

Effect of Zileuton and Celecoxib on Urinary LTE₄ and PGE-M Levels in Smokers

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Abstract

COX-2 and 5-lipoxygenase (5-LO) use arachidonic acid for the synthesis of eicosanoids that have been implicated in carcinogenesis and cardiovascular disease. The ability of celecoxib, a selective COX-2 inhibitor, to redirect arachidonic acid into the 5-LO pathway can potentially reduce its efficacy as a chemopreventive agent and increase the risk of cardiovascular complications. Levels of urinary prostaglandin E metabolite (PGE-M) and leukotriene E₄ (LTE₄), biomarkers of the COX and 5-LO pathways, are elevated in smokers. Here, we investigated the effects of zileuton, a 5-LO inhibitor, versus zileuton and celecoxib for 6 ± 1 days on urinary PGE-M and LTE₄ levels in smokers. Treatment with zileuton led to an 18% decrease in PGE-M levels ($P = 0.03$); the combination of zileuton and celecoxib led to a 62% reduction in PGE-M levels ($P < 0.001$). Levels of LTE₄ decreased by 61% in subjects treated with zileuton alone ($P < 0.001$) and were unaffected by the addition of celecoxib. Although zileuton use was associated with a small overall decrease in PGE-M levels, increased PGE-M levels were found in a subset (19 of 52) of subjects. Notably, the addition of celecoxib to the 5-LO inhibitor protected against the increase in urinary PGE-M levels ($P = 0.03$). In conclusion, zileuton was an effective inhibitor of 5-LO activity resulting in marked suppression of urinary LTE₄ levels and possible redirection of arachidonic acid into the COX-2 pathway in a subset of subjects. Combining celecoxib and zileuton was associated with inhibition of both the COX-2 and 5-LO pathways manifested as reduced levels of urinary PGE-M and LTE₄. *Cancer Prev Res*; 6(7); 646–55. ©2013 AACR.

Introduction

Cigarette smoking continues to be the leading cause of preventable death in the United States. The risk of lung cancer is increased in smokers with chronic lung injury (1). While primary prevention efforts through tobacco cessation are likely to be the most effective strategy for reducing disease, successful quit rates are at best around 30% (2). Given the recognized link between smoking, inflammation, and cancer, identifying inflammatory pathways that are

pharmacologically modifiable represents a bona fide approach to attempting to reduce the risk of smoking-related cancer.

Multiple lines of evidence suggest an important role for aberrant arachidonic acid metabolism in both inflammation and carcinogenesis. The current report is focused on 2 pro-inflammatory pathways involving the enzymes COX and 5-lipoxygenase (5-LO). COX-1, a constitutively expressed enzyme (3), and COX-2, an enzyme rapidly induced by inflammatory stimuli, tobacco carcinogens, and growth factors, catalyze the conversion of arachidonic acid to PGH₂ (4–7). PGH₂ is converted by microsomal prostaglandin E synthase to PGE₂ (8), which can regulate cell proliferation, apoptosis, angiogenesis, and immunosurveillance (9). Catabolism of PGE₂ is initiated by 15-hydroxyprostaglandin dehydrogenase and results in a stable end metabolite, 11- α -hydroxy-9, 15-dioxo-2, 3, 4, 5-tetranorprostane-1, 20-dioic acid (PGE-M) that is excreted in the urine (Fig. 1) and used as an index of systemic PGE₂ production (10–12).

Arachidonic acid can also be converted by 5-LO and its molecular partner, 5-LO-activating protein (FLAP), to leukotriene A₄ (LTA₄; ref. 13). LTA₄ is then conjugated to reduced glutathione by leukotriene C₄ synthase, forming LTC₄. The final biologically active metabolites of the 5-LOX pathway include LTB₄ and the cysteinyl-LTs (cys-LTs), LTC₄, LTD₄ and LTE₄. The cys-LTs are potent inflammatory

