Inhibition of Epidermal Growth Factor Receptor Tyrosine Kinase Fails to Suppress Adenoma Formation in Apc<sup>Min</sup> Mice but Induces Duodenal Injury<sup>1</sup>

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Abstract

A highly selective, p.o. bioavailable irreversible inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, N-[4-(3-chloro-4-fluorophenylamino)-quinazolin-6-yl]-acrylamide (CFPQA), was evaluated for its ability to prevent intestinal adenoma formation in Apc<sup>Min</sup> mice. Ten-week continuous dietary exposure to CFPQA at doses sufficient to abolish intestinal EGFR tyrosine phosphorylation failed to affect intestinal tumor multiplicity or distribution but induced flat mucosal lesions in the duodenum characteristic of chronic injury. Intestinal trefoil factor, an intestinal peptide that mediates antiapoptotic effects through an EGFR-dependent mechanism, was notably absent in adenomas but was highly expressed in flat mucosal lesions. We conclude that chronic inhibition of EGFR tyrosine kinase by CFPQA does not prevent adenomas in Apc<sup>Min</sup> mice but may induce duodenal injury.

Introduction

Because of its role in malignant transformation and growth, EGFR<sup>3</sup> is an attractive target for treatment and prevention of cancer (1–3). Preclinical studies show that inhibition of EGFR activation induces cytostasis and apoptosis in a variety of carcinoma cell lines in vitro and has antitumor activity against epidermoid, breast, ovarian, lung, and nasopharyngeal carcinoma in vivo (4–6), and Phase I studies in humans with advanced malignancies are under way.

Selective inhibitors of EGFR tyrosine kinase induce caspase-dependent apoptosis in colon cancer and adenoma cell lines (7–9), perhaps by preventing EGFR-mediated antiapoptotic signals through the phosphatidylinositol 3-kinase-Akt pathway (10–12). ITF/TFF3 also activates this antiapoptotic pathway and confers resistance of colon cancer cells to apoptosis induced by DNA-damaging agents, serum deprivation, or ceramide through an EGFR-dependent mechanism (13). Of particular interest in colorectal cancer, EGFR and its family members interact with and phosphorylate β-catenin after activation by EGFR ligands or ITF/TFF3 (14–16). β-Catenin accumulates early during colorectal carcinogenesis, following functional loss of the adenomatous polyposis coli (APC) gene product (17), and is considered to mediate the transforming and antiapoptotic effects of Apc loss through its actions as a transcriptional activator of antiapoptotic genes when complexed with the Leit/Tcf family of transcription factors (18, 19). The impact of EGFR signaling on the oncogenic function of β-catenin remains unknown. However, together, these observations suggest that EGFR may play a central role in antiapoptotic signaling during colorectal carcinogenesis generated by EGFR ligands, ITF/TFF3, and perhaps β-catenin. To determine whether EGFR-mediated signals are critical for adenoma formation induced by Apc loss, we examined the effects of a 10-week continuous exposure to a highly selective, p.o. bioavailable inhibitor of EGFR tyrosine kinase activity, CFPQA (PD179651; Ref. 20), at three different oral doses on tumor multiplicity and distribution in the C57BL/6J-Apc<sup>Min</sup> mouse model of intestinal tumorigenesis.

Materials and Methods

Mice. The Apc<sup>Min</sup> mouse is a genetic model of intestinal tumorigenesis driven by a germ-line mutation at codon 850 of the Apc gene analogous to FAP in human (21). Inbred C57BL/6J-Apc<sup>Min</sup> mice were produced by breeding from stock animals purchased from The Jackson Laboratory (Bar Harbor, ME) and screened for the Min<sup>+</sup> genotype using a previously described PCR assay (22). Study mice were housed in an AAALAC-approved specific pathogen-free facility using forced air Thoren microisolator cages (three mice/cage), monitored for health every 2–3 days, and weighed weekly. Mice were weaned onto the AIN-93G powdered diet at 25–30 days of age and assigned systematically to drug treatment and control groups to normalize the distribution of males and females and to avoid clustering of individual mice from single litters. At the end of the study, an intestinal tissue sample was taken from each animal and subjected to Min<sup>+</sup> screening PCR to verify the Min<sup>+</sup> genotype as described previously (22).

