Targeting Cyclooxygenase-2 and the Epidermal Growth Factor Receptor for the Prevention and Treatment of Intestinal Cancer

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Abstract
Clinical and animal studies indicate a role for cyclooxygenase-2 (COX-2) and the epidermal growth factor receptor (EGFR) in the development and progression of intestinal polyps and cancers. Although this combination of enzyme inhibition has shown synergy in intestinal polyp and tumor models, the exact mechanism for these effects remains undefined. Therefore, we sought to define the molecular mechanisms through which this process occurs. We observed a significant reduction in the number and size of small intestinal polyps in APC min mice treated with either celecoxib (a selective COX-2 inhibitor) or erlotinib (Tarceva, an EGFR inhibitor). However, in combination, there was an overall prevention in the formation of polyps by over 96%. Furthermore, we observed a 70% reduction of colorectal xenograft tumors in mice treated with the combination and microarray analysis revealed genes involved in cell cycle progression were negatively regulated. Although we did not observe significant changes in mRNAs of genes with known apoptotic function, there was a significant increase of apoptosis in tumors from animals treated with the combination. The inhibition of EGFR also induced the downregulation of COX-2 and further inhibited prostaglandin E2 formation. We observed similar effects on the prevention of intestinal adenomas and reduction of xenograft tumor volume when nonselective COX inhibitors were used in combination with erlotinib. Together, these findings suggest that the inhibition of both COX-2 and EGFR may provide a better therapeutic strategy than either single agent through a combination of decreased cellular proliferation and prostaglandin signaling as well as increased apoptosis. [Cancer Res 2007;67(19):9380–8]

Introduction
Colorectal cancer is the third most prevalent cancer in the world and most of the disease is thought to arise from the progression of intestinal adenomas (or polyps). Due to the large number of cases worldwide, the need for effective prevention is apparent, as the cost of worldwide endoscopic screening of every human over the age of 50 is prohibitive. Since the discovery that prostaglandin G/H synthase-2 or cyclooxygenase-2 (COX-2) is up-regulated in intestinal adenomas and cancers, there has been enormous interest in the use of selective COX-2 inhibitors as chemopreventive agents (1). Prostaglandin G/H synthases, often referred to as COX-1 and COX-2, are responsible for the biosynthesis of prostaglandins. COX-1 is constitutively expressed in most tissues, whereas COX-2 is an inducible form that is expressed in sites of inflammation as well as a wide array of cancers. Two large clinical trials that evaluated the use of celecoxib (a selective COX-2 inhibitor) in the prevention of colorectal polyp recurrence were recently completed (2, 3). Both trials provide significant evidence that the use of celecoxib is an effective agent for the prevention of colorectal adenoma recurrence. Likewise, the use of nonsteroidal anti-inflammatory drugs (NSAID) has been shown to inhibit colorectal tumor growth in patients with familial adenomatous polyposis (FAP; ref. 4). FAP patients have mutations in the APC gene, which leads to the formation of intestinal polyps. APC min mice serve as an animal model of FAP and will develop intestinal polyps by 6 weeks of age. Many reports have shown that NSAID use can inhibit the formation of adenomas in these mice (5). However, significant cardiovascular side effects are associated with the long-term use of selective COX-2 inhibitors (6). This highlights the need to either change current dosing/scheduling or investigate other options, including combinations with other agents.

Another important signaling pathway, which plays a role in colorectal cancer, is that mediated via the epidermal growth factor receptor (EGFR). This tyrosine kinase is one of four members of the HER/ErbB growth factor receptor family. Interestingly, increased EGFR activity has been associated with adenomas that occur in APC min mice (7) and disruption of EGFR signaling through either kinase inhibition or genetic mutation inhibits polyp formation as well as the growth of established tumors (8). In light of the cardiovascular effects associated with long-term COX-2 use and the recent appreciation for the role of EGFR signaling in colorectal cancer, the inhibition of both these pathways may provide a beneficial therapeutic window. Indeed, the use of COX-2 and EGFR inhibitors in combination has been observed in animal models of colorectal cancer (9–11). Moreover, a phase I clinical trial was recently completed, which evaluated the optimal biological dose of celecoxib in combination with erlotinib (an EGFR tyrosine kinase small-molecule inhibitor) in patients with advanced non–small cell lung cancer (12).