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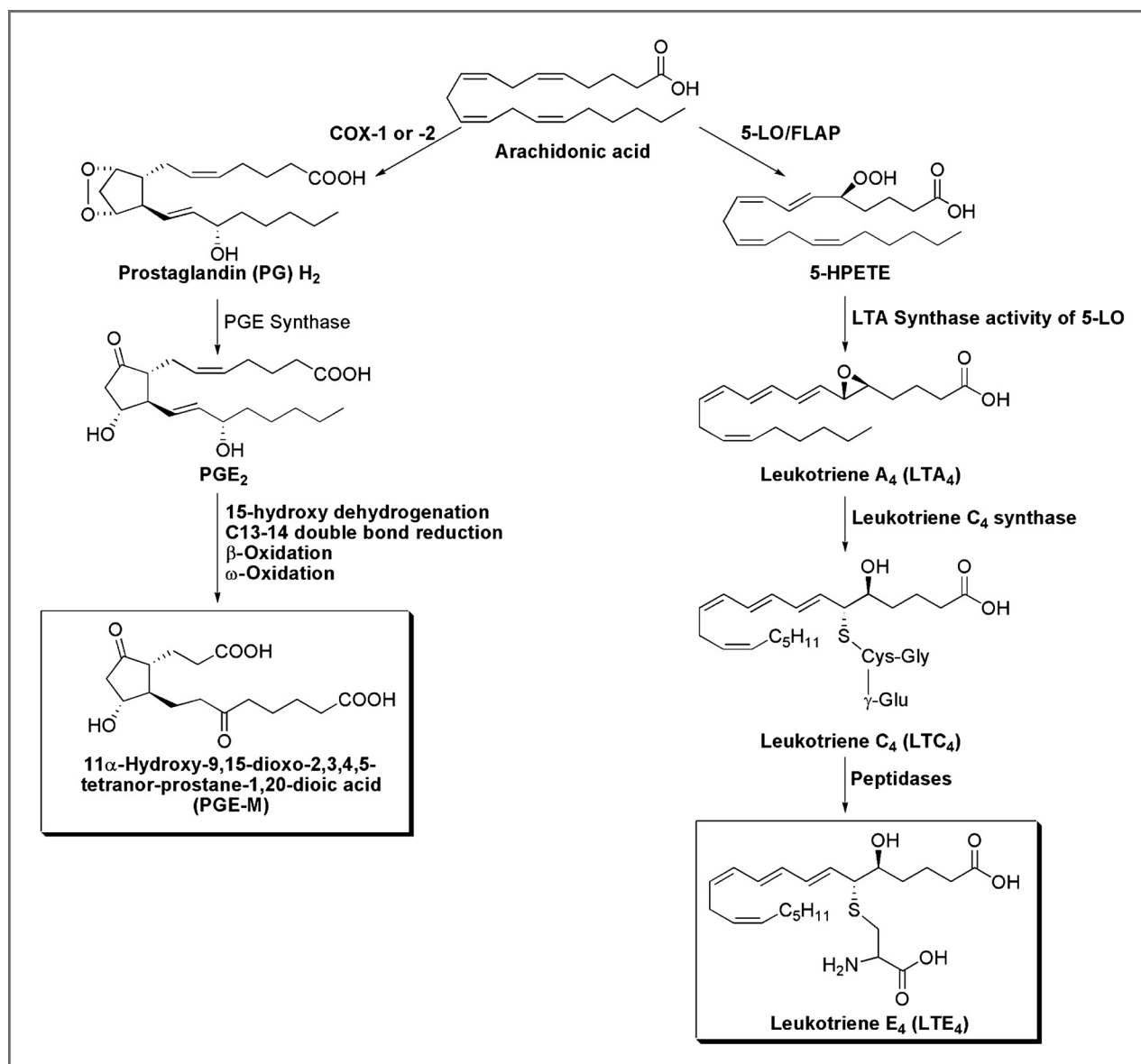


Figure 1. Synthesis of PGE-M and LTE₄ via COX and 5-LO pathways.

mediators that increase vascular permeability and stimulate smooth muscle contraction (14, 15). The cys-LTs, LTC₄ and LTD₄, are metabolized to the end product LTE₄, which is excreted in the urine without further modification (16, 17).

Selective inhibitors of both COX-2 and 5-LO possess chemopreventive activity in preclinical studies (9, 18, 19). Drug-induced shunting of arachidonic acid from the COX pathway into the 5-LO pathway or vice versa has been postulated to decrease the efficacy of these agents (20, 21). This possibility provided the rationale for combining agents that target each of the pathways to reduce shunting of arachidonic acid with the goal of improving drug efficacy. In fact, several preclinical studies have suggested that the combination of celecoxib, a selective COX-2 inhibitor, and zileuton, a 5-LO inhibitor, is more effective

than either agent alone in preventing tumor formation or inhibiting tumor growth (22–24). Translating these preclinical findings into the clinic represents a significant need. Mao and colleagues (25) showed that treatment of active smokers with celecoxib led to increased levels of LTB₄ in bronchoalveolar fluid, which suggested a drug-induced shunt of arachidonic acid into the 5-LO pathway. We previously reported that celecoxib suppressed COX-2 activity leading to reduced levels of urinary PGE-M in smokers; the reduction in urinary PGE-M was associated with increased levels of urinary LTE₄ in a subset of subjects (26).

The COX-2 and 5-LO pathways have been suggested to be important in the pathogenesis of cardiovascular disease in addition to carcinogenesis (27–33). The ability of celecoxib to redirect arachidonic acid into the 5-LO pathway has been

suggested to help explain the link between use of COX-2 inhibitors and cardiovascular complications (26, 34). Although both the COX-2 and 5-LO pathways are activated in smokers (26), the effects of celecoxib when combined with zileuton in human smokers are unknown. In the current study, we had 2 main objectives: (i) to determine whether zileuton redirects arachidonic acid into the COX pathway in association with suppression of urinary LTE₄ and (ii) to investigate whether combining celecoxib and zileuton suppresses shunting of arachidonic acid and thereby reduces both urinary PGE-M and LTE₄ levels. Our findings suggest that a COX-2 inhibitor can be combined with a 5-LO inhibitor to suppress the synthesis of both PGE-M and LTE₄ and thereby prevent the redirection of arachidonic acid into a competing pro-inflammatory pathway.

Materials and Methods

Study design

This was a randomized biomarker trial which evaluated the effects of 1,200 mg twice daily oral zileuton (Zyflo CR, Cornerstone Therapeutics) or this dose of zileuton in combination with 200 mg twice daily oral celecoxib (Celebrex, Pfizer) for 6 ± 1 days in healthy current smokers. The protocol was approved by the Weill Cornell Medical College Institutional Review Board and Clinical and Translational Science Center (New York, NY) and conducted in accordance with an assurance filed with and approved by the Department of Health and Human Services. All subjects provided written informed consent for participation.