Diet and Drug Treatment. The test drug, CFPQA, is a member of the quinazolin family of irreversible selective inhibitors of EGFR tyrosine kinase (1), and its structure, biochemical properties, and selectivity are comparable to those of the well-characterized analogue PD166393 (4, 20). CFPQA has an IC<sub>50</sub> of 0.75 nM against the isolated enzyme and an IC<sub>50</sub> of 3.1 nM against epidermal growth factor-stimulated receptor phosphorylation in A431 epidermoid cancer cells (20). Similar IC<sub>50</sub> values have been determined against murine EGFR<sup>4</sup> CFPQA has minimal activity against other receptor tyrosine kinases. Like another closely related quinazolin, PD153035, CFPQA induces cytostasis and apoptosis of colorectal cancer cell lines at concentrations near its IC<sub>50</sub> for inhibition of EGFR tyrosine kinase activity (7). However, unlike PD153035, CFPQA is well suited for in vivo chemopreventative studies because it has greater aqueous solubility and is p.o. bioavailable.

Food and water were freely available at all times for the duration of the study. Study mice were fed either powdered AIN-93G rodent diet alone (Dyets, Inc., Bethlehem, PA) or AIN-93G plus drug prepared immediately before use by extensive manual mixing of milled drug with powdered diet. Fresh diet was provided every 2–3 days, and all unused AIN-93G diet was stored at 4°C for a period not exceeding 3 months from the time of purchase. Both piroxicam and CFPQA are stable in dry chow at 22°C for over a week (22). All experimental groups were treated continuously from the time of weaning (25–30 days of age) until the time of sacrifice (99–111 days of age). Experimental groups included an AIN-93G vehicle control (200 ppm piroxicam; n = 6 mice; Ref. 23), and three CFPQA treatment groups (50, 200, and 500 ppm CFPQA; n = 10

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2 The abbreviations used are: EGFR, epidermal growth factor receptor; CFPQA, N-[4-(3-chloro-4-fluorophenylamino)-quinazolin-6-yl]-acrylamide; ITF, intestinal trefoil factor; TFF, SP, spasmolytic peptide; FAP, familial adenomatous polyposis; TNA, 10 mml Tris (pH 7.2), 150 mml NaCl, and 0.01% sodium azide.

4 William E. Karnes, Jr., unpublished observations.
mice/group). Based on the average food consumption per day and the average weight of mice in this study, approximate dosage conversions were as follows: (a) 500 ppm = 100 mg/kg/day; (b) 200 ppm = 40 mg/kg/day; and (c) 50 ppm = 10 mg/kg/day. Three additional supplementary studies were performed: (a) four Apc<sup>Min</sup> mice were treated with 500 ppm CFQPA for 80 days and then observed off treatment for 4 weeks before sacrifice to assess the reversibility of CFQPA-induced duodenal lesions (described in “Results”); (b) four Apc<sup>Min</sup> mice were treated with 500 ppm CFQPA for 80 days and then treated with 200 ppm piroxicam for 10 days before sacrifice to assess the effects of piroxicam on CFQPA-induced duodenal lesions; and (c) four wild-type C57BL/6J mice were treated with 500 ppm CFQPA for 80 days before sacrifice to assess whether duodenal lesions were dependent on the Apc<sup>Min</sup> genotype.

Tumor Enumeration. Methods used for determining intestinal tumor multiplicity and distribution in Apc<sup>Min</sup> mice have been described in detail (23). Briefly, the entire gastrointestinal tract was dissected, washed extensively with PBS, and fixed with methacarn (60% methanol, 30% chloroform, and 10% acetic acid), and tumors were evaluated by dark-field microscopy. The smallest tumors scored by this method were 0.2 mm in diameter, and the site of each tumor was recorded using a calibrated stage micrometer (Klarmann Rulings) to facilitate distribution analysis. For tumor distribution analysis, duodenum, jejunum, ileum, and colon were each divided into three equal segments.

Immunoprecipitation and Western Blot. Frozen tissue was homogenized in 10 volumes of ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM sodium EDTA, 1 mM sodium EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and supernatant representing 1 mg of total protein was rotated at 4°C for 2 h with 2 μg of EGFR antibody (clone 180,000 EGFR are indicated by the arrowheads in Fig. 2). Treatment groups labeled CQ/50, CQ/200, and CQ/500 represent CFQPA at 50, 200, and 500 ppm, respectively.

