### Title

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# Optimization of erlotinib plus sulindac dosing regimens for intestinal cancer prevention in an Apc-mutant model of Familial Adenomatous Polyposis (FAP)

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## Abstract

A clinical trial in Familial Adenomatous Polyposis (FAP) patients demonstrated that sulindac plus erlotinib (SUL+ERL) had good efficacy in the duodenum and colon, but toxicity issues raised concerns for long-term prevention. We performed a biomarker study in the polyposis in rat colon (Pirc) model, observing phosphorylated extracellular signal-related kinase (pErk) inhibition in colon polyps for up to 10 days after discontinuing ERL+SUL administration. In a follow-up study lasting 16 weeks, significant reduction of colon and small intestine (SI) tumor burden was detected, especially in rats given 250 ppm SUL in the diet plus once-a-week intragastric dosing of ERL at 21 or 42 mg/kg body weight (BW). A long-term study further demonstrated antitumor efficacy in the colon and SI at 52 weeks, when 250 ppm SUL was combined with once-a-week intragastric administration of ERL at 10, 21 or 42 mg/kg BW. Tumor-associated *matrix*

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Disclosure of potential conflicts

E. Vilar has a consulting and advisory role with Janssen Research and Development. No other disclosures are reported.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

*metalloproteinase-7* (*Mmp7*), *tumor necrosis factor* (*Tnf*) and *early growth response 1* (*Egr1*) were decreased at 16 weeks by ERL+SUL, and this was sustained in the long-term study for *Mmp7* and *Tnf*. Based on the collective results, the optimal dose combination of ERL 10 mg/kg BW plus 250 ppm SUL lacked toxicity, inhibited molecular biomarkers, and exhibited effective antitumor activity. We conclude that switching from continuous to once-per-week ERL, given at one-quarter of the current therapeutic dose, will exert good efficacy with standard of care SUL against adenomatous polyps in the colon and SI, with clinical relevance for FAP patients before or after colectomy.

**Keywords**

Adenoma; APC; colorectal cancer; FAP; Pirc

## Introduction

Mortality rates for colorectal cancer (CRC) vary according to geographic region, gender, age, ethnicity, and diet/lifestyle factors (1,2). Genetic influences also contribute to the etiology of CRC, for both sporadic cases and hereditary syndromes, such as Familial Adenomatous Polyposis (FAP) driven by germline mutation in *Adenomatous Polyposis Coli* (*APC*) (2–4,5). Patients with FAP develop hundreds to thousands of adenomatous polyps in the gut, necessitating risk-reducing surgical intervention coupled to long-term chemoprevention strategies (6–9).

Sulindac (SUL) and other nonsteroidal anti-inflammatory agents are effective in the suppression of adenomatous colon polyps (6–9), but not without toxicity, and they have lower efficacy in the duodenum, where tumor formation also is of concern (10). In patients with FAP, a randomized clinical trial combined standard of care SUL (150 mg *p.o.* twice daily) with erlotinib (ERL, 75 mg *p.o.* daily) for 6 months, reducing the duodenal polyp burden markedly compared with historical controls (11). Secondary analysis identified a reduction in polyp burden in those with an entire colorectum, as well as those with ileal pouch or retained rectum post-colectomy (12), suggesting broad-spectrum antitumor efficacy of the combination regimen in the gastrointestinal (GI) tract. However, Samadder et al. also noted that skin toxicity or other adverse events might limit the long-term use of ERL +SUL in combination at the doses tested in the clinical trial (11).

Efficacy has been reported for SUL in preclinical models of FAP, including the Apc-mutant polyposis in rat colon (Pirc) model (13, 14). The Pirc model has a high colon tumor burden and a lifespan suitable for biomarker, prevention, toxicity, and resistance studies (13–20). Importantly, the Pirc model mimics the tumor distribution in FAP patients; rats develop both duodenal polyps and adenomatous lesions in the colorectum (13–15, 21).

Antitumor activity of ERL also has been reported in preclinical models of CRC (22–24). Consistent with Wingless-related integration site (WNT) plus extracellular signal-related kinase (ERK) pathway interaction (25,26), WNT, epidermal growth factor receptor (EGFR) and prostaglandin E2 (PGE2)-related genes were identified as mechanistic targets modulated in FAP patients taking ERL+SUL (27). We utilized the Pirc model to define scheduling and

dosing regimens for ERL+SUL that would maintain antitumor efficacy while limiting toxicity. A secondary objective was to validate the above-mentioned mechanistic targets in the Pirc colon and small intestine (SI), with a view to future clinical trials in FAP patients using optimized combination regimens.

## Materials and Methods

### Preclinical studies

Experiments were approved by the Institutional Animal Care & Use Committee. Under a Taconic Breeding License, Pirc males were generated for the various preclinical studies and genotyped as reported (13), with AIN93 and custom diets from Research Diets, Inc. (New Brunswick, NJ). ERL (>99%) was supplied by LC laboratories (Woburn, MA), whereas SUL (>98%) was obtained from MilliporeSigma (St. Louis, MO). For intragastric (*i.g.*) oral gavage, the test agent was administered in sterile 30% Captisol®, a modified β-cyclodextrin facilitating drug solubilization (28).

**A) Short-term Biomarker Study:** As shown in Fig 1, six-month-old rats were provided with AIN93 basal diet, or switched to 250 parts per million (250 ppm) SUL in AIN93 diet, equivalent to 150 mg SUL twice daily in FAP patients (11). Based on a prior report (29), ERL was given in the first week by one of four treatment schedules: *i.g.* oral gavage every day at 6 or 12 mg/kg body weight (BW), *i.g.* at 21 mg/kg BW twice per week, or *i.g.* at 42 mg/kg BW once per week. Oral dosing of 6 mg/kg BW ERL for one week equates to the daily intake of 70 ppm ERL in rodent diet, or 75 mg ERL *q.d.* in FAP patients (11). ERL at 42 and 21 mg/kg BW (ERL42, ERL21) corresponds, respectively, to 'loading' and 'half-loading' bolus doses from one-week daily intake at 6 mg/kg BW *i.g.* (29). After the final ERL treatment, rats were sacrificed at 1, 2, 7, 10, and 14 days.

**B) Efficacy Study:** As shown in Fig 2, at 6 weeks of age, rats were given AIN basal diet or switched to AIN diet containing 250 ppm SUL. In the presence or absence of SUL, other groups comprised of ERL42 and ERL21 *qw* (*i.g.* once/wk), ERL 70 or 140 ppm in the diet on continuous weeks (ERL70c, ERL140c), and ERL 70 ppm in the diet on alternating weeks (ERL70a). Adenomatous polyps were tracked via colonoscopy (14–16) after 6, 10 and 14 weeks of ERL±SUL treatment, and 2 weeks thereafter rats were sacrificed, *i.e.,* at 16 weeks of drug treatment in total, coinciding with the approximate age of the rats in the Biomarker study.

**C) Long-term Efficacy/Resistance/Toxicity Study:** As shown in Fig 3, at 6 weeks of age, rats were maintained on AIN basal diet or switched to AIN diet containing 250 ppm SUL. Other groups included ERL10 *qw*, ERL21 *qw* and ERL42 *qw*, with or without SUL. The study was terminated at 52 weeks, facilitated by occasional polypectomy (16), mainly in the AIN controls (n=5 rats).

At necropsy, the entire GI tract was opened, cleaned of its contents, and subjected to gross examination, including for possible ulceration (see below). Colon and SI polyps were recorded for location, multiplicity, tumor volume, and total tumor burden. Tumor dimensions were measured using Vernier calipers. Tumor volume was calculated using the

formula: volume = length × width$^2$ × 0.5, and the total tumor burden was calculated as the sum of the individual tumor volumes of each polyp, expressed as mm$^3$. Tumors and adjacent normal-looking tissues were frozen for molecular analyses of protein and RNA expression changes, or fixed in 10% buffered formalin for histopathological diagnosis. Other tissues were examined for signs of toxicity or gross pathology, and organ weights were recorded. Blood samples at termination were collected for biomarker assessment. For the biochemistry panel, blood samples (0.5 mL) were collected in sterile lithium heparin tubes (# 13–680-62, BD Microtainer™, Fisher Scientific) and analyzed the same day using standardized protocols at the Texas A&M Veterinary Medical Diagnostic Laboratory (College Station, TX).