Participant selection

Eligible subjects were required to be 18 years of age or older, healthy [Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1] current smokers with ≥10 pack-years of smoking. The exclusion criteria included active cancer, history of cancer, chronic inflammatory conditions, ongoing or active infection, or use of certain medications (e.g., celecoxib, zileuton, corticosteroids, nonsteroidal anti-inflammatory drugs, drugs known to interact with celecoxib or zileuton, or other investigational drugs) within 30 days of consent, contraindication to celecoxib or zileuton (pregnancy, peptic ulcer disease, sulfa allergy, prior cardiovascular events), kidney or liver disease.

Study schema, treatment, and study assessments

After signing informed consent, participants underwent a baseline evaluation that included smoking and concomitant medication assessment and liver functions tests and a pregnancy test, if applicable. Upon confirmation of eligibility, participants were randomized and presented for the baseline full medical assessment including physical examination. A single void urine specimen was collected (Fig. 2A). The trial consisted of 2 arms (administration of zileuton 1,200 mg twice daily; combined administration of zileuton 1,200 mg twice daily plus celecoxib 200 mg twice daily) with subjects randomized at a 3:1 ratio to receive either zileuton alone or the combination of zileuton and

celecoxib, respectively. These doses of zileuton and celecoxib are the maximum recommended for the treatment of asthma and arthritis, respectively. After the initial urine sample was collected, subjects were randomized to 1 of the 2 treatment arms and received study agents for 6 ± 1 days. This length of treatment was chosen to be certain steady-state levels of drug were achieved. At day 6 (±1), urine and blood were collected. Toxicity was monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Urine was aliquoted into 2-mL cryovials and stored at -80°C. Plasma was prepared from blood and stored at -80°C.

Study endpoints

Urine was analyzed for PGE-M and LTE₄. Posttreatment plasma specimens were analyzed for zileuton and celecoxib levels as a measure of drug compliance. All measurements were carried out in a blinded manner.

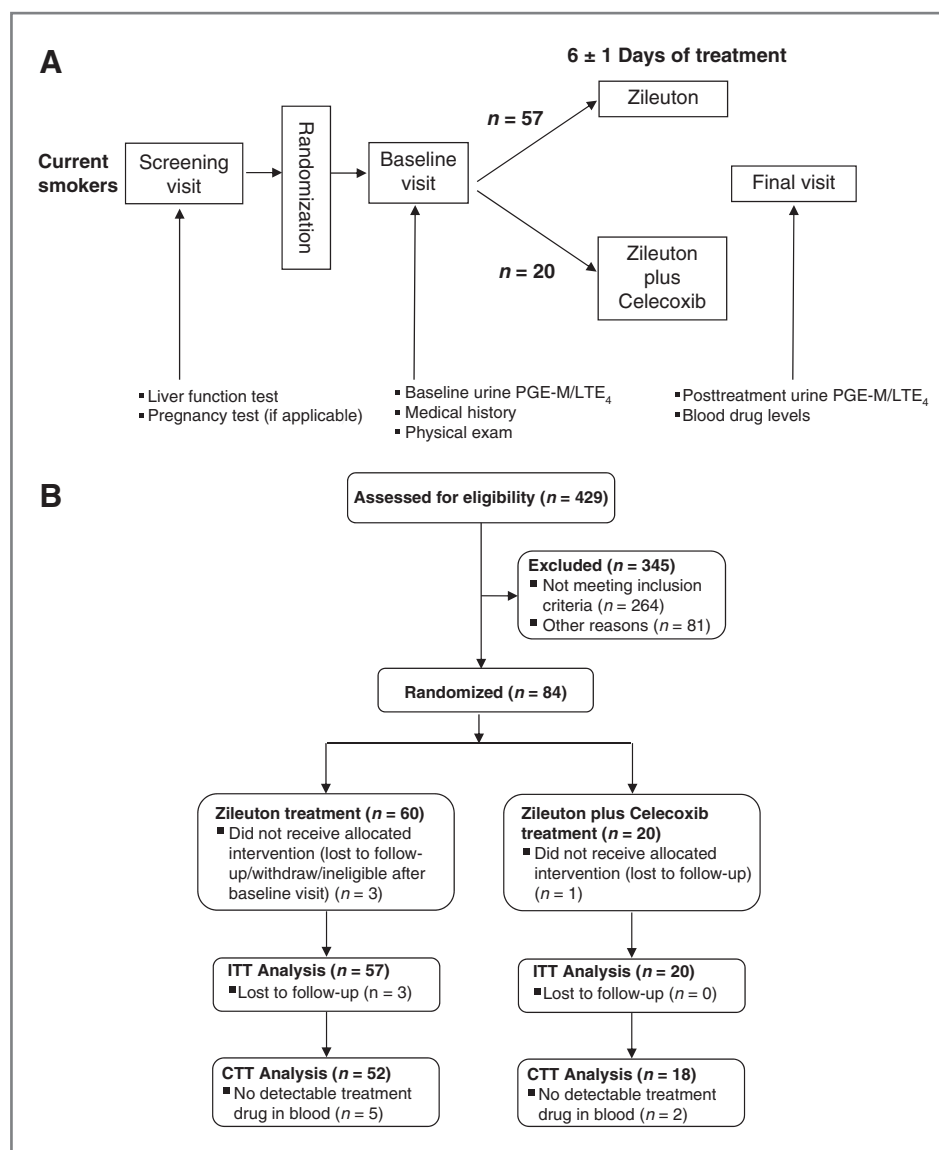
Urinary PGE-M

PGE-M and d₆-PGE-M were custom synthesized by Dr. Doug Taber and colleagues (35). C-18 Sep-Pak extraction cartridges were purchased from Waters Corporation. Methyloxime HCl was purchased from Sigma Aldrich. All other organic reagents were of high-performance LC quality and purchased from EM Sciences.