Immunohistochemistry. Expression of trefoil peptides was determined by immunohistochemistry using rabbit polyclonal antibodies kindly provided by Dr. Daniel Podolsky (Massachusetts General Hospital, Boston, MA) raised against rat ITF/TFF3 and human SP (SP/TFF2), both recognizing the murine forms. Sections (5 μm) of methacarn-fixed, paraffin-embedded tissues were dewaxed and rehydrated, treated with 2% hydrogen peroxide in 100% methanol for 10 min, and then rinsed in PBS (pH 7.4). Slides were then blocked in 5% FCS/PBS for 30–45 min, followed by a 1-h incubation with 100–200 μl of primary antibody diluted in 1/5000 PBS at a 1:100 ratio for IF and a 1:500 ratio for SP in a closed humidified chamber. Slides were then rinsed three times in PBS and incubated with 100–200 μl of secondary antibody conjugated to horseradish peroxidase secondary antibody (DAKO, Carpinteria, CA) diluted 1:1000 in PBS. Slides were then washed in several washes with PBS. Slides were then permount coverslipped and analyzed using a Zeiss Axiostar S100TV microscope and a Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were batch-processed for consistent color balance and contrast using Adobe Photoshop version 4.0.

Statistical Analysis. End points for efficacy included intestinal tumor multiplicity and distribution, body weight, serum drug levels, and EGFR phosphorylation level in intestinal mucosa. Statistical comparisons of tumor multiplicity between chemoprevention study groups were performed using Wilcoxon’s rank-sum or two-sample t tests. All P values <0.05 were considered significant.

Results

CFQPA Steady-State Serum Levels and Effects on Intestinal EGFR Tyrosine Phosphorylation. Chronic oral administration of CFQPA to Apc<sup>Min</sup> mice through a food/drink mixture produced serum levels of 11.4 ± 2.5 nm (for 50 ppm), 105 ± 26 (for 200 ppm), and 282 ± 69 nm (for 500 ppm). Serum exposure levels attained for 200 and 500 ppm CFQPA were sufficient to abolish detectable EGFR tyrosine phosphorylation in the intestinal mucosa of Apc<sup>Min</sup> mice by
immunoprecipitation and Western blot (Fig. 1). Therefore, the 200 and 500 ppm doses of CFPQA produced exposure levels sufficient to modulate the pharmacodynamic marker (EGFR autophosphorylation) in the target tissue (intestinal mucosa).

**CFPQA Does Not Affect Adenoma Multiplicity or Distribution in Apc\textsuperscript{Min} Mice.** Despite adequate systemic exposure and confirmed modulation of EGFR tyrosine phosphorylation, none of the tested doses of CFPQA caused significant changes in intestinal tumor multiplicity or distribution in Apc\textsuperscript{Min} mice (Fig. 2, A and B). An apparent increase in tumor multiplicity in the first portion of the duodenum (Fig. 2B, D1) was judged to be an artifact after histological analysis revealed nonadenomatous mucosal lesions confined to the duodenum in the CFPQA-treated animals (described below). In contrast, piroxicam (positive control) dramatically reduced adenoma multiplicity in the duodenum, jejunum, and ileum (>95% reduction; \(P < 0.01\) versus the AIN-93G control group). These findings suggest that adenoma multiplicity in the Apc\textsuperscript{Min} mouse is not dependent on EGFR tyrosine kinase activity.

**CFPQA Induces Duodenal Lesions.** No significant differences in body weights or overall health between any of the treatment groups were noted. However, all Apc\textsuperscript{Min} and wild-type mice treated with 200 or 500 ppm CFPQA for 10 weeks exhibited flat mucosal lesions restricted to the duodenum. Histologically, the duodenal lesions exhibited absent villi, expanded hyperchromatic proliferative zones with an increased frequency of mitotic figures and glandular crowding, and underlying Brunner’s gland hypertrophy without mucosal ulceration or erosion (Fig. 3). The duodenal changes induced by CFPQA were reversible on cessation of treatment (gross observation 4 weeks after the end of treatment) but were not affected by simultaneous treatment with piroxicam (200 ppm; data not shown). Taken together, these observations suggest that the CFPQA-induced duodenal lesions develop independently of Apc status and represent reactive rather than neoplastic changes.