Because both COX-2 and EGFR have been implicated in the progression of colorectal cancer and cross-talk between these signaling pathways has been reported (13), we sought to determine what effect celecoxib and erlotinib would have on intestinal adenomas. Furthermore, we observed that this combination effectively reduced the growth of colorectal tumors xenografted in mice. Evaluation of these models reveals the mechanism behind the inhibitory effects observed and emphasizes the importance of combination therapy.

Materials and Methods

Materials. Erlotinib (Tarceva) was kindly provided by Genentech, Inc. and celecoxib was a kind gift from Pfizer. Antibodies to COX-1 and COX-2...
were from Cayman Chemical. Antibodies to β-actin and iibuprofen were from Sigma. All other antibodies were from Santa Cruz Biotechnology.

**Cell culture.** HCA-7 cells were a kind gift from Dr. Susan Kirkland (Imperial College, London, United Kingdom). These cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a 5% CO2 atmosphere. Western blot analysis. Equal amounts of samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in TTBS (TBS with 0.1% Tween 20) containing 5% dry milk. Primary antibody incubations were done in TTBS with 5% dry milk overnight at 4°C. After washing, the membranes were incubated with the appropriate secondary peroxidase-conjugated antibody for 1 h in TTBS with 5% dry milk. Immunoreactive proteins were visualized using the enhanced chemiluminescence system from Amersham Pharmacia.

**Intestinal polyp formation.** C57BL/6j-Apc<sup>min/+</sup> mice were obtained from The Jackson Laboratory and housed in the Vanderbilt Animal Care Facility according to NIH and institutional guidelines for laboratory animals. An Institutional Animal Care and Use Committee approved all animal protocols. Erlotinib was given at 50 mg/kg (q.d, p.o.) in methylcellulose (vehicle). Celecoxib was given in the diet (ad libitum) at 1,000 mg/kg. After treatment as indicated, the mice were sacrificed by CO2 asphyxiation, and the intestine was dissected, washed in PBS, and immediately fixed in 10% neutral buffered formalin overnight at room temperature.

**Xenograft tumor growth.** Xenograft experiments were done essentially as described previously (14). HCA-7 cells were trypsinized, resuspended in sterile PBS, and then pelleted by brief centrifugation at 200 × g. The cell pellet was resuspended in sterile PBS and counted using a hemocytometer. Cells were resuspended in PBS to a final concentration of 5 × 10<sup>4</sup>/mL, and 100 µL of cell suspension were injected s.c. into the dorsal flank of athymic nu/nu mice (Harlan Sprague Dawley) using a 27-gauge needle. Mice were housed in the Vanderbilt Animal Care Facility according to NIH and institutional guidelines for laboratory animals. An Institutional Animal Care and Use Committee approved all animal protocols. Tumor volume was determined with digital calipers and calculated according to the equation \( V = (L \times W^2) / 2 \), where \( V \) is the volume, \( L \) is the length, and \( W \) is the width. After tumors were established, mice were randomly placed into treatment groups. Erlotinib was given at 50 mg/kg (q.d, p.o.) in methylcellulose (vehicle). Celecoxib was given in the diet (ad libitum) at 1,000 mg/kg. Tumors were measured twice weekly for the duration of the study.

**Measurement of prostaglandins and urinary prostaglandin E2 metabolite.** Prostaglandin profiles from tumor tissues and intestinal polyps were measured and quantified using a gas chromatography/negative ion chemical ionization mass spectrometric assay as reported previously (15). Urinary prostaglandin E2 (PGE2) metabolite (PGEM) was quantitated as described previously (16).