Sample preparation. Urine samples were removed from -80°C storage and allowed to thaw at room temperature. One milliliter of sample was then acidified to pH 3 with 1 mol/L HCl, and endogenous PGE-M was then converted to the O-methyloxime derivative by treatment with 0.5 mL of 16% (w/v) methyloxime HCl in 1.5 mol/L sodium acetate buffer (pH 5). Following 1-hour incubation, the methoximated PGE-M was diluted with 10-mL water adjusted to pH 3, and the aqueous sample was applied to a C-18 Sep-Pak that had been preconditioned with 5 mL methanol and 5 mL water (pH 3). The Sep-Pak was washed with 20 mL water (pH 3) and 10 mL heptane. PGE-M was then eluted from the Sep-Pak with 5 mL ethyl acetate, and any residual aqueous material was removed from the eluate by aspiration. The [²H₆]-O-methyloxime PGE-M internal standard (6.2 ng in 10 μL ethanol) was then added, and the eluate was evaporated under a continuous stream of nitrogen at 37°C. The dried residue was resuspended in 50 μL (20 μL injections) 95:4.9:0.1 (v/v/v) 5 mmol/L ammonium acetate:acetonitrile:acetic acid and was filtered through a 0.2-μm Spin-X filter (Corning).

Liquid chromatography/tandem mass spectrometry. Samples were analyzed using liquid chromatography/tandem mass spectrometry (LC/MS-MS) with slight modifications to the method previously described by Murphey and colleagues (12). LC was conducted on a 2.0 × 50 mm², 1.7-μm particle Acquity CSH Phenyl-Hexyl column (Waters Corporation). Mobile phase A was 95:4.9:0.1 (v/v/v) 5 mmol/L ammonium acetate:acetonitrile:acetic acid, and mobile phase B was 10.0:89.9:0.1 (v/v/v) 5 mmol/L ammonium acetate:acetonitrile:acetic acid. Gradient elution was

Figure 2. Study procedures and accrual. A, study schema. B, flow diagram of subjects who were accrued to the study. CTT, compliant-to-treatment; ITT, intent-to-treat.



conducted with 5% B for 1 minute, a linear increase to 11% B until 1.1 minute, a linear increase to 17% B until 30 minutes, a step to 60% B at 31 minutes, hold for 3 minutes at 60% B, a step to 100% B at 34.1 minutes, hold for 3 minutes at 100% B, and re-equilibration from 37.1 minutes to 40 minutes with 5% B. The flow rate was set to 200 μ L/min and the column was maintained at room temperature.

The analytes were detected on a Thermo Scientific Quantum Vantage triple quadrupole mass spectrometer fitted with an electrospray ion source operating in the negative ion mode using selected reaction monitoring (SRM). The following SRM reactions were monitored: PGE-M (m/z 385 \rightarrow 336) and d₆-PGE-M (m/z 391 \rightarrow 339) using collision energy of 20eV under 1.5 mT argon gas. Endogenous levels were calculated by comparing the ratio of the peak heights of endogenous PGE-M with that of the deuterated internal standard.

Urinary LTE₄

20,20,20-²H₃-LTE₄ was purchased from Enzo Life Sciences. Empore SD C-18 extraction cartridges (standard density, 6 mL capacity, 3M) were obtained from VWR International and Thermo Fisher Scientific. All organic reagents were of high-performance LC quality and purchased from EM Sciences.

Sample preparation. Urine samples were removed from -80°C storage and allowed to thaw at room temperature. Three milliliters of sample was then acidified to pH 3 with 1 mol/L HCl. To the acidified urine was added the internal standard, [²H₃]-LTE₄. The sample was next applied to an Empore C-18 solid-phase extraction column that had been prewashed with methanol (6 mL) and 0.001 N HCl (6 mL). The column was subsequently washed with 0.001 N HCl (6 mL), methanol/10 mmol/L ammonium acetate buffer (pH 5.6) (1/9, v/v, 6 mL), and ethyl acetate/heptane (1/1, v/v,

6mL). The analyte was eluted with methanol (1 mL). The eluate was evaporated under a continuous stream of dry nitrogen, then dissolved in 100 μ L methanol and filtered using a 0.2- μ m Spin-X filter (Corning). The sample was again dried under a stream of nitrogen and then dissolved in 40 μ L methanol (20 μ L injections) for analysis by UPLC/ESI-MS/MS.

UPLC/MS-MS. UPLC was conducted on a 2.1×50 mm², 1.7- μ m particle Acquity UPLC BEH C-18 column (Waters Corporation) attached to an Acquity UPLC (Waters Corporation; ref. 36). Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile. Gradient elution was conducted with 5% B for 1 minute, a linear increase to 53% B until 9.5 minutes, a linear increase to 76% B until 11 minutes, a step to 100% B until 11.1 minutes, hold for 1 minute at 100% B, and re-equilibration from 12.1 to 14 minutes with 5% B. The flow rate was set to 600 μ L/min, and the column temperature was maintained at 30°C.