**ITF/TFF3 Expression in Adenomas and Duodenal Lesions.** We then performed immunohistochemical analysis of adenomas, normal intestinal mucosa, and the duodenal lesions of CFPQA-treated and control mice for protein expression of ITF/TFF3, as well as SP (SP/TFF2), a trefoil peptide expressed primarily in the neck cells of gastric glands (24). Specific ITF/TFF3 (Fig. 4) but not SP/TFF2 (data not shown) immunoreactivity was present in cells of the crypts, rare villus goblet cells, and the overlying mucous layer of normal intestinal mucosa of treated and untreated animals. Adenomas exhibited no detectable immunoreactivity for either peptide (Fig. 4). However, CFPQA-induced duodenal lesions showed strong ITF/TFF3 immunoreactivity of isolated cells throughout the entire thickness of the lesions, consistent with the histological impression that these lesions represent expanded regenerative crypts (Fig. 4). The absence of ITF/TFF3 immunoreactivity in adenomas suggests that this peptide does not play a role in Apc\textsuperscript{Min} adenoma development or in the resistance of adenomas to chemoprevention by CFPQA.

**Discussion**

In the present study, chronic dietary exposure to 200 and 500 ppm of the selective EGFR tyrosine kinase inhibitor CFPQA achieved steady-state serum concentrations well above the \(\text{IC}_{50}\) for inhibition of EGFR tyrosine kinase activity and abolished detectable EGFR tyrosine phosphorylation in intestinal mucosa but had no effect on tumor multiplicity or distribution of adenomas in Apc\textsuperscript{Min} mice. Similar concentrations of CFPQA or its closely related analogue, PD153035, are sufficient to induce cytostasis and apoptosis in EGFR-
dependent colorectal cancer cell lines within 24 h (7). Together, these results suggest that EGFR-mediated proliferative and survival signals are not critical for early stages of colorectal carcinogenesis. We speculate that other signaling systems may be activated that compensate for or protect against the antitumor effects of EGFR inhibition or that EGFR is not involved in adenoma formation induced by Apc loss. Neither ITP/TTF3 nor SP/TTF2 was expressed in adenomas, suggesting that these antiapoptotic gut peptides (13) are not involved in adenoma formation and do not confer resistance of adenomas to chemoprevention by CFPQ.

Although the ApcMin mouse is considered to be a genetically relevant model of human colorectal carcinogenesis triggered by APC loss, there are important phenotypic differences between ApcMin mice and ApcMin mice with FAP. Compared with adenomas in FAP, ApcMin adenomas infrequently progress to frank carcinomas, they are distributed more densely in the small intestine than in the colon, and they lack the genetic complexity and instability of human colorectal neoplasms (25). Furthermore, in contrast to reports that ITP/TTF3 is expressed in human adenomas and colorectal cancers (26), we found no detectable ITP/TTF3 expression in the adenomas of ApcMin mice. Thus, the lack of antitumor activity for CFPQA in the ApcMin model cannot be generalized to human colorectal adenomas and carcinomas.

Although no gross toxicity was detected, doses of CFPQA sufficient to abolish mucosal EGFR tyrosine phosphorylation induced hyperproliferative duodenal lesions in both ApcMin and wild-type C57Bl6J mice. Unlike adenomas, these lesions expressed ITP/TTF3, developed in wild-type mice as well as ApcMin mice, and were reversible in mice held from CFPQA treatment for 4 weeks before sacrifice, suggesting that they are reactive and not neoplastic. The lesions showed characteristics of a regenerative response to injury with absent villi, glandular crowding, and expanded proliferative zone. Interestingly, piroxicam, which depletes mucosal cytoprotective prostaglandins through inhibition of cyclooxygenases I and II, did not exacerbate these lesions. Future studies using any of the several classes of EGFR tyrosine kinase inhibitors that are chemically distinct from the quinazolones will help determine whether these lesions are a response to a secondary (off-target) activity or to EGFR inhibition per se.

In summary, we have shown that the EGFR-selective tyrosine kinase inhibitor CFPQA does not inhibit intestinal tumor formation in the ApcMin mouse despite inhibition of the target enzyme EGFR in intestinal mucosa. Although no gross toxicities were detected, doses of CFPQA sufficient to abolish mucosal EGFR tyrosine phosphorylation induced reversible duodenal lesions suggestive of chronic injury in both ApcMin and wild-type C57Bl6J mice. These findings may have important implications regarding the role of EGFR-mediated signals in intestinal carcinogenesis and for the future development of EGFR-targeted therapies for chemoprevention of adenomas.

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