### Immunoblotting (IB), immunohistochemistry (IHC) and H&E staining

Published IB methodologies in the rat (14,15,29,30) used primary antibodies to anti-phospho-Erk1/2 (pErk, Sigma-Aldrich #05–797) and Erk1/2 (Erk, Cell Signaling #9107). Proteins were visualized by Western Lightning Plus-ECL (Perkin Elmer, Inc.) and quantified using a ChemiDoc imaging system (BioRad). For IHC, colon polyps and adjacent normal tissue were sectioned at 4–5 μm and processed with primary antibodies to β-catenin (BD Biosciences #61053) and Ki-67 (Dako #M7248), with Dako Universal Negative Control (N1699) as reported (19,31–33). Max PolyOne Polymer HRP Detection solution (Vector Laboratories) was applied at room temperature, followed by Nova Red as chromogen and Dako hematoxylin as counterstain. Epidermal tissues were sectioned at 4–5 μm and stained using hematoxylin and eosin (H&E).

### RNA analyses

As reported (31–33), RNA was extracted using an RNeasy kit (Qiagen), reverse-transcribed via SuperScript™ III (ThermoFisher) and quantified by qPCR, with target mRNAs normalized to *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)*. Primer sequences used for qPCR were *Tnf*: 5'-ATGGGCTCCCTCTCATCAGT-3' (Forward), 5'-GCTTGGTGGTTTGCTACGAC-3' (Reverse); *Egr1*: 5'-CGAGGAGCAAATGATGACCG-3' (Forward), 5'-CAGGGATCATGGGAACCTGG-3' (Reverse); *Mmp7:* 5'-ATGGTGAGGACTCAGGAGTGA-3' (Forward), 5'-TTGCTGGTGTCTGTCGTGTT-3' (Reverse); *Gapdh*: 5'-ATGGGAGTTGCTGTTGAAGTC-3' (Forward), 5'-CCGAGGGCCCACTAAAGG-3' (Reverse). Three or more independent experiments were performed for each sample.

### Ulcerogenicity

The stomach was examined microscopically for signs of toxicity, as reported (34). Ulcers were enumerated under a Nikon SMZ800N stereomicroscope. The ulcer index (UI) was scored as follows: 0 = no lesions/normal color; 0.5 = red coloration; 1 = spot lesions (<1 mm); 2 = 1–5 small ulcers (1–2 mm); 3 = >5 small (1–2 mm) or 1 large ulcer (>2 mm); 4 = >1 large ulcer (>2 mm).

### Skin toxicity

One to two skin biopsies were taken per animal, mainly from the facial area surrounding the eyes. Based on prior studies, H&E stained epidermal biopsies were examined for acanthosis, hyperkeratosis, perivascular inflammation, or other reported abnormalities (35).

### Statistical analyses

Unless indicated otherwise, tumor data were presented as bar graphs with mean±SD. Other data were presented as box-and-whisker plots or bar graphs with mean±SE. Analysis of variance (ANOVA) was used for group comparisons followed by Tukey's multiple comparisons test. In the figures and tables, significant outcomes were shown with P values (*P<0.05, **P<0.01, ***P<0.001), or with superscript letters designating no significant difference among groups.

## Results

### After discontinuing ERL dosing, pErk was inhibited for up to 10 days in Pirc colon polyps

Lubet et al. reported that pErk protein expression was inhibited in rat mammary tumors one week after a single bolus dose of 42 mg ERL/kg BW *i.g.*, but they did not examine the time-course for recovery (29). In the Biomarker Study (Fig 1A), there was effective inhibition of pErk in colon polyps between 1– 10 days post-ERL dosing, when daily SUL in the diet (250 ppm) was administered with ERL 6 mg/kg BW *i.g.* daily (Fig 1B, red boxes and associated densitometry, P<0.05 by ANOVA). Dose-response and time-course experiments revealed that pErk inhibition by 6 or 12 mg/kg BW *i.g.* daily lasted for up to 10 days post-ERL dosing (Figs 1C,D, P<0.05 by ANOVA), whereas pErk inhibition was detected for up to 7 days post ERL *i.g.* dosing at 21 mg/kg twice weekly or 42 mg/kg weekly (Figs 1E,F, P<0.05 by ANOVA), based on densitometry measurements of the corresponding immunoblots (Supplementary Fig 1). Although SUL is used as standard of care in FAP patients (6–10), SUL+ERL was more effective than SUL alone at inhibiting pErk between 24 h and 7–10 days after discontinuing ERL treatment (Figs 1C-F).

### Short-term ERL+SUL combinations provided good efficacy in the Pirc model of FAP

We next assessed antitumor outcomes of the following dosing regimens: (i) once per week *i.g.* oral gavage administration of ERL at 21 and 42 mg/kg BW (ERL21, ERL42); (ii) 6 mg/kg BW equivalent ERL, but given in the diet (70 ppm) either continuously or every other week; and (iii) 12 mg/kg equivalent ERL, given in the diet (140 ppm) on alternating weeks. Colonoscopy data revealed efficacy in multiple groups, especially for ERL+SUL in combination (Fig 2B), and assisted in determining the final termination date for the study. Statistical analyses of the colonoscopy data (Supplementary Table 1) indicated that antitumor efficacy started as early as 6 weeks after initiating drug treatments, especially in the ERL+SUL combination arms, and was sustained for the entire study duration. No deleterious effects were noted on body weight growth curves or food consumption in any of the groups (Supplementary Figs 2A,B).

When the Efficacy Study was terminated after 16 weeks of drug treatment, in animals at 22 weeks of age (Fig 2A), the number of polyps and the total tumor burden (*i.e.*, number plus

volume of all tumors combined) were determined in the colon (Figs 2C,D) and SI (Figs 2E,F). Key observations were as follows: (i) SUL at 250 ppm in the diet suppressed colon and SI tumor burden significantly (50–60% inhibition; P<0.001) compared with AIN basal diet (yellow *vs.* white bars in Figs 2D and 2F); (ii) ERL 70 and 140 ppm in the diet did not reduce colon polyp number significantly, but in combination with SUL it offered better protection than SUL alone (70–85% inhibition; P<0.001, Fig 2C); (iii) suppression of colon and SI polyp number and total tumor burden was prominent in rats given weekly *i.g.* doses of ERL21 and ERL42, alone (70–95% inhibition; P<0.001) or in combination with SUL (80–98% inhibition; P<0.001) (pink and purple bars, Figs 2C-F); (iv) a low dose 'window' for antitumor efficacy was defined for ERL70, given either continuously or on alternating weeks in combination with SUL (250 ppm), for which the individual test agents were less effective (Figs 2C-F, E70cS and E70aS *vs.* SUL, E70a, or E70c).

Based on the densitometry data for pErk/Erk, target modulation occurred in Pirc colon polyps for multiple treatment groups (Fig 2G). Unlike in the Biomarker study (Fig 1B), treatment with SUL alone for 16 weeks decreased pErk levels (Fig 2G, P<0.05 by ANOVA). Addition of SUL to ERL (ERL+SUL) led to even lower pErk levels, especially for ERL70a and ERL70c, which was in concordance with the antitumor efficacy of the combined agents at these doses (Figs 2B-F).

### Long-term ERL+SUL had sustained efficacy with minimal toxicity/resistance

A 1-year study was undertaken, beginning in rats at 6 weeks of age, and with drug treatments lasting 46 weeks (Fig 3A). Based on the short-term efficacy study, which indicated that oral gavage dosing regimens generally were superior to feeding ERL in the diet (Figs 2C-F), we selected once per week *i.g.* doses of ERL 21 and 42 mg/kg BW, and included one lower *i.g.* dose, *i.e.,* once weekly ERL 10 mg/kg BW, to seek the cut-off for efficacy. Monthly colonoscopy data identified good antitumor efficacy for once per week ERL10, ERL21 and ERL42 when combined with SUL (Fig 3B and Supplementary Table 2). At the termination of the study, compared with AIN controls, ERL10 had good efficacy in the SI and colon when combined with SUL. Indeed, ERL10+SUL suppressed significantly in both target tissues with respect to the number of polyps (60–85% inhibition; P<0.001; Figs 3C,E) and the total tumor burden (86–91% inhibition; P<0.001; Figs 3D,F). However, ERL10+SUL was less efficacious than ERL21+SUL and ERL42+SUL with respect to the number of polyps (81–99% inhibition; P<0.01; Figs 3C,E) and the total tumor burden (91–99% inhibition; P<0.05; Figs 3D,F). As in the short-term Efficacy study, pErk inhibition was observed in multiple treatment groups, including SUL, ERL10±SUL, ERL21±SUL and ERL42±SUL (Fig 3G), coinciding with antitumor activity after 52 weeks.