The analytes were detected on a Thermo Scientific Quantum Vantage triple quadrupole mass spectrometer fitted with an electrospray ion source operating in the negative ion mode using SRM. The following SRM reactions were monitored: LTE₄ (m/z 438 \rightarrow 333) and d₃-LTE₄ (m/z 441 \rightarrow 336) using a collision energy of 20 eV under 1.5 mT argon gas. Endogenous levels were calculated by comparing the ratio of the peak heights of endogenous LTE₄ with that of the deuterated internal standard.

Urine creatinine

Creatinine (Cr) in the urine was measured by a chemical assay based on Jaffe's reaction according to the manufacturer's instructions (Enzo Life Sciences) for normalization of PGE-M and LTE₄. Levels of both PGE-M and LTE₄ are expressed per mg Cr.

Drug levels

Celecoxib was provided by the National Cancer Institute and celecoxib-d4 was obtained from TRC. Zileuton was obtained from Sigma-Aldrich and zileuton-d4 was obtained from Santa Cruz Biotechnology. Blank human plasma was obtained from Innovative Research. Solvents were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific.

Sample preparation. Plasma samples were removed from -80°C storage, allowed to thaw on ice, and vortexed. One hundred microliters of thawed sample was added to 250 μ L acetonitrile containing both deuterated internal standards and immediately vortexed. The samples were then centrifuged at 5,000 r.c.f. for 2 minutes. The supernatants were transferred to autosampler vials, diluted 1:1 (v:v) with H₂O, and analyzed via LC-MS.

LC/MS-MS. Analytes were chromatographed on a Supelco C18 column (50 \times 2 cm², with a C18 guard cartridge) using the following gradient: 50% B to 90% B over 4.0 minutes followed by a 0.4-minute hold at 90% B. The column was re-equilibrated at 50% B for at least 3 minutes before each injection. The flow rate was 0.375

mL/min. Component A was 10 mmol/L ammonium acetate (aqueous) and B was 1:1 methanol: acetonitrile with 10% A.

The analytes were detected on a Thermo Quantum triple quadrupole mass spectrometer via SRM. The mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive ion mode. The following SRM reactions were used: celecoxib (m/z 380 \rightarrow 316); celecoxib-d4 (m/z 384 \rightarrow 320); zileuton (m/z 237 \rightarrow 161); zileuton-d4 (m/z 241 \rightarrow 165; the Q1 masses represent [M + H]⁺ complexes). Unknown samples were quantitated against a standard curve prepared in blank human plasma and extracted and analyzed concurrently with the unknown samples. The standard curves were constructed by plotting the analyte response (ratio of the analyte peak area to its corresponding deuterated internal standard peak area) against the concentration of the standards.

Statistical analysis

Demographic and smoking characteristics of the subjects were compared between treatment arms using methods appropriate for the type of data. For age, the 2-sample Student *t* test was used to compare the means. For smoking intensity measured by pack-years, the nonparametric Wilcoxon rank-sum test was used. For the categorical variables, Fisher exact test was used to compare the differences in proportions.

PGE-M and LTE₄ values were analyzed primarily using nonparametric tests and reported in terms of median (range). Differences in baseline levels of PGE-M and LTE₄ between 2 treatment arms were examined using the Wilcoxon rank-sum test. Pre/postchange in PGE-M and LTE₄ levels following a given treatment for subjects in a specific treatment arm was evaluated using Wilcoxon signed rank test. Magnitude of change between 2 treatment arms was compared using Wilcoxon rank-sum test.

Consistent results were obtained for log-transformed data when corresponding parametric methods were used. Further analyses adjusting for age, gender, and race were carried out using multiple regression for log-transformed PGE-M and LTE₄ data. Age, gender, and race had no effect on any of the reported results.

Results

The trial opened in April 2010 with the last participant completing treatment in September 2011. The screening and accrual to this study has been described previously (ref. 37; Fig. 2B). A total of 84 subjects were enrolled in the trial and randomized at a 3:1 ratio to receive either zileuton alone or the combination of zileuton and celecoxib. Four subjects withdrew for different reasons before beginning study medication. Eighty subjects were started on the study medication(s). Seventy-seven subjects completed the entire study. The 3 subjects who failed to complete the study withdrew for personal reasons. The study medications were undetectable in the plasma of 7 of 77 subjects (9%) who completed the trial. This indicated noncompliance with the

