Supra-additive Growth Inhibition by a Celecoxib Analogue and Carboxyamido-triazole Is Primarily Mediated through Apoptosis

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Abstract

Combination studies of celecoxib and chemotherapeutic agents suggest that combining cyclooxygenase-2 inhibitors with other agents may have supra-additive or synergistic effects on tumor growth inhibition. Carboxyamido-triazole (CAI), a voltage-independent calcium channel inhibitor, has been shown to induce growth inhibition and apoptosis in cancer cells. We found that continuous exposure to cytostatic doses of CAI and LM-1685, a celecoxib analogue, reduced the proliferation and survival of seven human cancer cell lines by at least one log (P ≤ 0.001) over either agent alone. To explore the mechanism of action of this combination, we further studied the effects of LM-1685/CAI on CCL-250 colorectal cancer cells. We found that the supra-additive antiproliferative effects occurred throughout a range of LM-1685 doses (5-25 μmol/L) and paralleled a decrease in COX-2 activity as measured by prostaglandin E2 production. In these cells, treatment with LM-1685/CAI suppressed the extracellular signal-regulated kinase pathway within the first hour but ultimately results in high, sustained activation of ERK over a 9-day period (P = 0.0005). Suppression of cyclin D1 and phospho-AKT, and cleavage of caspase-3 and PARP were concomitant with persistent ERK activation. Addition of PD98059, a MEK-1 inhibitor, suppressed ERK activation and significantly but incompletely reversed these signaling events and apoptosis. Flow cytometry experiments revealed that the CAI/LM-1685 combination induced a 3-fold increase in apoptosis over control (P = 0.005) in 3 days. We show that the combination of CAI and LM-1685 produces a cytotoxic effect by suppressing proliferation and triggering apoptosis. (Cancer Res 2005; 65(9): 3853-60)

Introduction

Biological systems are complex networks of diverse and frequently multifunctional sets of signals that interact to drive or inhibit growth, apoptosis, and invasion. Elucidation of these systems has revealed pathway redundancy, synergy and adaptability (1, 2), signal amplification, diversity and cross-talk (3–6), and the existence of autocrine, paracrine, and other types of feedback loops (1, 7). Single pathway–targeted agents have been shown to be very effective in vitro and in animals but not in clinical trials (8, 9). This suggests that effective targeting of signaling pathways must come from multiple directions and impact multiple components. The use of a combination of biochemically based agents to target signaling has been the subject of recent research interest (10–12). The goal of biochemically based combination therapy is higher therapeutic success accompanied by less toxicity, and requires strategic selection of agents.

Cyclooxygenase-2 (COX-2) is an inducible enzyme that mediates the cleavage of arachidonic acid to prostaglandins, bioactive lipid molecules implicated in tumor cell survival and proliferation (13). Autocrine feedback systems have been identified in which prostaglandins induce COX-2 expression. Bradbury et al. (14) show that prostaglandin E2 (PGE2) binding to its receptors E-prostanoids 2 and 4 (EP2 and EP4) induces COX-2 expression by generating cyclic AMP (cAMP) signal leading to cAMP-responsive element binding protein and cAMP-responsive element activation of the COX-2 promoter. There is also evidence of other autocrine systems that involve receptor tyrosine kinase pathways such as the epidermal growth factor receptor (EGFR) system (15, 16). PGE2, a COX-2-derived prostaglandin, has been shown to transactivate EGFR through a mechanism involving release of endogenous ligands such as transforming growth factor-α (TGF-α) from the cell membrane (17). In turn, EGFR activation has been shown to induce COX-2 expression in a number of cell systems (15). Taken together, these observations suggest a relationship between COX-2, PGE2, and EGFR that may be autocrine in nature in some cell systems.

The EGFR system is a well characterized network of interrelated pathways, the deregulation of which has been associated with neoplastic growth. Ligand binding and EGFR phosphorylation activate a number of intracellular signal pathways, including the phosphatidylinositol-3-kinase/Akt axis and ERK1/2 pathways (16, 17). COX-2 inhibitors such as celecoxib have been shown to suppress these pathways and induce growth inhibition and apoptosis (18–21). Celecoxib is a widely marketed Food and Drug Administration–approved and generally well-tolerated drug, although recent studies suggest that long-term use of coxibs may be associated with an increased risk of cardiovascular events (22). One strategy to improve the balance of benefits and risks associated with use of coxibs is to identify combinations of drugs that are effective at doses lower than the effective doses of either agent alone.