Cumulative data for lesions from the long-term Efficacy study (Supplementary Fig 3) revealed that the largest polyps in the colon and SI were in the range 0.5–1.2 $cm^3$ and 1–3 $cm^3$, respectively. The final data included lesions that were resected periodically via colonoscopy-polypectomy (16), to prevent occlusion of the colon and premature termination of the study. The AIN controls required the most resections, with one large polyp removed from each of five separate rats. The Pirc model typically is not associated with invasion (13), and although histopathological analysis was not performed in the current study, drug

treatments lowered β-catenin and Ki-67 overexpression in the colon tumors (Supplementary Fig 4A), consistent with effective tumor interception.

No deleterious effects were noted on body weight growth curves in any of the groups in the long-term study (Fig 4A). The Kaplan-Meier plot revealed lower overall survival in the AIN group, intermediate survival for SUL, and better survival for all other groups – especially for ERL+SUL in combination (Fig 4B). Compared with AIN controls, an increase in the ulcer index was observed for ERL21±SUL and ERL42±SUL, but not in SUL, ERL10, or ERL10+SUL groups (Figs 4C). In contrast to AIN, SUL, ERL10 and ERL10+SUL groups, 30–40% percent of rats given ERL21±SUL or ERL42±SUL had porphyrin staining around the eye (Fig 4D). Biopsies at the time of necropsy revealed no overt skin pathology, such as acanthosis, hyperkeratosis, perivascular inflammation, or other abnormalities (Supplementary Fig 4B).

Organ weights that were altered in tumor-bearing Pirc controls fed AIN basal diet were normalized by drug treatments (Supplementary Figs 5A-C), except for lung (Supplementary Fig 5D). Reduced splenomegaly and increased hematocrit with SUL±ERL (Supplementary Figs 5E,F) resembled prior studies in the *Apc*$^{Min/+}$ mouse treated with SUL and tea (36,37). An inverse correlation was noted for spleen/body weight (%) *vs.* % hematocrit (Supplementary Fig 6A), as well as for the number of polyps in the colon and SI *vs.* % hematocrit (Supplementary Figs 6B,C).

A biochemistry panel identified few changes in the circulation (Supplementary Fig 7). Higher doses of ERL±SUL improved total protein levels to within the normal mean value for wild type age-matched control F344 male rats (blue dotted line). Albumin was higher for ERL21+SUL, ERL21 produced an increase in ALT, and glucose levels were elevated by ERL42±SUL. No other statistically significant changes were noted.

A summary is provided of key clinical observations (Table 1). Diarrhea and blood in the rectum were observed mainly for AIN, SUL and ERL10 groups harboring a greater tumor burden and increased mortality/morbidity, whereas higher doses of ERL±SUL produced an eye and/or mild skin phenotype. An apparent low-dose ‘window’ – representing the lowest threshold dose for effective tumor suppression – was defined for ERL10+SUL, exhibiting no skin toxicity, minimal diarrhea/blood in the rectum (Table 1), and sustained antitumor activity (Figs 3C-F).

## Gene expression was altered by ERL+SUL in tumors from the Pirc model

Differential gene expression profiling in duodenal polyps from FAP patients treated with ERL+SUL *vs.* placebo prioritized WNT, EGFR and PGE2 related pathways as mechanistically-relevant molecular targets (27), with top genes in each category including *MMP7*, *EGR1*, and *TNF,* respectively. The corresponding murine genes *Mmp7*, *Egr1* and *Tnf* were examined in Pirc colon polyps from the Efficacy Study at 16 weeks (Fig 5A). Higher doses of ERL+SUL inhibited all three genes significantly compared with AIN controls. Notably, ERL70 continuous or alternating dietary treatment plus SUL in the diet (ERL70cS, ERL70aS) also inhibited *Mmp7* significantly. Prior studies identified *Mmp7* as a

key mechanistic target in the Pirc model following treatment with nonsteroidal anti-inflammatory agents (14).

Gene expression analyses were extended to drug treatment groups at 52 weeks in the long-term study, except for ERL42SUL, which had scarce tissue availability after robust tumor suppression (Figs 3C-F). In colon polyps at 52 weeks (Fig 5B), *Mmp7* expression was inhibited significantly by ERL+SUL treatment, including ERL10S, but not by ERL or SUL alone. In the SI tumors at 52 weeks (Fig 5C), overall trends for *Mmp7* paralleled the antitumor outcomes, with higher doses of ERL plus SUL being more effective at lowering *Mmp7* levels.

For *Tnf*, trends also paralleled the antitumor outcomes, with higher doses of ERL+SUL being more effective than SUL alone. Inhibition of *Tnf* expression was noted in colon tumors from the short-term Efficacy study (Fig 5A), and in colon and SI polyps from the long-term study at 52 weeks (Figs 5B,C, respectively).

Whereas *Egr1* paralleled *Mmp7* and *Tnf* in the short-term Efficacy Study (Fig 5A), no significant inhibition of *Egr1* was observed in colon polyps at 52 weeks (Fig 5B), and an apparent statistically significant increase was noted for *Egr1* in the SI tumors after ERL21+SUL treatment *vs.* AIN alone (Fig 5C). We are cautious not to over-interpret the latter findings, given that outcomes could not be corroborated at higher dose combinations of ERL+SUL, due to scarce tumor tissue availability after robust antitumor activity (as noted above).

## Discussion

Collectively, the findings from this report identified ERL 10 mg/kg + SUL 250 ppm (ERL10+SUL) as the lowest, most efficacious dose combination with the least toxicity in the Pirc model. Dose selection was guided by each iterative experiment, as well as by prior work that reported ERL to affect antitumor outcomes and pErk inhibition when continuous daily oral gavage administration was switched to once a week cumulative ‘loading’ and ‘half-loading’ *i.g.* doses of ERL (29). We also determined, for the first time, the temporal recovery of pErk in rat colon polyps, observing pErk inhibition for up to 10 days after discontinuation of ERL dosing in the Pirc model.

Our initial goal was to determine the point at which pErk started an upward trajectory, timing the next ERL dose to maintain pErk inhibition in Pirc colon and SI polyps. In terms of clinical translation, a personalized approach to ‘precision medicine’ in FAP patients might involve ERL given less frequently, precisely targeting pErk reappearance in adenomatous polyps, while enabling acne-like rash more time for recovery (11). However, ERL10, ERL21 and ERL42 inhibited pErk expression to a similar extent in Pirc colon polyps (Fig 3G), and pErk levels did not faithfully reflect the degree of antitumor efficacy. Cyclooxygenase-1/cyclooxygenase-2 (Cox1/Cox2) enzymatic activities and PGE2 quantification in target tissues might clarify the role of Cox signaling in the Pirc model.

Notably, SUL alone inhibited pErk expression in adenomatous polyps (Figs 2G,3G), consistent with COX-independent actions (38–42) and the crosstalk between WNT and ERK

signaling pathways (25,26,36,37,42). This was less evident in the Biomarker Study (Fig 1B), probably due to the shorter duration of SUL treatment. These results recommend against the routine use of pErk as a predictive biomarker of response to SUL+ERL combination treatments in future clinical trials. Previous studies in the *Apc*$^{Min/+}$ mouse demonstrated tumor promotion by SUL in the colon (43,44), unlike the colon tumor suppression observed for SUL in the Pirc model (14,45). This discrepancy between rat and mouse models most likely relates to species, strain, and gender differences. The Apc-mutant mouse develops tumors mostly in the SI, with few in the colon, whereas the Apc-mutant rat has a significant tumor burden both in the SI and in the colon, more closely mimicking FAP in humans (13). However, the degree of inhibition of gross tumors in the Pirc model may be an overestimation, due to the potential presence of microscopic tumors that can only be detected histologically (Supplementary Fig 4A).