Table 1. Patient characteristics

	Compliant-to-treatment sample			P
	All (N = 70)	Zileuton (n = 52)	Zileuton + celecoxib (n = 18)	
Age, y				
Mean ± SD	43.6 ± 9.1	43.8 ± 9.6	43.1 ± 7.6	0.76
Gender				0.58
Female	27 (39%)	19 (37%)	8 (44%)	
Male	43 (61%)	33 (63%)	10 (56%)	
Race, n (%)				0.07
Asian	2 (3%)	0 (0%)	2 (11%)	
Black	35 (50%)	28 (54%)	7 (39%)	
White	33 (47%)	24 (46%)	9 (50%)	
Smoking (pack-years)				
Median (range)	19.5 (10–68)	19.5 (10–68)	19.5 (10–40)	0.59
Baseline PGE-M, ng/mg Cr				
Median (range)	12.6 (1.4–50.4)	12.8 (1.4–50.4)	10.2 (2.3–35.1)	0.61
Baseline LTE ₄ , pg/mg Cr				
Median (range)	102.5 (4–268)	107 (4–268)	86.5 (37–250)	0.42

study medication and led to exclusion of these 7 subjects in the analysis of treatment effect. Hence, data from 70 subjects who completed the treatment phase of the study and had measurable levels of drug in plasma were included in the treatment effect analyses. The urine of 2 subjects in the zileuton + celecoxib arm contained interfering substances possessing the same *m/z* as urinary PGE-M precluding analysis; these 2 study subjects were excluded in the analysis related to PGE-M. A description of the characteristics of the 70 subjects who were compliant to treatment is shown in Table 1. Subjects in the 2 arms of the study were well matched by age, gender, and pack-years of smoking.

Effect of celecoxib and zileuton versus zileuton alone on urinary PGE-M

Treatment with zileuton led to a small (18%) but statistically significant decrease in median levels of PGE-M ($P = 0.03$; Fig. 3A). The combination of celecoxib and zileuton led to a 62% reduction in median levels of PGE-M ($P < 0.001$; Fig. 3B). As shown in Fig. 3C, the magnitude of the decrease in PGE-M was greater in subjects who received celecoxib combined with zileuton versus zileuton alone ($P < 0.001$).

Effect of celecoxib and zileuton versus zileuton alone on urinary LTE₄

Median levels of LTE₄ decreased by 61% in participants treated with zileuton alone ($P < 0.001$; Fig. 4A). Treatment with the combination of zileuton and celecoxib led to approximately a 65% reduction in median levels of urinary LTE₄ ($P < 0.001$; Fig. 4B). The combination of zileuton and celecoxib was no more effective than zileuton alone in suppressing levels of urinary LTE₄ (Fig. 4C).

Celecoxib protects against an increase in urinary PGE-M in a subset of zileuton-treated subjects

As mentioned above, arachidonic acid is a substrate for both the COX and 5-LO pathways (Fig. 1). In theory, zileuton, a 5-LO inhibitor, might divert arachidonic acid into the COX pathway resulting in elevated levels of urinary PGE-M in a subset of subjects. Overall zileuton led to small decrease in urinary PGE-M levels ($n = 52$; Fig. 3A). However, a subset of subjects had increased levels of PGE-M following treatment where the median (range) of the increase was 3.4 (0.13–17.1) ng/mg Cr. In addition, the proportion of subjects with increased PGE-M levels following treatment was significantly higher among those treated with zileuton alone, 37% (19 of 52), than that among those treated with celecoxib in combination with zileuton, 6.2% (1 of 16), $P = 0.03$ (Fig. 5). Thus, the addition of celecoxib to zileuton protected against the increase in urinary PGE-M. Treatment with celecoxib has been reported to increase levels of urinary LTE₄ (26). Hence, we hypothesized that the addition of zileuton to celecoxib would protect against this effect. Levels of LTE₄ were quantified at baseline and following treatment with the combination of celecoxib and zileuton. Interestingly, LTE₄ levels increased in only 1 of 18 subjects following treatment with this combination regimen.

Safety and tolerability

Both zileuton and the combination of zileuton and celecoxib were well-tolerated with no reports of serious adverse events, and no statistically significant differences between the adverse event profiles of the 2 treatment arms. One grade III adverse event (sore throat), which was felt to be unlikely to be related to study drug, occurred in the zileuton arm. Thirteen adverse events that were deemed to be potentially related to drug treatment were reported in the