Recent studies show that COX-2 inhibitors may also interfere with calcium signaling (23). Evidence indicates that COX-2 inhibitors perturb intracellular Ca2+ by blocking endoplasmic reticulum Ca2+-ATPases (24, 25). The consequence of inhibiting this Ca2+ reuptake mechanism is Ca2+ mobilization from the endoplasmic reticulum stores and extracellular calcium influx leading to cytosolic Ca2+ elevation (26). Sustained cytosolic Ca2+ elevation has been shown to impose adverse effects on the cell (27).
Mitochondrial uptake of excess cytosolic Ca\(^{2+}\) yields cytochrome c release and triggers apoptosis via caspase activation (27, 28).

Calcium signaling occupies a vital position in signal transduction systems of the cell by virtue of its participation in a wide range of physiologic functions. The role of calcium as a universal second messenger in cell cycle progression, control of cell proliferation, and onset of apoptosis is well recognized (26, 29–31). Calcium modulates many of the signaling cascades that drive the malignant phenotype, affecting many of the pathways influenced by COX-2-modulates many of the signaling cascades that drive the malignant messenger in cell cycle progression, control of cell proliferation, physiologic functions. The role of calcium as a universal second messenger is well recognized (26, 29–31). Calcium modulates many of the signaling cascades that drive the malignant phenotype, affecting many of the pathways influenced by COX-2.

We hypothesized that the combination of a COX-2 inhibitor and a calcium influx inhibitor at pharmacologically relevant doses could cooperate to produce a cytotoxic, antitumor effect. LM-1685 is a selective COX-2 inhibitor derived from the tricyclic sulfonyl series which includes the commercially available drug celecoxib (32). LM-1685 and carboxamido-triazole (CAI), a voltage-independent calcium channel inhibitor (33), were used to challenge the survival of a series of human cancer cell lines. The sum of our findings shows that the CAI/LM-1685 at cytostatic doses for each agent produces a cooperative anticancer effect that may be in part mediated by the persistent activation of ERK.

**Materials and Methods**

**Cell culture.** Cancer cell lines (CCL-250, SK-OV-3, HeLa, HT-1080, PANC-1, NCI-H23, and HCT-116) were obtained from American Type Cell Collection (Manassas, VA) and were grown according to the instructions provided with them.

**Antibodies and reagents.** Antibodies used yielded a single band on Western blot and were affinity-purified, rabbit polyclonal: EGFR, MEK1/2, phospho-MEK1/2, ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), AKT, phospho-AKT (Ser472, 473), and β-actin (Cell Signaling, Beverly, MA); COX-2 and cdc5 D1 (Upstate Biotechnology, Inc., Lake Placid, NY). The MEK1 inhibitor PD98059 was purchased from Cell Signaling. CAI was acquired from the National Cancer Institute Chemotherapeutics Repository. LM-1685 was obtained from Calbiochem (San Diego, CA). Epidermal growth factor (EGF) and forskolin were obtained from Schuell Bioscience, Inc. (Keene, NH). Mild Re-Blot Plus was purchased from Chemicon Int'l (Temecula, CA). I-Block and Tropix chemiluminescence system were purchased from Applied Biosystems (Foster City, CA). Catalyzed signal amplification reagents were obtained from Tropix (Bedford, MA). Protein extraction reagents and bicinchoninic acid protein assay kit were obtained from Pierce Biotechnology (Rockford, IL). FAST slides were obtained from Schleicher & Schuell Bioscience, Inc. (Keene, NH). Mild Re-Blot Plus was purchased from Chemicon Int'l (Temecula, CA). I-Block and Tropix chemiluminescence system were purchased from Applied Biosystems (Foster City, CA). Catalyzed signal amplification reagents were obtained from DakoCytomation (Carpinteria, CA). Prostaglandin E\(_2\) correlate-enzyme immunoassay kit was purchased from Assay Designs, Inc. (Ann Arbor, MI). Other reagents were acquired from Sigma (St. Louis, MO).

**Time course assays.** Cancer cells were grown to 80% confluency and serum-starved for 24 hours. Cells were treated with 100 ng/mL EGF, 10 μM PGE\(_2\), 10 μM PD98059 (MEK1 inhibitor), and/or drugs at selected doses (25 and 5 μM/L. LM-1685 and CAI, respectively) were grown in medium containing 100 ng/mL EGF. Cells grown in vehicle (0.05% DMSO) + EGF were used as a control. Time points were harvested with a protein extraction buffer (1:1 solution of tissue protein extraction reagent/2× SDS sample buffer with β-mercaptoethanol and protease inhibitor cocktail). Cell lysates were immediately transferred to a chilled tube.