The short-term efficacy study set the stage for the long-term study by establishing antitumor activities for various ERL+SUL combinations in the colon (Figs 2C,D) and SI (Fig 2E,F). Notably, ERL10+SUL was the lowest dose combination that had antitumor efficacy at 52 weeks in the colon (Figs 3C,D) and SI (Figs 3E,F), with little or no signs of toxicity (Fig 4, Table 1). Moreover, antitumor effects in the SI were attributed, in large part, to suppression of duodenum polyps at 16 and 52 weeks (Supplementary Fig 8). These findings could be pertinent for FAP patients who have undergone a total colectomy, when duodenal cancer becomes the focus of prevention strategies (6,11,12). After allometric scaling, ERL10+SUL equates to one-quarter of the current weekly intake of ERL, combined as a single bolus dose and given once a week with standard of care SUL. This work has started to address the need (11,12) for ERL+SUL dose combinations that retain efficacy in the setting of duodenal cancer prevention, while minimizing toxicity concerns.

Toxicity readouts at higher doses of ERL±SUL included increased gastric ulceration (Fig 4C) and porphyrin staining near the eye, with no rash or other skin pathology (Supplementary Fig 4B), which is a common side effect in cancer patients treated with ERL (46,47). Whereas patients given ERL can present with diarrhea (46–48), in the current investigation diarrhea was related to a high-tumor burden rather than ERL dosing (Table 1, dashed box), in AIN and SUL groups that exhibited reduced overall survival after 52 weeks (Fig 4B).

Other notable observations from the long-term study included a modest impact on blood biochemistry parameters (Supplementary Fig 7), and the normalization of hematocrit and organ weights, such as liver, kidney, heart, and spleen (Supplementary Fig 5). One cautionary note concerned the diminished lung weights at 52 weeks (Supplementary Fig 5D). In some patients given ERL, interstitial lung disease has been reported, although this typically involves a pre-existing pulmonary condition and high-dose drug treatment (49–51). Further studies are needed to corroborate ERL-induced changes in the lung of the Pirc model.

Gene expression analyses in the rat were in general accordance with prior observations from FAP patients given ERL+SUL, in which WNT, EGFR and PGE2 signaling pathways were prioritized (27). Thus, *Mmp7*, *Egr1* and *Tnf* levels were downregulated by the various drug

treatments in Pirc colon polyps at the end of the short-term Efficacy Study (Fig 5A), and trends were somewhat indicative of antitumor outcomes. *Tnf* remained inhibited in colon and SI polyps after 46 weeks of drug treatment (Figs 5B,C), reflecting sustained actions on the PGE2 pathway (27), despite the fact that SUL – a drug known to target COX/PGE2 – was kept constant and *Tnf* changes paralleled the ERL dose applied. In the colon and SI polyps obtained after one year, ERL+SUL remained effective at downregulating *Mmp7*, but ERL as single agent did not attenuate *Mmp7* levels in colon polyps (Fig 5B). One interpretation is that SUL rather than ERL is the primary driver of Wnt-associated gene changes, perhaps via downregulation of β-catenin or β-catenin/Tcf-dependent transcription (8,36,37,42). Although *Egr1* was unexpectedly increased by ERL21+SUL treatment in SI polyps at 52 weeks (Fig 5C), we are cautious not to over-interpret these data. Additional work is needed to verify these observations and any possible drug resistance mechanisms arising via the Egfr pathway. Exome sequencing and transcriptomic analyses of large *vs.* small lesions (Supplementary Fig 3) might help to prioritize genetic changes and critical signaling pathways related to efficacy *vs.* resistance (8,14,27,36–42,52,53).

In summary, we defined an optimized low-dose strategy in the Pirc model, involving *i.g.* ERL at 10 mg/kg BW once per week plus 250 ppm dietary SUL. ERL10+SUL produced minimal toxicity (Table 1), normalized hematocrit and splenomegaly (Supplementary Fig 5), inhibited tumor-associated pErk (Fig 3G), *Mmp7* and *Tnf* (Figs 5B,C), and had significant antitumor activity in the colon (Figs 3C,D) and SI (Figs 3E,F). Clinical translation to FAP patients would entail ERL administration at 10 mg/kg BW equivalent, which is one-quarter of the current recommended therapeutic dose, as a once per week regimen with standard of care SUL. Expected outcomes would include efficacy against adenomatous polyps in the colon, and in particular the SI, while minimizing the likelihood for skin toxicity, gastric ulceration, or other deleterious outcomes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1). Torre LA, Siegel R, Ward EM, Jemal A. Global cancer incidence and mortality rates and trends – an update. Cancer Epidemiol Biomarkers Prev 2016;25:16–27. [PubMed: 26667886]

2). Mork ME, You YN, Ying J, Bannon SA, Lynch PM, Rodriguez-Bigas MA, Vilar E. High prevalence of hereditary cancer syndromes in adolescents and young adults with colorectal cancer. J Clin Oncol 2015;33:3544–9. [PubMed: 26195711]

3). Borras E, San Lucas FA, Chang K, Zhou R, Masand G, Fowler J, et al. Genomic landscape of colorectal mucosa and adenomas. Cancer Prev Res 2016;9:417–27.

4). Fu Y, Zheng S, An N, Athanasopoulos T, Popplewell L, Liang A, et al. beta-Catenin as a potential key target for tumor suppression. Int J Cancer 2011;129:1541–51. [PubMed: 21455986]

5). Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 1997;275:1787–90. [PubMed: 9065402]

6). Lynch PM. Chemoprevention of familial adenomatous polyposis. Fam Cancer 2016;15:467–75. [PubMed: 27083160]

7). Weng W, Feng J, Qin H, Ma Y. Molecular therapy of colorectal cancer: progress and future directions. Int J Cancer 2015;136:493–502. [PubMed: 24420815]

8). Li N, Xi Y, Tinsley HN, Gurpinar E, Gary BD, Zhu B, et al. Sulindac selectively inhibits colon tumor cell growth by activating the cGMP/PKG pathway to suppress Wnt/beta-catenin signaling. Mol Cancer Ther 2013;12:1848–59. [PubMed: 23804703]

9). Cruz-Correa M, Hylind LM, Romans KE, Booker SV, Giardiello FM. Long-term treatment with sulindac in familial adenomatous polyposis: a prospective cohort study. Gastroenterology 2002;122:641–5. [PubMed: 11874996]

10). Brosens LA, Keller JJ, Offerhaus GJ, Goggins M, Giardiello FM. Prevention and management of duodenal polyps in familial adenomatous polyposis. Gut 2005;54:1034–43. [PubMed: 15951555]

11). Samadder NJ, Neklason DW, Boucher KM, Byrne KR, Kanth P, Samowitz W, et al. Effect of sulindac and erlotinib vs placebo on duodenal neoplasia in familial adenomatous polyposis: a randomized clinical trial. JAMA 2016;315:1266–75. [PubMed: 27002448]

12). Samadder NJ, Kuwada SK, Boucher KM, Byrne K, Kanth P, Samowitz W, et al. Association of sulindac and erlotinib vs. placebo with colorectal neoplasia in Familial Adenomatous Polyposis: secondary analysis of a randomized clinical trial. JAMA Oncol 2018;4:671–7. [PubMed: 29423501]

13). Amos-Landgraf JM, Kwong LN, Kendziorski CM, Reichelderfer M, Torrealba J, Weichert J, et al. A target-selected Apc-mutant rat kindred enhances the modeling of familial human colon cancer. Proc Natl Acad Sci USA 2007;104:4036–41. [PubMed: 17360473]

14). Ertem FU, Zhang W, Chang K, Dashwood W, Rajendran P, Sun D, et al. Oncogenic targets Mmp7, S100a9, Nppb and Aldh1a3 from transcriptome profiling of FAP and Pirc adenomas are downregulated in response to tumor suppression by Clotam. Int J Cancer 2017;140:460–8. [PubMed: 27706811]

15). Rajendran P, Johnson G, Li L, Chen Y-S, Dashwood M, Nguyen N, et al. Acetylation of CCAR2 establishes a BET/BRD9 acetyl switch in response to combined deacetylase and bromodomain inhibition. Cancer Res 2019;79:918–27. [PubMed: 30643017]

16). Ertem F, Dashwood WM, Rajendran P, Raju G, Rashid A, Dashwood R. Development of a murine colonoscopy polypectomy model (with videos). Gastrointest Endosc 2016;83:1272–6. [PubMed: 26658879]