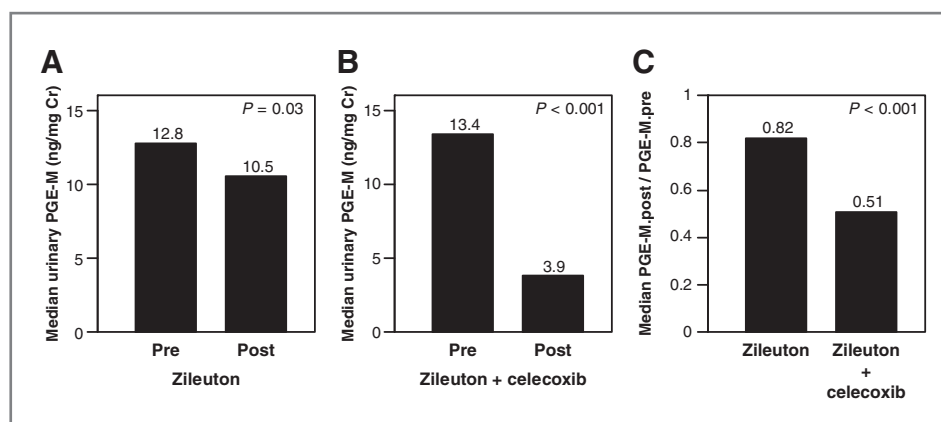


Figure 3. Combining celecoxib and zileuton is more effective than zileuton alone in reducing levels of urinary PGE-M. A, treatment with zileuton alone led to a small yet statistically significant decrease in urinary PGE-M levels [median (range)] (ng/mg creatinine) [pre PGE-M = 12.8 (1.4–50.4) vs. post PGE-M = 10.5 (2.0–34.6); $P = 0.03$, Wilcoxon signed rank test] among study participants compliant to treatment ($n = 52$). B, treatment with zileuton and celecoxib led to significant decrease in urinary PGE-M levels [pre PGE-M = 13.4 (2.3–35.1) vs. 3.9 (1.2–10.1); $P < 0.001$, Wilcoxon signed rank test] among study participants compliant to treatment and with evaluable urine samples at both time points ($n = 16$). C, significantly greater decrease in urinary PGE-M levels in terms of post/pre-fold change [median (range)] was observed in subjects treated with zileuton and celecoxib combined therapy compared with subjects treated with zileuton alone [0.51 (0.11–1.04) vs. 0.82 (0.31–6.82); $P < 0.001$, Wilcoxon rank-sum test].

2 arms (9 in the zileuton arm and 4 in the combination arm). One grade II headache occurred in the zileuton arm. All other reported adverse events were grade I in severity and included abdominal pain, diarrhea, nausea, dizziness, fatigue, headache, increased lacrimation, and postnasal drip.

Discussion

Numerous studies have suggested a role for both COX-2 and 5-LO in inflammation and carcinogenesis (9, 14, 18–24, 38–40). Previously, we carried out a study in human subjects and showed that celecoxib could redirect arachidonic acid into the 5-LO pathway resulting in enhanced urinary LTE₄ levels (26). In theory, both the anti-inflammatory and chemopreventive properties of celecoxib may be compromised in cells and tissues in which arachidonic

acid is shunted into the 5-LO pathway. Therefore, co-administration of an inhibitor of 5-LO, which inhibits the synthesis of leukotrienes, may increase the utility of a COX-2 inhibitor in conditions in which this type of shunt occurs.

In the current study, we compared the effects of zileuton versus the combination of zileuton and celecoxib in smokers. As predicted, zileuton was an effective inhibitor of 5-LO and markedly suppressed urinary LTE₄ levels (Fig. 4A). However, the effects of zileuton on urinary PGE-M were more complex. Overall, zileuton led to a small but statistically significant decrease in urinary PGE-M levels (Fig. 3A). This finding fits with prior evidence that zileuton suppressed PGE₂ levels in bronchoalveolar fluid following exposure to an allergen (41). Recently, zileuton was reported to inhibit the translocation of phospholipase A₂

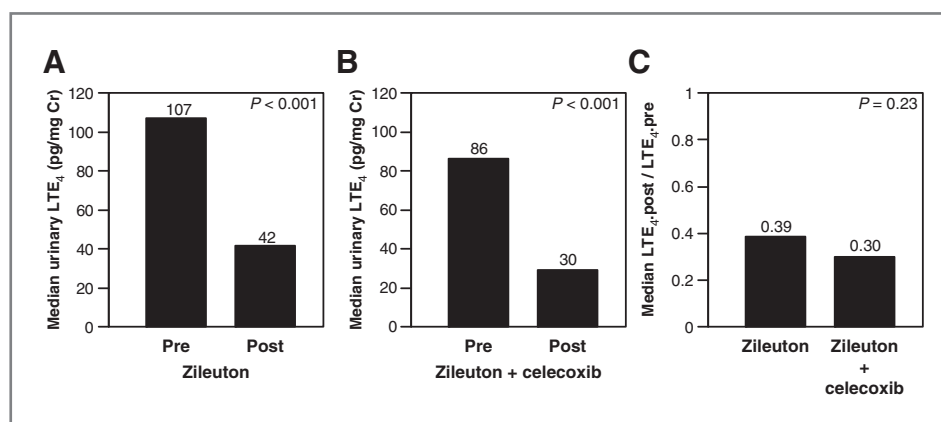


Figure 4. Adding celecoxib to zileuton does not enhance the suppression of urinary LTE₄. A, treatment with zileuton alone led to significant decrease in urinary LTE₄ levels [median (range)] (ng/mg creatinine) [pre LTE₄ = 107 (4–268) vs. post LTE₄ = 42 (4–292); $P < 0.001$, Wilcoxon signed rank test] among study participants compliant to treatment ($n = 52$). B, treatment with zileuton and celecoxib led to significant decrease in urinary LTE₄ levels [pre LTE₄ = 86 (37–250) vs. post LTE₄ = 30 (10–84); $P < 0.001$, Wilcoxon signed rank test] among study participants compliant to treatment ($n = 18$). C, similar magnitude of decrease in urinary LTE₄ levels in terms of post/pre-fold change [median (range)] was observed in subjects treated with zileuton and celecoxib combined therapy compared with subjects treated with zileuton alone [0.30 (0.12–1.01) vs. 0.39 (0.05–4.00); $P = 0.23$, Wilcoxon rank-sum test].