**Analysis of gene expression by microarray.** Total RNA was isolated from HCA-7 xenograft tissue using the RNeasy Mini kit and RNase-Free DNase kit from Qiagen. RNA was submitted to the Vanderbilt Microarray Shared Resource Core facility for microarray analysis. Microarray analysis was done on Affymetrix U133 PLUS 2.0 arrays. Data were analyzed using GeneSpring software (Stratagene).

**Results**

**Effect of COX-2 and EGFR inhibition on small intestinal polyps.** To evaluate the role of COX-2 and EGFR on the development and progression of intestinal polyps, we used a well-established mouse model of intestinal polyp formation. APC<sup>min/+</sup> mice have a heterozygous mutation of the APC gene, which results in the formation of intestinal polyps beginning at 6 to 7 weeks old. APC<sup>min/-</sup> mice serve as a useful animal model for a human disease known as FAP. These patients harbor mutations in the APC gene and will develop numerous intestinal polyps by early adulthood that eventually progress into intestinal cancers. We treated APC<sup>min/-</sup> mice with celecoxib (a selective COX-2 inhibitor, ad libitum in diet) and/or erlotinib (EGFR inhibitor, 50 mg/kg q.d, p.o.) starting at 7 weeks of age and continuously treated for 6 weeks. After treatment, small intestines were collected and polyps were counted according to size. Because this treatment regimen began at the time when mice were just beginning to develop detectable intestinal polyps, the chemopreventive properties of these compounds could be better evaluated. Vehicle-treated mice showed an average of 33 small-sized (<1 mm), 37 average-sized (1–2 mm), and 25.5 large-sized (>2 mm) small intestinal polyps (Fig. 1A). As previously reported (17), the inhibition of COX-2 had a pronounced effect on the prevention of intestinal polyps. Celecoxib as a single agent led to a 60% reduction in the number of <1-mm polyps and an 80% reduction in the number of 1- to 2-mm polyps, whereas no polyps >2 mm in diameter were observed. An overall 78% inhibition of total polyp number was observed in mice treated with celecoxib alone. Interestingly, the use of the EGFR inhibitor erlotinib also had a pronounced effect on the development of small intestinal polyps. Mice that received 50 mg/kg (q.d, p.o.) erlotinib showed a 43% and 46% reduction in the number of <1-mm-sized and 1- to 2-mm-sized polyps, respectively. There was also a 96% reduction in the number of large-sized (>2 mm) polyps as well as a 57% inhibition in total polyp number. However, the use of celecoxib and erlotinib in combination had a dramatic effect on the development of small intestinal polyps in APC<sup>min/-</sup> mice (Fig. 1A). We observed an average of only 3.33 polyps per mouse, all of which were <1 mm in size. This resulted in over a 96% inhibition in the development of small intestinal polyps. We have not observed any intervention as effective as this one in our previous studies.

Because the chemopreventive effect of COX-2 and EGFR inhibition had such a drastic effect on the prevention of the development of small intestinal polyps in APC<sup>min/-</sup> mice, we next sought to determine the effect of this combination on established polyps. Therefore, APC<sup>min/-</sup> mice were treated in combination with celecoxib and erlotinib from 13 to 14.5 weeks of age. As shown in Fig. 1B, a shift to smaller-sized polyps was observed in the combination-treated group. Small intestinal polyps that were >2 mm regressed to <2 mm in size, and although there was no significant difference in the total number of polyps during this short treatment period, a 53% increase in the number of small polyps (<1 mm) was observed. Because mice at 13 weeks of age already have established polyps of varying size, the effects of combination therapy observed are due to the regression of already established polyps.