17). Yun C, Dashwood WM, Li L, Yin T, Ulusan AM, Shatzer K, et al. Acute changes in colonic -PGE2 levels as a biomarker of efficacy after treatment of the Pirc rat with celecoxib. Inflamm Res 2020;69:131–7. [PubMed: 31797003]

18). Luceri C, Femia AP, Tortora K, D'Ambrioso M, Fabbri S, Fazi M, Caderni G. Supplementation with phytoestrogens and insoluble fibers reduces intestinal carcinogenesis and restores ER-beta expression in Apc-driven colorectal carcinogenesis. Eur J Cancer Prev 2020;29:27–35. [PubMed: 31651566]

19). Okonkwo A, Mitra J, Johnson GS, Li L, Dashwood WM, Hegde ML, et al. Heterocyclic analogs of sulforaphane trigger DNA damage and impede DNA repair in colon cancer cells: interplay of HATs and HDACs. Mol Nutr Food Res 2018;62:e1800228. [PubMed: 29924908]

20). Irving AA, Duchow EG, Plum LA, DeLuca HF. Vitamin D deficiency in the ApcPirc/+ rat does not exacerbate colonic tumorigenesis, while low dietary calcium might be protective. Dis Model Mech 2018;11:dmm032300. [PubMed: 29590632]

21). Jones JE, Busi SB, Mitchem JB, Amos-Landgraf JM, Lewis MR. Evaluation of a tumor-targeting, near-infrared fluorescent peptide for early detection and endoscopic resection of polyps in a rat model of colorectal cancer. Mol Imaging 2018;17:1536012118790065.

22). Buchanan FG, Holla V, Katkuri S, Matta P, DuBois RN. Targeting cyclooxygenase-2 and epidermal growth factor receptor for the prevention and treatment of intestinal cancer. Cancer Res 2007;67:9380–8. [PubMed: 17909047]

23). Pagán B, Isidro AA, Cruz ML, Ren Y, Coppola D, Wu J, Appleyard CB. Erlotinib inhibits progression to dysplasia in a colitis-associated colon cancer model. World J Gastroenterol 2011;17:4858–66. [PubMed: 22171126]

24). Buck E, Eyzaguirre A, Brown E, Petti F, McCormack S, Haley JD, et al. Rapamycin synergizes with epidermal growth factor receptor inhibitor erlotinib in non-small cell lung cancer, pancreatic, colon and breast tumors. Mol Cancer Ther 2006;5:2676–84. [PubMed: 17121914]

25). Zeller E, Hammer K, Kirschnick M, Braeuning A. Mechanisms of RAS/β-catenin interactions. Arch Toxicol 2013;87:611–32. [PubMed: 23483189]

26). Jeong WJ, Ro EJ, Choi KY. Interaction between Wnt/β-catenin and RAS-ERK pathways and an anti-cancer strategy via degradations of β-catenin and RAS by targeting the Wnt/β-catenin pathway. NPJ Precis Oncol 2018;2:5. [PubMed: 29872723]

27). Delker DA, Wood AC, Snow AK, Samadder NJ, Samowitz WS, Affolter KE, et al. Chemoprevention with cyclooxygenase and epidermal growth factor receptor inhibitors in familial adenomatous polyposis patients: mRNA signatures of duodenal neoplasia. Cancer Prev Res (Phila) 2018;11:4–15. [PubMed: 29109117]

28). Devasari N, Dora CP, Singh C, Paidi SR, Kumar V, Sobhia ME, Suresh S. Inclusion complex of erlotinib with sulfobutyl ether-beta-cyclodextrin: preparation, characterization, in silico, in vitro and in vivo evaluation. Carbohydr Polym 2015;134:547–56. [PubMed: 26428157]

29). Lubet RA, Szabo E, Iwata KK, Gill SC, Tucker C, Bode A, et al. Effect of intermittent dosing regimens of erlotinib on methylnitrosourea-induced mammary carcinogenesis. Cancer Prev Res (Phila) 2013;6:448–54. [PubMed: 23531447]

30). Rajendran P, Dashwood WM, Li L, Kang Y, Kim E, Johnson G, et a. Nrf2 status affects tumor growth, HDAC3 gene promoter associations, and the response to sulforaphane in the colon. Clin Epigenetics 2015;7:102. [PubMed: 26388957]

31). Wang R, Dashwood WM, Nian H, Löhr CV, Fischer KA, Tsuchiya N, et al. NADPH oxidase overexpression in human colon cancers and rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Int J Cancer 2010;128:2581–90. [PubMed: 20715105]

32). Wang R, Kang Y, Löhr CV, Fischer KA, Bradford CS, Johnson G, et al. Reciprocal regulation of BMF and BIRC5 (Survivin) linked to eomes overexpression in colorectal cancer. Cancer Lett 2016;381:341–8. [PubMed: 27539959]

33). Wang R, Löhr CV, Fischer KA, Dashwood WM, Greenwood JA, Ho E. Epigenetic inactivation of endothelin-2 and endothelin-3 in colon cancer. Int J Cancer 2013;132:1004–12. [PubMed: 22865632]

34). Tammara VK, Narurkar MM, Crider AM, Khan MA. Morpholinoalkyl ester prodrugs of diclofenac: synthesis, in vitro and in vivo evaluation. J Pharm Sci 1994;83:644–8. [PubMed: 8071813]

35). Brown AP, Dunstan RW, Courtney CL, Criswell KA, Graziano MJ. Cutaneous lesions in rats following administration of an irreversible inhibitor of erbB receptors, including the epidermal growth factor receptor. Toxicol Pathol 2008;36:410–419. [PubMed: 18467688]

36). Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Dashwood RH. Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of beta-catenin signaling by a combination of tea plus sulindac. Carcinogenesis 2003;24:263–7. [PubMed: 12584176]

37). Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Al-Fageeh M, et al. Response of Apc(min) and A33(Delta N beta-cat) mutant mice to treatment with tea, sulindac, and 2-amino-1-methylimidazo[4,5-b]pyridine (PhIP). Mutat Res 2002;506–507:121–7.

38). Liggett JL, Min KW, Smolensky D, Baek SJ. A novel COX-independent mechanism of sulindac sulfide involves cleavage of epithelial cell adhesion molecule protein. Exp Cell Res 2014;326:1–9. [PubMed: 24859349]

39). Gurpinar E, Grizzle WE, Piazza GA. COX-independent mechanisms of cancer chemoprevention by anti-inflammatory drugs. Front Oncol 2013;3:181. [PubMed: 23875171]

40). Dell'Omo G, Crescenti D, Vantaggiato C, Parravinci C, Borroni AP, Rizzi N, et al. Inhibition of SIRT1 deacetylase and p53 activation uncouples the anti-inflammatory and chemopreventive actions of NSAIDs. Br J Cancer 2019;120:537–546. [PubMed: 30739913]

41). Babbar N, Ignatenko NA, Casero RA, Gerner EW. Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. J Biol Chem 2003;278:47762–75. [PubMed: 14506281]

42). Rice PL, Kelloff J, Sullivan H, Driggers LJ, Beard KS, Kuwada S, et al. Sulindac metabolites induce caspase- and proteasome-dependent degradation of beta-catenin protein in human colon cancer cells. Mol Cancer Ther 2003;2:885–92. [PubMed: 14555707]

43). Greenspan EJ, Nichols FC, Rosenberg DW. Molecular alterations associated with sulindac-resistant colon tumors in ApcMin/+ mice. Cancer Prev Res (Phila). 2010;3:1187–97. [PubMed: 20716632]

44). Yang K, Fan K, Kurihara N, Shinozaki H, Rigas B, Augenlicht L, Kopelovich L, Edelmann W, Kucherlapati R, Lipkin M. Regional response leading to tumorigenesis after sulindac in small and large intestine of mice with Apc mutations. Carcinogenesis. 2003;24:605–11. [PubMed: 12663524]

45). Femia AP, Soares PV, Luceri C, Lodovici M, Giannini A, Caderni G. Sulindac, 3,3'-diindolylmethane and curcumin reduce carcinogenesis in the Pirc rat, an Apc-driven model of colon carcinogenesis. BMC Cancer. 2015;15:611. [PubMed: 26335331]