**Reverse-phase protein arrays.** This method of proteomic analysis was described previously (34). Briefly, cell lysates were arrayed on nitrocellulose-coated FAST slides. Each sample was spotted in a serial 1:2 dilution curve with three replicates of each dilution. Extraction buffer alone was spotted as a negative control. Slides were prepared for signal development by incubating for 10 minutes in a 10% solution of Mill Re-Blot Plus followed by incubation in I-Block, a casein-based blocking solution, for at least 1 hour. Signal was developed using the catalyzed signal amplification system based on enzyme-mediated deposition of biotin-tyramide conjugates at the site of a biotinylated antibody-ligand complex. Arrays were analyzed with ImageQuant version 5.2 software (Molecular Dynamics, Amersham, United Kingdom). The spot intensity after background correction was proportional to the concentration of the target protein (34). Protein loading was normalized for phospho- and nonphospho-proteins with the total self-protein and β-actin, respectively (phospho-X / total X or X / actin).

**Proliferation assay.** Growth inhibition of cancer cells by CAI and LM-1685 was measured by crystal violet nuclear stain as previously described (33). Briefly, cells were grown in medium containing 100 ng/mL EGF and either vehicle (0.05% DMSO) or inhibitors(s) in DMSO and stained with crystal violet dye (0.5% crystal violet in 20% methanol) at selected time points. Bound dye was eluted from cells and quantified spectrophotometrically.

**Prostaglandin E\(_2\) measurement.** PGE\(_2\) was measured in conditioned culture medium using an enzyme immunoassay system (Assay Designs). Assays were done according to the manufacturer’s instructions. Results are expressed as picograms of PGE\(_2\) per milliliter of a 2 \(×\) 10\(^5\) cell culture supernatant and represent the mean ± SD of duplicate determinations.

**Flow cytometry.** Cell cycle progression and apoptosis were determined by flow cytometric analysis as previously described (35). Briefly, CCL-250 cells were treated for 3 days with EGF plus inhibitor alone, the CAI/LM-1685 combination, or the combination with PD98059. Cells treated with vehicle (0.5% DMSO) plus EGF were used as a control. Cells were trypsinized, washed, and fixed with cold ethanol (70% v/v). Immediately prior to analysis, the cells were centrifuged at 2,000 \(×\) g at 4 °C and resuspended in PBS. Cells were analyzed for cell cycle distribution and DNA content by the Beckton FACStar flow cytometer after the addition of 0.05% propidium iodide. Quantitative analysis of apoptotic cells was done by analyzing FACStar data with ModFit software.

**Micro-Western blotting.** Lysates to be analyzed by micro-Western blot (36) were evaluated for protein concentration with the bicinchoninic acid protein assay kit to normalize protein loading. Signal was developed using Tropix chemiluminescence system.

**Statistical analysis.** Student’s \(t\) test was used to determine the statistical difference between various experimental and control groups. A \(P\) value of ≤ 0.05 was considered significant.

**Results**

**Prostaglandin E\(_2\) transactivates epidermal growth factor receptor and induces cyclooxygenase-2 expression.** The ability of PGE\(_2\) to activate EGFR in CCL-250 cells was determined, and the mechanism through which this occurs was examined. After verifying antibody specificity by Western blot (Fig. 1A), we assessed the activation of EGFR in samples treated with EGF, PGE\(_2\), or PGE\(_2\) after pretreatment with anti-TGF-α antibody. Figure 1B and C show images of reverse-phase arrays and results of spot analysis of the same arrays. Our results showed that PGE\(_2\) activated EGFR through TGF-α in a statistically significant fashion. This transactivation significantly increased COX-2 expression in these cells (Fig. 1D). The known role of COX-2 in stimulating production of PGE\(_2\), and the downstream expression of COX-2 after EGFR activation by PGE\(_2\) in these cells, implicates COX-2 as a component of an EGFR-PGE\(_2\) feedback loop. With these data, we identified COX-2 as a target for abrogating EGFR signaling.