**Effect of COX-2 and EGFR inhibition on colorectal xenografts.** HCA-7 cells, a human colorectal cancer cell line, were injected s.c. into the flank region of athymic nu/nu mice. Once established (~ 300 mm<sup>3</sup>), mice were randomly separated into four treatment groups: control (vehicle), celecoxib (ad libitum in diet), erlotinib (50 mg/kg, q.d, p.o.), and celecoxib in combination with erlotinib. Treatment began on day 26 and continued through the end of the study (day 74). The control-treated group progressed in size to >800 mm<sup>3</sup> by study end point (Fig. 1C). As reported (18), we also observed inhibition of growth with the use of celecoxib compared with placebo in mice bearing HCA-7 xenografts. For the entire 52-day treatment, we did not observe any significant growth or regression of these tumors. However, a regression of 31.4% in tumor volume was initially observed in mice treated with erlotinib alone that did not regress significantly further during the course of study. Surprisingly, in mice treated with celecoxib and erlotinib, we observed an overall reduction of tumor volume by 70.6% that primarily occurs within the first 10 days of treatment.
Because the majority of the effects on tumor volume occurred within the first 2 weeks of treatment, we repeated this study and treated the mice for only 14 days. Similar to the study shown in Fig. 1C, HCA-7 cells were injected s.c. into the flank of nu/nu mice. Mice were allocated into the following treatment groups: control (vehicle), celecoxib (ad libitum), erlotinib (50 mg/kg p.o., qd), and celecoxib/erlotinib (ad libitum, 50 mg/kg p.o., qd). Mice were treated from day 36 to day 50. Tumors were measured using the formula $V = \frac{(L \times W^2)}{2}$, where $V =$ volume, $L =$ length, and $W =$ width. Points, mean tumor volume of five individual mice; bars, SD.

Figure 1. Celecoxib- and erlotinib-induced reduction of small intestinal polyps and effect on xenograft growth. A, APC$^{min/C0}$ mice were treated as indicated from 7 to 12 wk of age: control (vehicle), celecoxib (ad libitum), erlotinib (50 mg/kg p.o., qd), and celecoxib/erlotinib (ad libitum, 50 mg/kg p.o., qd). Columns, polyp numbers are the mean of six mice and are subdivided by polyp diameter; bars, SD. B, APC$^{min/C0}$ mice were treated as indicated from 13 to 14.5 wk of age: control (vehicle) or celecoxib/erlotinib (ad libitum, 50 mg/kg p.o., qd). Columns, polyp numbers are the mean of five individual mice and are subdivided by polyp diameter; bars, SD. C and D, HCA-7 cells were injected s.c. into the flank of nu/nu mice. Mice were allocated into the following treatment groups: control (vehicle), celecoxib (ad libitum), erlotinib (50 mg/kg p.o., qd), and celecoxib/erlotinib (ad libitum, 50 mg/kg p.o., qd). Mice were treated from day 26 to day 74 (C) or from day 36 to day 50 (D). Tumors were measured using the formula $V = \frac{(L \times W^2)}{2}$, where $V =$ volume, $L =$ length, and $W =$ width. Points, mean tumor volume of five individual mice; bars, SD.
Microarray analysis of COX-2 and EGFR inhibition in HCA-7 xenograft tumors. From the data presented in Fig. 1C and D, the effect of tumor regression occurred early and had a prolonged effect on tumor growth. We therefore sought to determine if changes in the expression of specific genes following celecoxib and erlotinib treatment might play a role in the initial regression and delay of subsequent growth. Therefore, another group of nu/nu mice was inoculated in the s.c. flank with HCA-7 cells and allowed to reach \( \approx 500 \text{ mm}^3 \). These tumors were size matched at a larger initial volume so that there would be ample tumor left after regression to do the studies of interest. These mice were randomly divided into the four treatment groups described in Fig. 1C and treated for 7 days. At the end of the study, the animals were sacrificed and tumors were excised. The tumors were divided equally so that RNA and protein could be isolated as well as prostaglandin levels were determined and immunohistochemical analysis was done on these samples. During the 7-day treatment period, as observed in Fig. 1C and D, the celecoxib-treated tumors became static in their growth, the erlotinib-treated tumors slightly regressed, whereas the combination treatment with celecoxib and erlotinib induced a 48% reduction in tumor volume (Fig. 2A). Microarray analysis of mRNAs isolated from the treated groups indicated the significant differential regulation of \( \approx 150 \) genes (>2-fold up-regulation or down-regulation). About 35 mRNAs were