46). Townsley CA, Major P, Siu LL, Dancey J, Chen E, Pond GR, et al. Phase II study of erlotinib (OSI-774) in patients with metastatic colorectal cancer. Br J Cancer 2006;94:1136–43. [PubMed: 16570047]

47). Wacker B, Nagrani T, Weinberg J, Witt K, Clark G, Cagnoni PJ. Correlation between development of rash and efficacy in patients treated with the epidermal growth factor receptor tyrosine kinase inhibitor in two large phase III studies. Clin Cancer Res 2007;13:3913–21. [PubMed: 17606725]

48). Yang Z, Hackshaw A, Feng Q, Fu X, Zhang Y, Mao C, Tang J. Comparison of gefitinib, erlotinib and afatinib in non-small cell lung cancer: a meta-analysis. Int J Cancer 2017;140:2805–19. [PubMed: 28295308]

49). Kashiwabara K, Semba H, Fujii S, Tsumura S. Outcome in advance non-small cell lung cancer patients with successful rechallenge after recovery from epidermal growth factor receptor tyrosine kinase inhibitor-induced interstitial lung disease. Cancer Chemother Pharmacol 2017;79:705–10. [PubMed: 28258422]

50). Qi WX, Sun YJ, Shen Z, Yao Y. Risk of interstitial lung disease associated with EGFR-TKIs in advanced non-small cell lung cancer: a meta-analysis of 24 phase III clinical trials. J Chemother 2015;27:40–51. [PubMed: 24724908]

51). Fujita K, Hirose T, Kusumoto S, Sugiyama T, Shirai T, Nakashima M, et al. High exposure to erlotinib and severe drug-induced interstitial lung disease in patients with non-small cell lung cancer. Lung Cancer 2014;86:113–4. [PubMed: 25123334]

52). Larsen CA, Dashwood RH. (−)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells. Arch Biochem Biophys 2010;510:52–7.

53). Blum CA, Xu M, Orner GA, Fong AT, Bailey GS, Stoner GD, et al. beta-Catenin mutation in rat colon tumors initiated by 1,2-dimethylhydrazine and 2-amino-3-methylimidazo[4,5-f]quinoline, and the effect of post-initiation treatment with chlorophyllin and indole-3-carbinol. Carcinogenesis 2001;22:309–14. [PubMed: 11181453]

**Prevention Relevance Statement:**

This investigation concludes that switching from continuous to once-per-week erlotinib, given at one-quarter of the current therapeutic dose, will exert good efficacy with standard of care sulindac against adenomatous polyps in the colon and small intestine, with clinical relevance for Familial Adenomatous Polyposis (FAP) patients before or after colectomy.

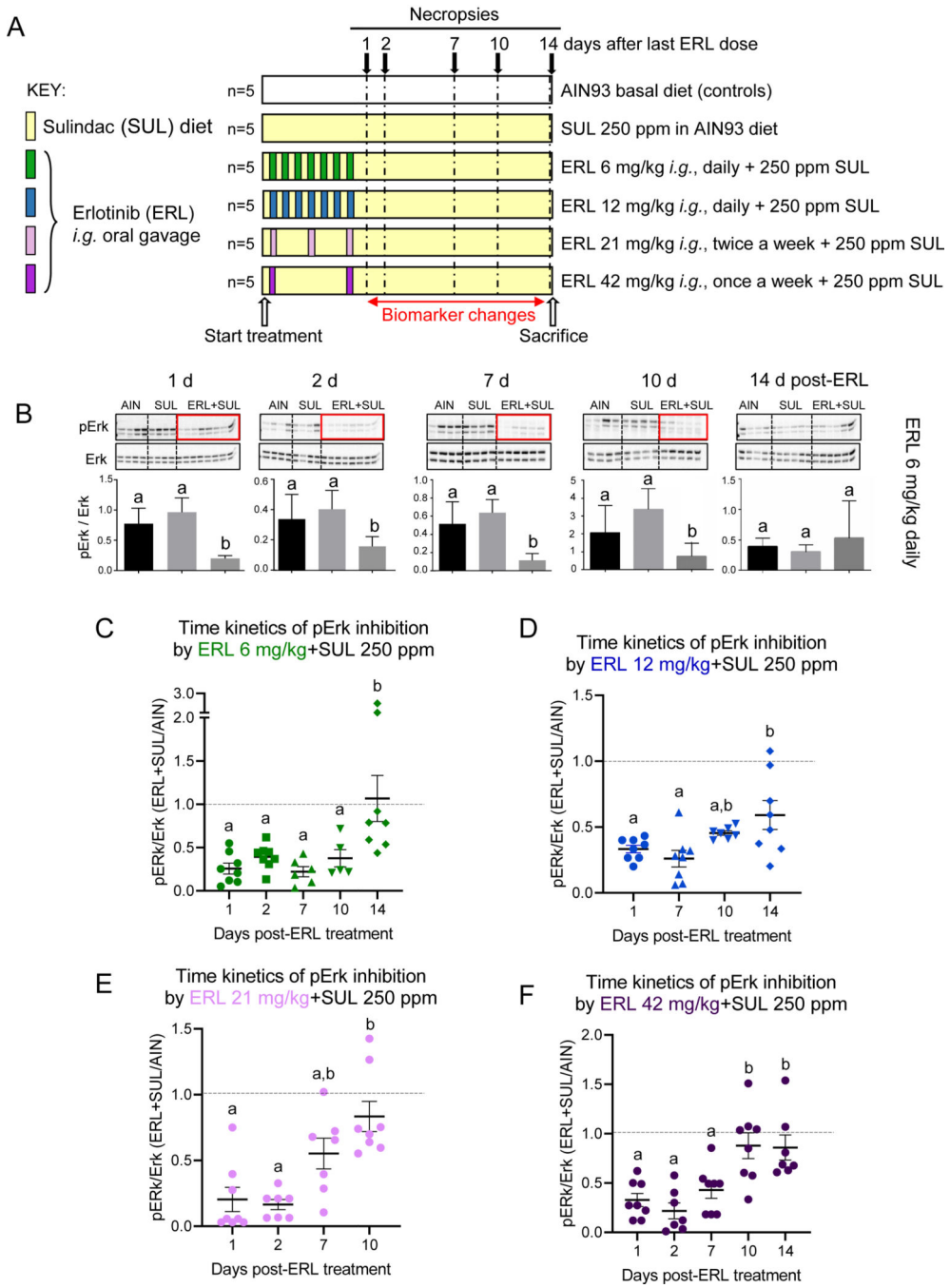


**Figure 1.**

Dose-response and time-course outcomes for biomarker modulation in Pirc colon tumors. A) Biomarker Study protocol, with n=5 rats per group. B) Immunoblotting of pErk normalized to Erk in Pirc colon tumors after animals were fed with AIN or SUL diets, or treated with dietary SUL plus intragastric (*i.g.*) ERL at 6 mg/kg body weight (ERL+SUL). Representative findings from two or more independent experiments. The corresponding densitometry data in panel B are shown as mean±SE; data bars sharing the same superscript letter were not significantly different by one-way analysis of variance (ANOVA). C-F)

Densitometry data were further analyzed at 6, 12, 21 and 42 mg/kg ERL±SUL, to determine the kinetics of pErk inhibition for up to 14 days post drug dosing, plotted as a ratio of the AIN control group in the corresponding immunoblot. Dotted line, normalized pErk/Erk level in AIN controls, given an arbitrary value of 1.0. Original uncropped immunoblotting images are presented in Supplementary Fig 1.