**Effects of epidermal growth factor, prostaglandin E\(_2\), and inhibitors on the epidermal growth factor receptor pathway.** We examined the downstream effects of EGFR activation. Figure 2 illustrates the sequence, duration, and intensity of the signal generated by the activated pathway components after EGF stimulation. Signal intensity of phospho-EGFR reached maximum...
We assessed the dose response of LM-1685 alone or in combination with different biological outcomes. Growth factor stimulation coincides with an acute, transient activation of ERK, whereas treatment with the CAI/LM-1685 combination is concomitant with marked, persistent ERK activation that is partially attenuated by the addition of PD98059. These findings show that the strength and duration of ERK activation varies with different stimuli and may be associated with different biological outcomes.

LM-1685 and carboxamido-triazole supra-additively inhibit growth of cultured cancer cells expressing cyclooxygenase-2 protein. We assessed the dose response of LM-1685 alone or in combination with a constant dose of CAI in CCL-250 colorectal carcinoma cells. The dose chosen for CAI was 5 μmol/L, a physiologically attainable dose within the range measured in patient serum in a phase I clinical trial (37). Figure 3A shows that the maximum difference of net cell quantity in CCL-250 cells treated with the combination versus LM-1685 occurs at 25 μmol/L. A cooperative effect is also achieved at lower doses. A parallel decrease in PGE2 production by cells treated with the CAI/LM-1685 combination is seen throughout the same range of LM-1685 doses. The 25 μmol/L LM-1685 dose and the 5 μmol/L CAI dose were chosen for further study in order to maximize the cellular effects of the drug combination.

Six additional cell lines, HeLa cervical adenocarcinoma, NCI-H23 non–small cell lung cancer, SKOV-3 ovarian carcinoma, PANC-1 pancreatic carcinoma, HT-1080 fibrosarcoma, and HCT-116 colorectal carcinoma, had similar growth responses to the CAI/LM-1685 combination. The combination decreased net cell proliferation by at least one log in all cell lines over a 10-day treatment period (P < 0.0001; Fig. 3B). HCT-116 cells showed no significant inhibition by LM-1685 alone but still showed supra-additive sensitivity to the CAI/LM-1685 combination. We then tested the cell line panel for expression of COX-2 ± 100 ng/mL EGF for 72 hours (Fig. 3C). All seven cell lines expressed COX-2 protein in the presence of EGF to varying degrees. To further investigate our observation that treatment with the CAI/LM-1685 combination induces high, sustained ERK activation, we looked at the effect of addition of PD98059 to the combination in CCL-250 cells to determine if the antiproliferative response to the CAI/LM-1685 combination could be decreased by suppression of the MEK-ERK pathway (Fig. 3D). No significant effect on net growth of the CCL-250 cells was observed upon addition of PD98059 to the combination.
Cell cycle and protein expression changes induced by carboxamido-triazole/LM-1685 combination. We examined cell cycle and protein expression changes induced by CAI/LM-1685 and/or PD98059 by flow cytometry and reversed phase protein array. Induction of apoptosis was shown by the appearance of a sub-G1 peak and quantitatively measured by flow cytometry. Fluorescence histograms of cells synchronized by serum starvation, treated for 3 days with inhibitors, and stained with propidium iodide showed a 3-fold increase in apoptotic cells with exposure to CAI/LM-1685 (P = 0.0001) and >2-fold increase in apoptotic cells with exposure to CAI/LM-1685 / PD98059 (P = 0.003) over untreated cells. Cells treated with a single inhibitor showed a small, nonsignificant increase in apoptotic cells. These data show that the CAI/LM-1685 combination induces cytotoxicity at doses that are predominantly cytostatic for either agent alone. This effect is significantly attenuated by the addition of PD98059 (P = 0.023). The seemingly discordant results in which addition of PD98059 does not significantly increase net cell proliferation inhibition (Fig. 3D) but inhibits apoptosis (Fig. 4F) suggests that an increase in cell death is most likely to be the major cause of the differential response to cells treated with the combination versus either agent alone.

Carboxamido-triazole/LM-1685 induces cell signaling changes mediating cell cycle arrest and apoptosis. We assessed protein expression changes to investigate the mechanism for the antiproliferative and apoptotic effects induced by CAI/LM-1685. Consistent with the proapoptotic activity observed by flow cytometry, the CAI/LM-1685 combination induced caspase 3 and PARP cleavage, hallmarks of apoptosis and DNA damage, respectively. Addition of PD98059 again significantly attenuated the effect (4A). Suppression of the pro-survival protein phospho-AKT and the cell cycle regulator protein cyclin D1 was shown in cells treated with the CAI/LM-1685 combination; the effect was significantly reversed by the addition of PD98059 (4B). These results, taken together with the marked but incomplete reversal of ERK activation and attenuation of apoptosis by PD98059, support the involvement of ERK in mediating apoptosis and may be necessary but not sufficient for full activity.