Figure 2. Combinational treatment effect on cellular proliferation. A, HCA-7 cells were injected s.c. into the flank of nu/nu mice. Mice were allocated into the following treatment groups: control (vehicle), celecoxib (ad libitum), erlotinib (50 mg/kg p.o., qd), and celecoxib/erlotinib (ad libitum, 50 mg/kg p.o., qd). Mice were treated from day 33 to day 40. Tumors were excised and microarray analysis was done on isolated mRNA (Table 1) and confirmed by quantitative PCR (B) and Western blot analysis (C).
Table 1. Microarray analysis of celecoxib- and erlotinib-regulated genes

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<thead>
<tr>
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<th>Celecoxib</th>
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Abbreviation: ND, not determined.
*Genes involved in cell proliferation.
†Genes involved in the biosynthesis and metabolism of PGE₂.

dysregulated in the erlotinib-treated group, whereas no significant change in the mRNAs from the celecoxib alone–treated group was found. Analysis of these results revealed that a large subset of genes involved in cell cycle progression or cellular proliferation was differentially regulated. The effect of celecoxib and erlotinib on mRNA levels of these genes is summarized in Table 1. Fourteen genes, which have a positive effect on proliferation (cyclin D1, cyclin B1, etc.), were down-regulated, whereas 1 gene (p57, Kip2) that has an inhibitory effect on cellular proliferation was up-regulated.

Interestingly, the expression patterns of some genes were mainly altered by the presence of erlotinib (cyclin D1), whereas others were only significantly affected following treatment with celecoxib and erlotinib (cyclin B1). Validation of the mRNA levels from the microarray analysis was done by quantitative PCR and an example of two genes is provided in Fig. 2B. A reduction in the relative expression of cyclin D1 mRNA is mediated mainly by the inhibition of EGFR alone, whereas the reduction in relative expression of cell division cycle associated 1 is only observed when both COX-2 and EGFR are inhibited (Fig. 2B; Table 1). Furthermore, Western blot analysis was done to confirm that the decrease/increase in mRNA expression was observed in protein expression as well. Figure 2C shows an example of two proteins that are down-regulated in xenograft tumors on celecoxib and erlotinib treatment. β-Actin was also visualized to ensure equal protein was loaded in each lane.

**Immunohistochemical analysis of treated xenografts and small intestines.** Due to the pronounced effect of COX-2 and EGFR inhibition on the mRNA level of genes involved in cell cycle progression, we did immunohistochemical staining to quantitate the effect on proliferation within the tumors. Sections of treated tumors were stained with Ki-67, a marker for cell proliferation, and the results are shown in Fig. 3A. Vehicle-treated animals from the control group produced tumors that stained highly for Ki-67, indicating a significant degree of cellular proliferation. Decreased staining was observed in tumors from mice treated with either celecoxib or erlotinib as a single agent. However, the biggest decrease in Ki-67 staining was observed in the tumors following combination treatment. Quantitation of the immunohistochemical results was done by counting the number of positive cells per 20× field from 10 random fields (Fig. 3B). A significant difference (P < 0.001) was detected in all treatment groups when compared with control. In addition, an additive inhibitory effect of celecoxib and erlotinib on the degree of cellular proliferation was observed. Immunohistochemical analysis of cellular proliferation [proliferating cell nuclear antigen (PCNA)] was also completed on rolled sections from small intestines of APC<sup>min/+</sup> mice treated with either vehicle or celecoxib plus erlotinib from Fig. 1A. The vehicle-treated mice had large well-organized polyps, which showed a high degree of PCNA staining (data not shown). Polyps from the celecoxib/erlotinib-treated mice were smaller and showed fewer positively stained nuclei, indicating a reduction in cellular proliferation.