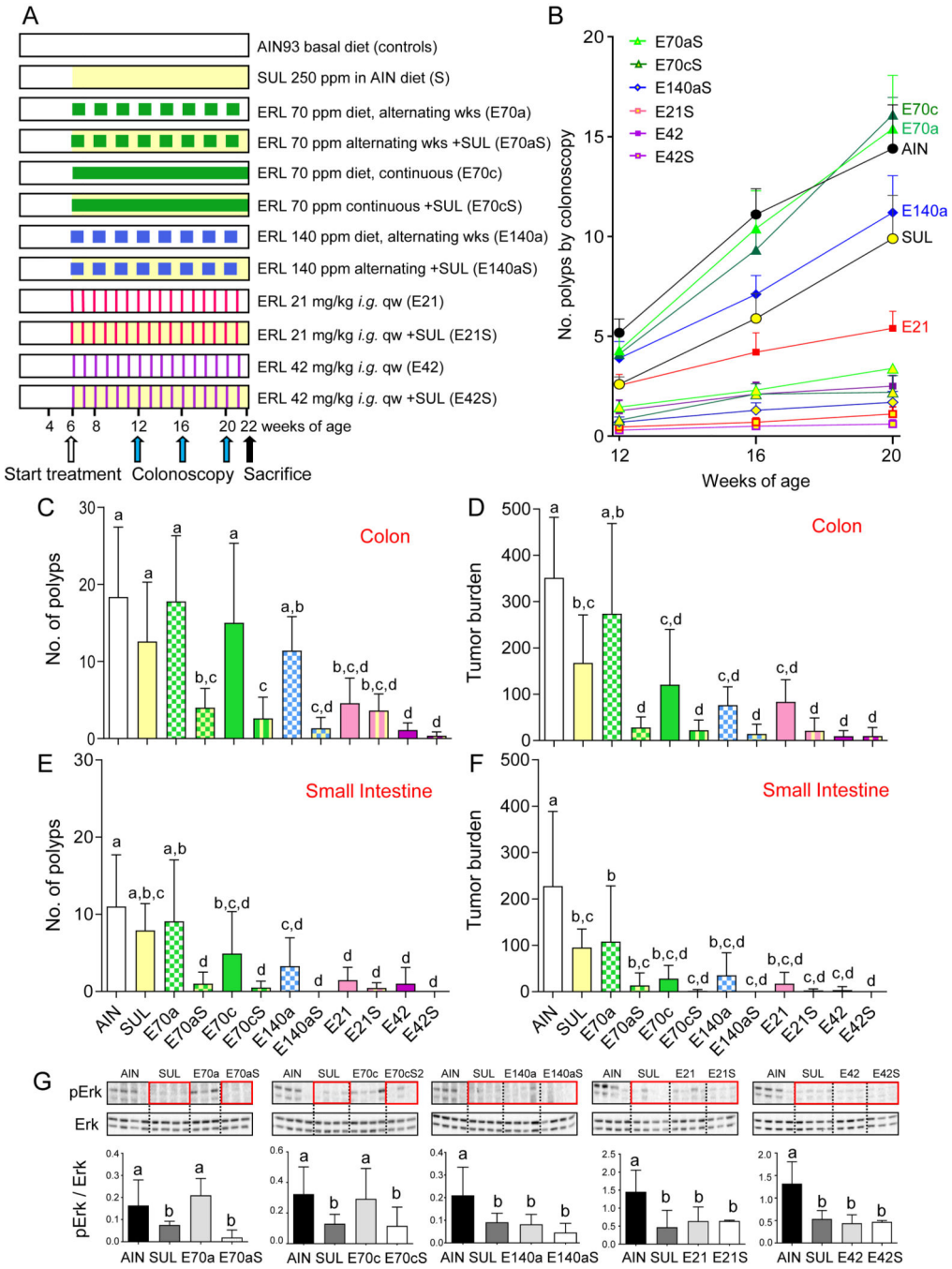


**Figure 2.**

Short-term assessment of antitumor efficacy of ERL±SUL in the Pirc model. A) Efficacy Study protocol, with n=10 rats per group. B) Temporal tracking of antitumor response by colonoscopy (mean±SE). C-F) Tumor outcomes determined at 16 weeks (mean±SD). G) Immunoblotting of pErk/Erk in colon tumors. Representative findings from two or more independent experiments, assaying the same set of negative and positive controls (AIN n=4, SUL n=4) within each panel. The corresponding densitometry data indicate mean±SE. In C-

G, bars sharing the same superscript letter were not significantly different by one-way ANOVA.

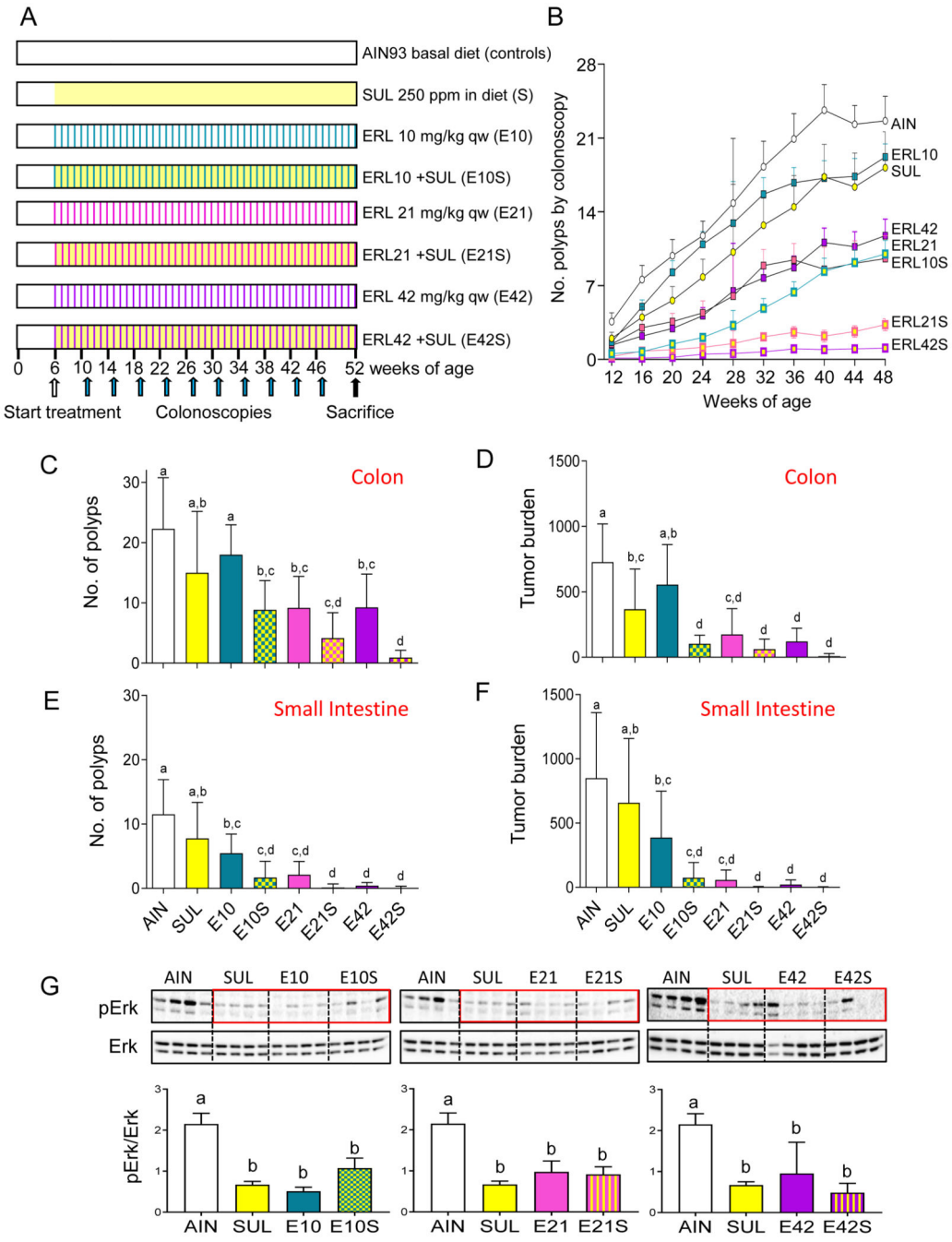


**Figure 3.**

Long-term assessment of antitumor efficacy in the Pirc model. A) Study protocol, with n=22 rats per group. B) Temporal tracking of antitumor response by colonoscopy (mean±SE). C-F) Tumor outcomes determined at 52 weeks (mean±SD). G) Immunoblotting of pErk/Erk in colon tumors. Representative findings from two or more independent experiments. The corresponding densitometry data indicate mean±SE. In C-G, bars sharing the same superscript letter were not significantly different by one-way ANOVA.