Discussion
An advantage to using multiple agents may be that the cooperative or synergistic nature of a given combination could produce increased therapeutic efficacy at lower doses than could be achieved by either agent alone, reducing the potential for
toxicity. CAI and selective COX-2 inhibitors are generally well-tolerated, orally available agents reported to have minimal toxicity in a majority of human subjects at therapeutic doses when given as a single agent. These qualities suggest that the combination of CAI and a selective COX-2 inhibitor may be investigated for chemotherapeutic applications, where perhaps much lower levels of each could be employed, while maintaining the desired therapeutic benefit. At cytostatic doses, the CAI/LM-1685 combination cooperatively induced cell cycle arrest and apoptosis in cancer cells by creating a combined blockade of mitogenic and antiapoptotic signaling. Within 3 days, the combination induced a 3-fold decrease in net cell proliferation in CCL-250 cells over control cells. In the same time period, apoptosis increased 3-fold in these cells as measured by flow cytometry. By day 10, all seven cell lines in our study showed at least a 10-fold decrease in net cell proliferation in response to the CAI/LM-1685 combination.

Because of the complexity of cell signaling systems, the effects of targeted agents can be unpredictable. The mitogenic MEK-ERK cascade is one of the primary pathways activated by EGFR phosphorylation and was found to be activated in cancer cells (38–41). We thus anticipated that the CAI/LM-1685 combination would suppress the MEK-ERK pathway. Paradoxically, our experiments showed that the CAI/LM-1685 combination, rather than inhibiting the MEK-ERK pathway, resulted in sustained activation of ERK in this experimental system. This prolonged activation of the MEK-ERK pathway was concomitant with antiproliferative and apoptotic events. In our study, suppression of phospho-AKT and cyclin D1 coincided with sustained ERK activity. Inactivation of AKT has been shown to block the stimulation of the antiapoptotic Bcl-2 gene promoter function (42). Cyclin D1 plays a key role in cell cycle regulation by serving as a checkpoint at the G0/G1 transition phase (43); down-regulation of cyclin D1 expression has been shown to induce cell cycle arrest and apoptosis in cancer cells (44). In addition, sustained ERK activation was associated with increased cleavage of caspase-3 and PARP, events which are indicators of caspase-dependent apoptosis activation and DNA damage, respectively (45). Addition of the selective MEK inhibitor, PD98059,

Figure 3. LM-1685 and CAI supra-additively inhibit the growth of cancer cells expressing COX-2 protein. For all experiments (except top Western blot in C), cells were grown in serum-free media + 100 ng/mL EGF. Cells treated with EGF + 0.05% DMSO served as a vehicle control. A, CCL-250 cells were treated with doses of LM-1685 ranging from 0 to 100 μmol/L, ± 5 μmol/L CAI, for 10 days. On day 11, cells were stained and bound dye was eluted and measured on a spectrophotometer at 540 nm. Points, mean; bars, ± SE. Inset, PGE2 production by CCL-250 cells is measured by enzyme immunoassay (see Materials and Methods) after treatment for 72 hours with the CAI/LM-1685 combination (5 μmol/L CAI + 5-50 μmol/L LM-1685). Treatment with vehicle alone (no CAI) served as a control. Columns, mean; bars, ± SE; P values compare the PGE2 levels after treatment with the lowest dose of the CAI/LM-1685 combination compared with control cells. B, seven cancer cell lines were treated for 10 days with CAI, LM-1685, or the combination. On day 11, cells were stained and bound dye was eluted and measured on a spectrophotometer at 540 nm. Columns, mean; bars, ± SE; P values compare the combination with either drug alone. C, cells treated with ± 100 ng/mL EGF for 72 hours were harvested and lysed. Samples of total cellular protein (20 μg) were subjected to Western blot analysis using anti-COX-2. Results are representative of three separate experiments. D, dye from stained wells containing CCL-250 cells treated with CAI, LM-1685, PD98059, or a combination of inhibitor wells was eluted and measured on a spectrophotometer at 540 nm. Columns, mean; bars, ± SE.
reduced ERK activation and partially reversed these signaling events, although no significant reversal of growth inhibition was seen. This suggests a possible role for ERK in cell cycle arrest and apoptosis in our experimental system, and that the predominant mechanism for the anticancer effects of the combination is through apoptosis rather than growth inhibition.