The rate of cellular proliferation (cell growth) and apoptosis (cell death) regulates tumor size. Although we did not identify obvious genes that serve a role in the regulation of apoptosis from our microarray analysis of treated xenografts, we did terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining to measure the rate of apoptosis within these tumors (Fig. 3C). Very little apoptotic staining was observed in the control-, celecoxib-, and erlotinib-treated groups. However, we did observe a significant increase in the number of apoptotic cells in the combination-treated group. This was confirmed by counting apoptotic-positive cells per 20× field. The average of 10 random fields is shown in Fig. 3D. There was no significant increase in apoptotic cells from the tumors treated with single agent when compared with control. However, the tumors exposed to celecoxib and erlotinib showed over a 4-fold increase in the amount of apoptosis. Together, these results indicate that the use of either celecoxib or erlotinib can induce a reduction in the rate of proliferation. However, only with combination treatment did we detect a significant increase in apoptosis.

**Effect of celecoxib and erlotinib on prostaglandin synthesis and degradation.** As mentioned above, the use of celecoxib and erlotinib had a pronounced effect on the expression of genes involved in cell proliferation. Analysis of the microarray data also indicated an effect of EGFR inhibition on genes that are involved in the production and/or inactivation of prostaglandins. As shown in Table 1, both cytosolic phospholipase A2 (cPLA2) and COX-2 mRNAs were significantly down-regulated in tumors treated with erlotinib. cPLA2 is one of the major phospholipases that generates arachidonic acid, the substrate of COX-2. A decrease in protein expression of COX-2 and not COX-1 was also confirmed through Western blot analysis (Fig. 4A). We also observed an increase in hydroxyprostaglandin dehydrogenase 15-NAD (PGDH) mRNA in
the presence of erlotinib. PGDH is primarily responsible for the metabolism of PGE2 to an inactive metabolite. The regulation of these mRNAs from Table 1 was verified by quantitative PCR analysis (data not shown). The ability of erlotinib to stimulate the expression of PGDH in vitro and an inhibitory effect of EGF on PGDH enzymatic activity has been previously reported by our group (16). Therefore, aside from the inhibitory effects of celecoxib on COX-2 activity, in the presence of erlotinib, we observed a decrease in two of the key enzymes responsible for the production of prostaglandins as well as an increase in an enzyme responsible for the breakdown of PGE2.

To determine the combinational effect of COX-2 and EGFR inhibition on the concentration of PGE2 within the tumors, we submitted samples for gas chromatography-mass spectrometry analysis. As shown in Fig. 4B, we observed a 60% reduction in the level of PGE2 in the presence of celecoxib when compared with control (*, P < 0.05). Interestingly, we also observed a reduction of 63% in PGE2 concentration with erlotinib alone when compared with vehicle (*, P < 0.05). However, in combination, the use of celecoxib and erlotinib induced an 81% decrease in the level of PGE2 within the tumors when compared with control animals (**, P < 0.001). We also analyzed polyps from APC\textsuperscript{min+/−} mice treated from Fig. 1A and found similar results (Fig. 4C). In normal small intestinal tissue, we observed that celecoxib and erlotinib both inhibited PGE2 production with a much larger effect in combination. Furthermore, we found that intestinal polyps of all sizes contained up to 2-fold increases in PGE2 concentration. In addition, a similar pattern of inhibitory effect was observed in the polyps. However, PGE2 was not detected in polyps >2 mm in diameter.

We have shown previously that APC\textsuperscript{min+/−} mice treated with erlotinib undergo a 6-fold increase in the amount of urine PGEM (19), indicating an increase in the catabolism of PGE2 following EGFR inhibition. We also found that APC\textsuperscript{min+/−} mice treated with celecoxib have a reduction in the level of PGEM presumably due to a decrease in PGE2 production (Fig. 4D). However, we observed a 3-fold increase in the level of PGEM from the erlotinib-treated mice. Although these mice also had decreased concentrations of PGE2, erlotinib induced the expression of PGDH. In the presence of celecoxib and erlotinib, we found a 3-fold increase in urinary PGEM due to the additive effect on the inhibition of PGE2 formation.