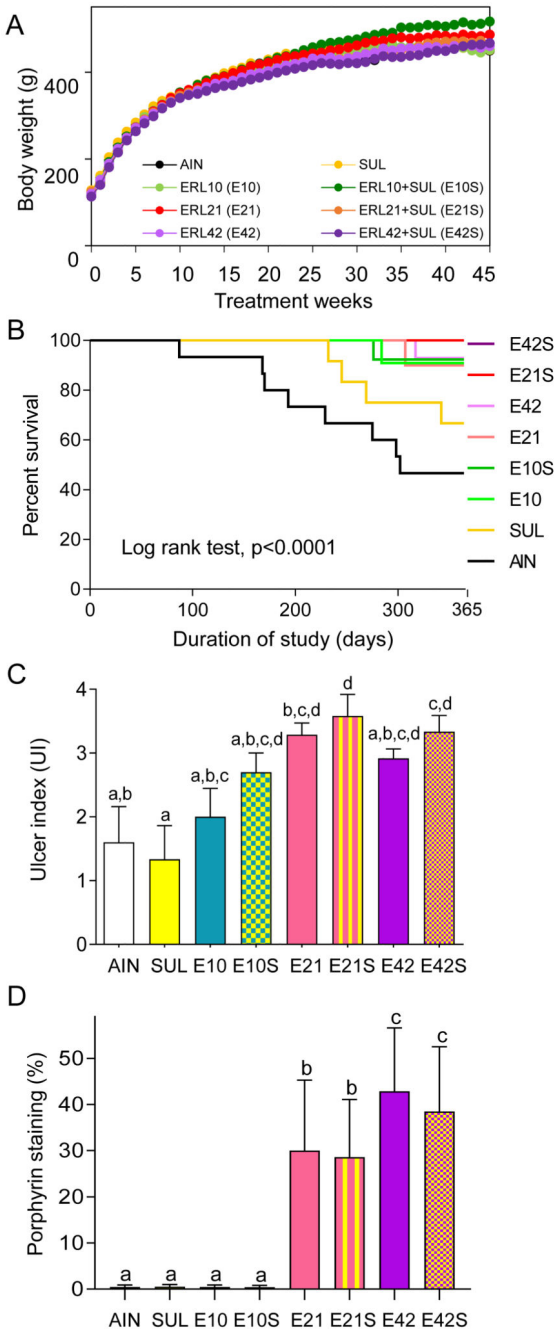


**Figure 4.**

Toxicity assessment for ERL±SUL in the Pirc model. A) Body weights in the various treatment groups. B) Kaplan-Meier curves for ERL±SUL treatment groups. C) Gastric ulceration (mean±SE) following drug treatments, with the Ulcer Index determined as described in Methods. D) Porphyrin staining around the eye, summarized as a percentage of animals in each group (mean±SE). In C) and D), bars sharing the same superscript letter were not significantly different by one-way ANOVA.

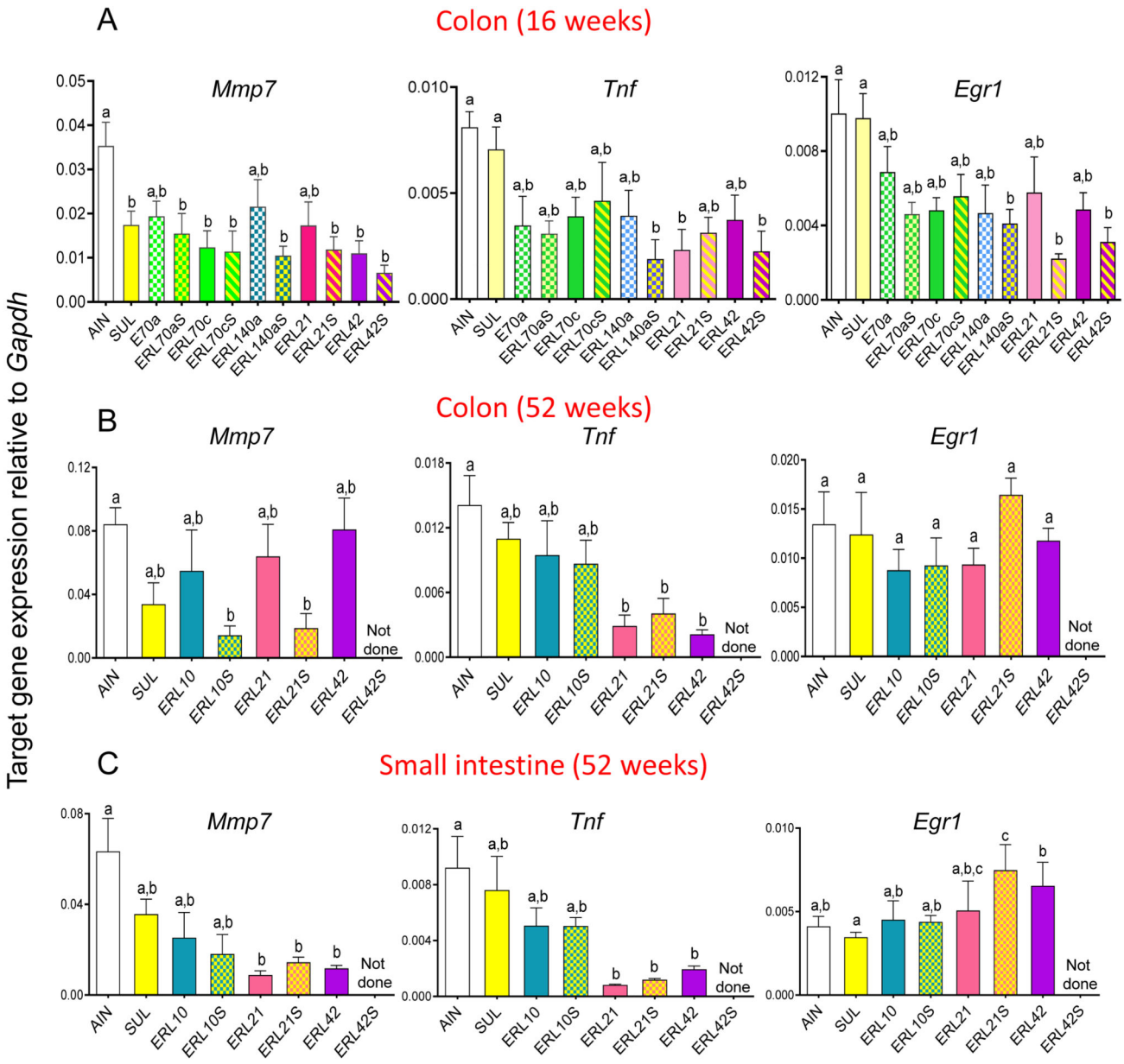


**Figure 5.**

Tumor-associated gene expression changes in the Pirc model following ERL±SUL treatment. A) Colon tumors from the Efficacy Study. B) Colon tumors and C) small intestine tumors from the Toxicity/Resistance Study. Representative RT-qPCR data (mean±SE) from two or more independent experiments, with each target normalized to *Gapdh*. In A) the data were obtained from n=6–9 polyps per group, whereas in B) and C) there were n=8 biological replicates, except for ERL21, ERL21+SUL and ERL42 groups (n=4 tumors per group). The ERL42+SUL group was not determined ('Not done'), due to scarce tissue availability after robust tumor suppression, and prioritization of pErk/Erk assessment (Figure 3G). Bars sharing the same superscript letter were not significantly different by one-way ANOVA.

**Table 1**

Clinical observations after 42 weeks of drug treatment

| Group | Rats in study | Rats at 1 yr | Eye/skin phenotype | Diarrhea | Blood in rectum | Weight loss (>10%) | Mortality |
|---|---|---|---|---|---|---|---|
| AIN | 14 | 7 | 0 | 4 | 7 | 7 | 7 |
| SUL | 14 | 10 | 0 | 2 | 7 | 5 | 4 |
| E10 | 14 | 10 | 0 | 2 | 6 | 1 | 1 (3*) |
| E10+SUL | 13 | 10 | 0 | 1 | 3 | 1 | 1 (2*) |
| E21 | 13 | 9 | **1** | 1 | 2 | 0 | 1 (3*) |
| E21+SUL | 14 | 14 | **5** | 0 | 3 | 0 | 0 |
| E42 | 14 | 12 | **2** | 1 | 2 | 1 | 1 (1*) |
| E42+SUL | 14 | 13 | **4** | 0 | 3 | 0 | 0 (1*) |

* Operator error during repeated *i.g.* dosing. Dashed box, GI-related toxicity due to high tumor number in the Apc-mutant genetic background; Bold, drug-related toxicity (see Figure 4).