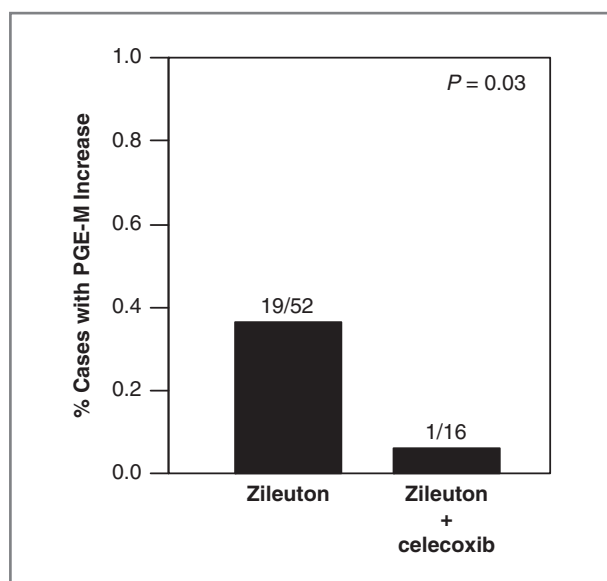


Figure 5. Celecoxib protects against an increase in urinary PGE-M in a subset of zileuton-treated subjects. Proportion of cases with a posttreatment increase in urinary PGE-M levels was significantly smaller in those treated with zileuton and celecoxib combined therapy compared with those treated with zileuton alone [6% (1 of 16) vs. 37% (19 of 52); $P = 0.03$, Fisher's exact test].

to cellular membranes resulting in decreased arachidonic acid release, which can explain the small decrease in urinary PGE-M that we observed (42). Alternatively, if zileuton had an anti-inflammatory effect, the small decrease in PGE-M might be explained by a decrease in the number of inflammatory cells in the lungs of smokers. Originally, we had hypothesized that zileuton might shunt arachidonic acid into the COX pathway resulting in increased levels of urinary PGE-M in a subset of patients. Although treatment with zileuton led to an overall reduction in urinary PGE-M levels, use of zileuton was associated with increased urinary PGE-M levels in 19 of 52 subjects, thereby suggesting that a shunt may occur in a minority of individuals (Fig. 5). Future studies are warranted to elucidate the mechanistic basis for this heterogeneous effect.

Neither imaging nor pulmonary function tests were carried out in this study. Hence, one possible contributor to the observed heterogeneity could be variability in the severity of underlying lung disease. Additional possibilities include genetic differences in key enzymes involved in arachidonic acid metabolism [e.g., single-nucleotide polymorphisms (SNP)] as well as effects of bioactive food components on these pathways. For instance, Norris and Dennis recently reported the effects of omega-3 fatty acids on inhibition of COX and shunting of arachidonic acid to lipoxygenase pathways (43). This raises the possibility that various dietary constituents affecting arachidonic acid metabolism could be partially responsible for the heterogeneity seen in this study. Regardless of the underlying mechanism, the addition of celecoxib to zileuton protected against the increase in urinary PGE-M found in individuals who

received zileuton alone (Fig. 5). Consistent with its known mechanism of action, the selective COX-2 inhibitor celecoxib when combined with zileuton suppressed urinary PGE-M levels (Fig. 3C) without modulating LTE₄ levels (Fig. 4C). Taken together, it appears that combining a COX-2 inhibitor with a 5-LO inhibitor prevents the shunting of arachidonic acid that can be observed in a subset of individuals when either a COX-2 inhibitor or 5-LO inhibitor is used alone. Selected cytochrome P450s can also metabolize arachidonic acid to eicosanoids (44). On the basis of the current results, it will be of considerable interest to determine whether inhibition of the COX-2 and 5-LO pathways alters P450-mediated arachidonic acid metabolism. In theory, targeting 2 pathways that use arachidonic acid as a substrate could result in increased levels of free arachidonic acid. Increased levels of free arachidonic acid can stimulate apoptosis in model systems but whether this occurs in humans is uncertain (45).

Use of selective COX-2 inhibitors has been associated with an increased risk of cardiovascular complications (27, 28). A number of different mechanisms may be important for understanding this risk. One possibility is that selective COX-2 inhibitors block the production of cardioprotective prostacyclin without inhibiting COX-1–dependent platelet thromboxane A₂ synthesis, supporting a prothrombotic mechanism (29, 46). Drug-induced redirection of arachidonic acid into the 5-LO pathway could also be important for understanding the cardiovascular toxicity associated with selective COX-2 inhibitors. Both COX-2 and 5-LO are expressed in atherosclerotic plaques (47, 48). 5-LO has been linked to atherosclerosis in some mouse models (30). Recently, disruption of the 5-LO pathway was found to attenuate the proatherogenic effects of COX-2 deletion in hyperlipidemic mice (49). This finding lends further support for the idea that the cardiovascular risk associated with use of COX-2 inhibitors might be reduced by co-administering an agent that targets the 5-LO pathway. Another recent preclinical study found that treatment with a selective 5-LO inhibitor protected against the reduction of renal blood flow that was induced by COX-2 inhibition (50). In the current study, we provide clear evidence in human smokers that celecoxib and zileuton can be combined to reduce both COX-2 and 5-LO activity.

In summary, preclinical evidence suggests that targeting the 5-LO pathway may both augment the antitumor activity of COX-2 inhibition and potentially reduce the cardiovascular toxicity associated with COX-2 inhibition. The results of the current study highlight the feasibility of using a 5-LO inhibitor in combination with a selective COX-2 inhibitor to inhibit the activities of both pro-inflammatory pathways. Future studies will be required to determine whether inhibitors of the 5-LO pathway can either increase the chemopreventive activity or decrease the cardiovascular risk associated with use of COX-2 inhibitors. This strategy could be relevant in both former and current smokers (40).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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