**Use of nonselective NSAIDs in combination with erlotinib.** Due to the cardiovascular side effects found with prolonged use of selective COX-2 inhibitors in humans (6), we also examined the effect of nonselective NSAIDs in combination with erlotinib.

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**Figure 3.** Immunohistochemical analysis of xenograft tumors and small intestinal polyps. A, Ki-67 staining of treated xenograft tumors from Fig. 2A. Brown-stained nuclei indicate proliferating cells. **B**, quantitation of Ki-67 staining. Columns, mean number of positive cells per 20 × field from 10 random fields; bars, SD. **C**, TUNEL staining of treated xenograft tumors from Fig. 2A. Brown-stained nuclei indicate apoptotic cells. **D**, quantitation of TUNEL staining. Columns, mean number of positive cells per 20 × field from 10 random fields; bars, SD.
As shown in Fig. 5A, the use of ibuprofen in combination with erlotinib in APC\textsubscript{min/+}/C0 mice with established polyps produced a similar regression of polyp size and number as that with celecoxib and erlotinib (Fig. 1B). Furthermore, the use of ibuprofen in combination with erlotinib was also examined in the HCA-7 xenograft model. As shown in Fig. 5B, the use of ibuprofen alone induced inhibition of tumor growth compared with controls, whereas erlotinib treatment resulted in a slight initial regression and then inhibition of further growth. However, in combination, the use of ibuprofen and erlotinib induced a 65% regression in tumor volume. These results are consistent with those observed in Fig. 1C, indicating that the use of nonselective NSAIDs alone or in combination with EGFR inhibition provides similar efficacy to that observed with selective COX-2 inhibition.

Discussion

In this study, we evaluated the potential benefit of inhibiting COX-2 in combination with the EGFR for the prevention of intestinal polyps and treatment of tumor xenografts. In light of the recent clinical trials that show a significant reduction of polyp reoccurrence in patients taking the selective COX-2 inhibitor celecoxib (2, 3), our data indicate that a combination of celecoxib with the EGFR inhibitor erlotinib may provide an enhanced effect. This combination of inhibitors when given to APC\textsubscript{min/+}/C0 mice almost completely inhibited the formation of small intestinal polyps. Surprisingly, two mice in our treated study group had no visible polyps at all, whereas age-matched control mice had an average of 95 polyps. One potential drawback to the long-term use of selective COX-2 inhibitors is an increase in cardiovascular side effects. One possible avenue might be to use a nonselective NSAID in combination with erlotinib. In our mouse models, we were able to recapitulate the effects of celecoxib + erlotinib with either ibuprofen (Fig. 5) or aspirin (data not shown) with erlotinib. Therefore, the use of nonselective NSAIDs in combination with erlotinib may provide a similar benefit with fewer cardiovascular issues. Likewise, the combination of celecoxib + erlotinib may allow for a reduced dose of celecoxib to be given while in combination with erlotinib while obtaining a similar efficacy.

Although the use of COX-2 and EGFR inhibitors for the treatment of cancer is currently in phase I clinical trials (12), a role for this combination has been established. In a colorectal cancer xenograft model (GEO) the combination of COX-2 and EGFR inhibition was more efficacious than either agent alone (10).

