental models of epithelial cell neoplasia as well as in human cancers (2,3). There is increasing evidence to support the concept that the malignant behavior of some tumors is sustained by deregulated activation of certain growth factor receptors. In many different malignancies, including colorectal cancer, the EGFR signaling pathways become hyperactivated or dysregulated by a range of mechanisms, including overproduction of ligands, overproduction of the receptor, and/or constitutive activation of the receptor.

It is becoming increasingly evident that blocking of EGFR signaling pathways is an effective therapeutic approach for treatment and prevention of many epithelial malignancies, including colorectal neoplasia. Indeed, a number of small molecule inhibitors such as gefitinib (Iressa) and erlotinib (Tarceva) as well as monoclonal antibodies to EGFR (cetuximab/Erbilux) and HER-2 (trastuzumab/Herceptin) have been developed and utilized for treatment of colorectal and other epithelial cancers but have met...
with only limited success (35). The latter could be due to the fact that growth of most solid tumors, including those in the colon, express more than one member of the EGFR family, and coexpression of multiple EGFR family members leads to an enhanced transforming potential and worsened prognosis (5,6). Therefore, identification of inhibitor(s) targeting multiple members of the EGFR family is likely to provide a therapeutic potential to a broad range of patient population. We have reported that ERRP, which we isolated and characterized as a pan-erbB inhibitor, targets multiple members of the EGFR family and that it could be a potential therapeutic agent for a wide variety of epithelial cancers, including colorectal cancer (7–13). This inference is supported by the results derived from in vitro studies utilizing colon and a variety of epithelial cancer cells as well as from investigation using SCID mice xenograft models of colon and pancreatic cancer cells (7–13). Our current observation that ERRP inhibits growth and stimulates apoptosis in both HCT-116 and HT-29 colon cancer cell lines that exhibit varying levels of EGFR and its family members further supports our contention that ERRP is a pan-erbB inhibitor (8).

However, it is becoming increasingly evident that in addition to EGFR and/or its family member(s), several other members of the receptor tyrosine kinases, specifically IGF-1R, may play a role in the development and progression in colorectal cancer (23–26). It has been demonstrated that IGF-1R is frequently overexpressed in colon cancer and that blockade of the IGF/IGF-1R axis by chemical inhibitors attenuates IGF-1-induced activation of Akt and also inhibits the growth of human colon cancer xenografts in mice (36,37). In view of these, it is important to devise a therapeutic strategy that will interfere with the signal transduction pathways induced by EGFR and/or its family member(s) as well as by IGF-1R.

Our current data, for the first time, demonstrate that curcumin, which has earlier been shown to inhibit EGFR signaling (22), also inhibits IGF-1R activation. Our observation of curcumin-induced inhibition of growth of colon cancer cells with a concomitant reduction of the constitutive activation of IGF-1R is similar to what has been reported for EGCG and N-acetyl-L-cysteine, both of which have been shown to inhibit the growth of colon cancer cells in vitro by attenuating IGF-1R (38,39).

The current observation that the combined treatment of colon cancer cells with curcumin and ERRP causes marked growth inhibition and apoptosis suggests this may be a more effective approach for inhibition of colon cancer cell growth than either agent alone. That the combined therapy is more effective could be attributed to inhibition of both EGFR and IGF-1R signaling pathways. The basis for this inference comes from the observation that whereas ERRP is effective against EGFR and its family member(s), curcumin inhibits activation of both EGFR and IGF-1R. Further support for this inference comes from the observation that downstream signaling events of EGFR and IGF-1R are also similarly affected by the combinatorial treatment. This is evidenced by a markedly greater reduction in the levels of phosphorylated (activated) form of Akt and Bad than that noted with either agent alone,

Figure 6. A: Electrophoretic mobility shift assay (EMSA) showing the DNA binding activity of NF-κB in nuclear fractions from untreated (control), curcumin-, EGF-R related protein (ERRP)-, or curcumin + ERRP-treated HCT-116 cells. Treatment time and doses were the same as in Fig. 4. Cold probe (×100, unlabeled double-stranded DNA probe; NF-κB antibody, anti-p65 antibodies. B: Western-blot showing changes in the levels of phosphorylated form of IKK-α (p IKK-α) in untreated (control) or treated HCT-116 cells with indicated agents. Dose and time of treatments were the same as in Fig. 4.
when compared with the controls. Non-phosphorylated Bad is known to promote apoptosis by binding to and neutralizing anti-apoptotic Bcl-2 proteins, whereas phosphorylated Bad is sequestered by 14–3–3 preventing Bad from blocking the anti-apoptotic action of Bcl-2 (40).

Activation of Akt has been shown to induce the expression of pro-survival genes, which is thought to be the result of cross-talk between Akt and NF-κB pathways (42). Akt is known to phosphorylate and activate IkB kinase (IKK) leading to degradation of IkB allowing NF-κB to translocate to the nucleus (33,34,40). Our current observation of a markedly greater reduction in the DNA binding activity of NF-κB in colon cancer cells HCT-116 cells in response to the combinatorial treatment than that noted with either agent alone suggests that NF-κB remains bound to IkB resulting in decreased activation of NF-κB. The marked reduction in DNA binding activity of NF-κB in response to the combinatorial treatment could partly be attributed to decreased activity of IkB kinase as evidenced by the reduction in the levels of phosphorylated form of IKK. These changes are thought to be responsible for the increased apoptosis or decreased survival of colon cancer cells in response to the combinatorial treatment.

In summary, our results demonstrate that exposure of colon cancer cells to curcumin together with ERRP causes a greater inhibition of growth than either agent alone. The effect of combinatorial treatment on inhibition of growth and stimulation of apoptosis could be attributed to inhibition of constitutive activation of EGFRs and IGF-1R signaling. Because neither curcumin nor ERRP are known to exert toxic effects, the current combinatorial therapy with curcumin and ERRP could potentially be a superior, nontoxic treatment strategy for colorectal cancer.

**Acknowledgments and Notes**

This work was supported by grants from the National Institutes of Health/National Institute on Aging R01 AG14343 (APNM), Susan G. Komen Foundation for Breast Cancer (AKR), and the Department of Veterans Affairs (VA Merit Review) to APNM and AKR. Address correspondence to A. P. N. Majumdar, John D. Dingell VA Medical Center, 4646 John R; Room: B-4238, Detroit, MI 48201. Phone: 313–576–4460. FAX: 313–576–1112. E-mail: a.majumdar@wayne.edu.

Submitted 8 March 2006; accepted in final form 11 May 2006.

**References**