Kinetic studies have shown that the strength and duration of ERK signaling regulate cell fate in different cell types (46). This phenomenon has been documented in other studies, and several mechanisms have been described to explain how subtle differences in signaling kinetics can be translated into vastly different biological outcomes, including mitogenesis, transformation, cell cycle arrest, and apoptosis (46–51). Transient ERK activation has been shown by us and others to result from growth factor stimulation; in contrast, high, sustained ERK activation is thought to be a consequence of phosphatase inhibition or calcium perturbation (47). Moreover, it has been previously shown that agents that induce antiproliferative activity, such as IFN-α, require ERK phosphorylation for full biological effect (52). Studies suggest that in some cases, drug-induced sustained ERK activity may modulate biological outcome, whereas in other instances, it does not (53–55). Elder et al. showed that colorectal cancer cells treated with a COX-2 inhibitor showed an increase in apoptosis that was preceded and accompanied by activation of ERK, and that a selective MEK inhibitor dose-dependently protected these cells from the apoptotic effects of the inhibitor (56). This report and our current study showing partial reversal of antiproliferative and apoptotic activity by a MEK1 inhibitor point to the involvement of MEK/ERK signaling in mediating the proapoptotic effects of COX-2-selective inhibitors in some cell systems. Downstream effectors of ERK that potentially mediate its proapoptotic effect after activation by COX-2 inhibitors include the nuclear factor-κB signaling pathway (57, 58) and the proapoptotic protein Par-4 (59, 60).

In our experimental system, the addition of a MEK1 inhibitor neither fully abrogated ERK activity nor completely reversed the antiproliferative and apoptotic effects of the combination.
Therefore, whereas ERK activation seems to be at least partially mediating cell cycle arrest and apoptosis, other parallel processes must be occurring which tip the balance toward cell death. It is possible that treatment of cells with the CAI/LM-1685 combination may influence multiple apoptosis-related pathways. In addition to mediation by ERK, two additional mechanisms that may be involved in apoptosis may be (a) an additive perturbation of Ca2+ homeostasis by CAI and LM-1685, placing prolonged stress on the endoplasmic reticulum and culminating in cytochrome c release from the mitochondria, and (b) an additive accumulation of toxic levels of arachidonic acid by prevention its of calcium-induced cleavage by CAI (33) and COX-2 inhibition (13, 61).

Combinatorial therapy becomes the new focus for chemotherapeutic and molecularly targeted drug development because drugs which, at safe doses, are ineffective alone may prove to have supra-additive or synergistic anticancer effects in combination. Identification of promising candidates for combinatorial therapeutics requires strategic selection. Celecoxib has been found to have antitumor activity in tumor cells and tissues that have little or no COX-2 enzyme (62). It has been suggested that COX-2 inhibitors exert their chemopreventive and therapeutic activity through mechanisms that are independent of their COX-2 inhibitory activity (62). Proposed non-COX cellular targets are AKT, IκB, and kinase β, the peroxisome proliferator-activated receptor family of nuclear hormone receptors, and the proapoptotic gene Bax (21, 63, 64). In our study, although some cell lines showed little or no constitutive COX-2 protein expression, all cell lines showed some degree of EGF-inducible COX-2 protein expression. HCT-116 cells have been shown to have a hyper-methylated COX-2 promoter (65). Although methylated genes are thought to be “silenced”, about one-half of tumors with methylated COX-2 promoters still express COX-2 protein. Therefore, whereas COX-2 inhibitors may have COX-independent effects, COX-2 inhibition remains a potential mechanism through which COX-2 inhibitors such as celecoxib exert their anticancer effects.

In summary, we report a number of new findings on the cellular effects of a novel drug combination of a calcium inhibulin and a COX-2 inhibitor. Calcium and COX-2 signaling intersect and diverge from the complex network of EGFR pathways (24, 66–68) and may provide effective targets for disrupting a multitude of cancer-related signaling pathways. The agents used in our study to target these pathways had profound anticancer effects, and the implication by our study that two agents used at cytostatic doses can produce cytotoxic effects is compelling. Although further studies are required to clarify the mechanism of action of this combination, this study provides an experimental basis for preclinical studies designed to determine whether a COX-2 inhibitor combined with a calcium inhibulin could be useful in the treatment or chemoprevention of cancer.

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