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Figure 4. Effect of celecoxib and erlotinib on PGE\textsubscript{2}. Western blot analysis of COX-1 and COX-2 (A) and PGE\textsubscript{2} concentrations (B) from xenograft tumor samples from Fig. 2A. PGE\textsubscript{2} concentration (C) and urinary PGEM levels (D) from normal intestine and polyps in mice treated in Fig. 1A. *, no data.
The mean of five individual mice and are subdivided by polyp diameter; $W_1 \times W_2 = \text{width}$. HCA-7 cells were injected s.c. into the flank of B, ibuprofen/erlotinib (50 mg/kg p.o., qd). Mice were treated from day 24 to day 44. Tumors were measured using the formula $V = (L \times W^2)/2$, where $V = \text{volume}$, $L = \text{length}$, and $W = \text{width}$. Points, mean tumor volume of five individual mice; bars, SD.

**Figure 5.** Use of ibuprofen in combination with erlotinib. A. APC$^{min/+}$ mice were treated as indicated from 18 to 24 wks of age: control (vehicle) or ibuprofen/erlotinib (ad libitum, 50 mg/kg p.o., qd). Columns, polyp numbers are the mean of five individual mice and are subdivided by polyp diameter; bars, SD. B. HCA-7 cells were injected s.c. into the flank of nu/nu mice. Mice were allocated into the following treatment groups: control (vehicle), ibuprofen (ad libitum), erlotinib (50 mg/kg p.o., qd), and ibuprofen/erlotinib (ad libitum, 50 mg/kg p.o., qd). Mice were treated from day 24 to day 44. Tumors were measured using the formula $V = (L \times W^2)/2$, where $V = \text{volume}$, $L = \text{length}$, and $W = \text{width}$. Points, mean tumor volume of five individual mice; bars, SD.

This combination with protein kinase A antisense gave even better results through both antitumor and antiangiogenic effects. The inhibition of COX-2 and EGFR has proven effective in squamous cell carcinoma of the head and neck (SCCHN). In vitro data suggest that this combination alone or in the presence of docetaxel synergistically inhibited the growth of several SCCHN cell lines (20, 21). This effect was observed through G1 arrest and increased apoptosis. The synergistic combination of COX-2 and EGFR inhibition was also observed in a xenograft model of SCCHN (11). Furthermore, COX-2, EGFR signaling, and matrix metalloproteinases have been shown to play a vital role in the metastasis of breast cancer to the lung (22). Although effects on apoptosis, proliferation, and angiogenesis have been observed, we have now identified the possible mechanism responsible for the synergistic effect at a molecular level. We treated xenograft tumors with either celecoxib, erlotinib, or celecoxib in combination with erlotinib for 1 week and then did microarray analysis on the excised tumors. We have identified ~15 genes involved in cell cycle progression with significantly altered expression patterns. The majority of which only occurs when exposed to the combination of celecoxib and erlotinib. The effect on these genes was verified by immunohistochemical analysis in which we observed a significant decrease in cellular proliferation in the treated tumors. Interestingly, we also observed a substantial increase in the rate of apoptosis in the treated xenografts. However, mRNA levels for genes known to be associated with apoptosis did not change by microarray analysis. Of course, this type of analysis does not measure changes in post-translational modification that could dramatically affect cell death rates. Our observation highlights the possibility that decreased proliferation and increased apoptosis may only be part of the effects of combinational therapy in the tumor microenvironment.

Another interesting observation from this work is the effect of erlotinib on the tissue level of prostaglandins. The treatment of xenograft tumors with erlotinib alone decreased the expression of enzymes involved in the biosynthesis of prostaglandins (cPLA2 and COX-2) while increasing the expression of an enzyme responsible for the metabolism of prostaglandins (PGDH). Therefore, in combination, we observed a significant decrease in available prostaglandins over and above that observed with celecoxib alone. If the additional decrease of prostaglandins is enough to drive part of the synergistic effect of combination therapy, it will be interesting to address this issue in future studies.

In summary, we have observed a substantial synergistic effect of celecoxib and erlotinib in the prevention and regression of intestinal polyps. Furthermore, we have identified an important subset of genes that may play a role in these effects. Our data support the further targeting of the COX-2 and EGFR pathways for the prevention and treatment of intestinal cancers.

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**References**


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