

Clinical safety and efficacy of NG440: a novel combination of rho iso-alpha acids from hops, rosemary, and oleanolic acid for inflammatory conditions¹

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Abstract: In this report, we examine the clinical safety and efficacy of NG440, a phytochemical-based antiinflammatory formula consisting of a combination of rho iso-alpha acids from hops, rosemary, and oleanolic acid. In a previous study, we demonstrated that NG440 significantly decreased pain by 50% in patients with osteoarthritis. Consistent with these data, results from a multicentre trial indicate that NG440 reduced pain scores in patients with joint discomfort, as measured by VAS (visual analog scale) methodology. As demonstrated in an ex vivo clinical study, these effects on pain relief may be due to reduced inflammatory cytokine production including lower prostaglandin E₂ formation. Finally, strong data exist to suggest that NG440 is a safe formula for human consumption. Animal toxicity data revealed no adverse effects of NG440 at dosages ≤ 250 mg·kg⁻¹·day⁻¹ for 21 days. Furthermore, human trial data suggest that NG440 does not negatively impact cardiovascular and gastrointestinal markers normally affected by selective COX-2 enzyme inhibitors, including platelet function, blood pressure, blood cell count, or fecal calprotectin, a measure of gastrointestinal injury. In conclusion, NG440 may serve as a safe and efficacious alternative in some areas where specific COX-2 inhibitors have been traditionally used.

Key words: antiinflammatory, prostaglandin E₂ inhibition, cyclooxygenase, hops, rho iso-alpha acids, rosemary, oleanolic acid.

Résumé : Dans le présent article, nous examinons l'innocuité et l'efficacité clinique de NG440, une préparation anti-inflammatoire d'inspiration phytochimique consistant en une combinaison d'acides rho iso-alpha, de romarin et d'acide oléanolique. Dans une étude antérieure, nous avons démontré que NG440 diminue la douleur de 50% chez les patients souffrant d'arthrose. En accord avec ces données, les résultats provenant d'un essai multicentrique indiquent que NG440 a réduit les cotations de douleurs chez des patients éprouvant un inconfort articulaire, tel que mesuré par la méthodologie VAS. Une étude clinique ex vivo a démontré que la diminution de la douleur pouvait être liée à une diminution de la production de cytokines inflammatoires de même qu'à une diminution de la formation de prostaglandine E₂. Finalement, des résultats incitent fortement à penser que la consommation de NG440 est sans danger pour l'être humain. Les résultats de toxicité chez les animaux n'ont révélé aucun effet néfaste à des doses ≤ 250 mg/jour administrées pendant 21 jours. De plus, les résultats d'essais cliniques chez les humains donnent à penser que NG440 n'a pas d'effet négatif sur les marqueurs cardiovasculaires et gastrointestinaux normalement affectés par les inhibiteurs sélectifs des enzymes COX-2, comme la fonction plaquettaire, la pression artérielle, la numération globulaire ou la calprotectine fécale, une mesure de lésions gastrointestinales. En conclusion, NG440 pourrait être une solution de rechange sécuritaire et efficace dans certaines affections où les inhibiteurs spécifiques des enzymes COX-2 sont traditionnellement utilisés.

Mots-clés : antiinflammatoire, inhibition de la prostaglandine E₂, cyclooxygénase, houblon, acides rho iso-alpha, romarin, acide oléanolique.

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Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs), including selective cyclooxygenase (COX)-2 inhibitors (coxibs), have a long history as the premier treatment for inflammatory conditions. Unfortunately, widespread use of traditional NSAIDs (e.g., aspirin, diclofenac, and ibuprofen) is associated with serious gastrointestinal complications such as gastric bleeding and small bowel complications such as perforation and obstruction (Adebayo and Bjarnason 2006). In fact, Wolfe et al. (1999) estimated that NSAID-related complications lead to 103 000 hospitalizations, incurring an estimated US\$2 billion in annual direct costs in the United States (Singh and Triadafilopoulos 1999). Moreover, conservative estimates of 16 500 NSAID-related annual deaths have been cited for patients with arthritides, constituting the 15th most common cause of death in the United States (Wolfe et al. 1999).

It has long been thought that the gastrototoxic effects of NSAIDs are primarily related to their inhibition of the COX-1 enzyme, which is responsible for the production of beneficial, “housekeeping” prostaglandins in several tissues (Warner et al. 1999). Hence, coxibs were developed in the 1990s with the aim of vastly improving gastrointestinal safety. COX-2 appeared to be the isoform of choice to target, since it is upregulated in virtually all tissues when induced by a stressor and is primarily responsible for producing inflammatory compounds such as prostaglandin (PG) E₂ (Dubois et al. 1998; Morita 2002; Warner and Mitchell 2004; Wolfe et al. 1999). In subsequent years, however, it was revealed that the COX-2 dogma may be scientifically flawed. Later research indeed confirmed that COX-2 plays a role in maintaining the integrity of the small intestine (Sigthorsson et al. 2002). Work in mice deficient in COX-1- and COX-2 showed that celecoxib resulted in intestinal damage similar to that seen with the NSAID indomethacin (Sigthorsson et al. 2002). Furthermore, criticisms have been raised regarding the clinical data from the CLASS trial, a study to verify long-term safety of celecoxib. Re-analysis of the data suggests that the number of ulcer complications among patients using celecoxib is not significantly different from that of patients using diclofenac (Jüni et al. 2002).

In addition to the potential, unforeseen, and long-term gastrointestinal safety issues of coxibs, recent data from large prospective trials and epidemiological studies have shown that their use may lead to increased cardiovascular events such as myocardial infarctions and strokes (Bjarnason et al. 2003; Sanghi et al. 2006). These results may not be entirely unexpected considering that COX-2 produces PGI₂, a potent inhibitor of platelet aggregation and a stimulator of vasodilation (Chandrasekharan and Simmons 2004; Morita 2002). These alarming findings have raised serious concerns about the coxibs, fueling dramatic public health actions taken by regulatory bodies. For example, in the European Union, coxibs are contraindicated for patients with cardiovascular disease, and more stringent warning statements on coxibs have been introduced (Heim and Broich 2006). Furthermore, much press has been generated on the 2 coxibs that have been withdrawn from the American market, representing the largest prescription drug withdrawal in history (Topol 2004).

To avoid the shortcomings of targeting the inhibition of COX-1 or COX-2 enzyme activity directly, we sought an alternative solution—to identify novel, natural products that confer high efficacy and low toxicity in humans by selectively inhibiting induction of inflammatory genes, a process that occurs in a tissue-specific manner as a consequence of inflammatory stimuli. This strategy should result in a therapeutic agent that has no effect either on preformed COX-1 or COX-2 enzyme activity, or on COX-2 present constitutively in tissues (e.g., kidney, brain, gastrointestinal tract, endothelial cells). Stated directly, it should address inflammation in specific tissues.

The result of our *in vitro* screen of over 200 natural products was NG440, a phytochemical combination of rho iso-alpha acids (RIAs) from hops, rosemary, and oleanolic acid, all of which are food-grade substances. *In vitro* cell culture studies indicate that each of these individual active ingredients inhibits PGE₂ activity, and in combination, a more potent inhibition is achieved (Hall et al. 2006; Tripp et al. 2003, 2005). It was also demonstrated that these actives did not significantly inhibit preformed COX-1 or COX-2 enzyme activity on the basis of extensive *in vitro* studies (Konda et al. 2006; Tripp et al. 2005) and cell-free enzyme assays (Darland et al. 2004). Additionally, NG440 was shown to inhibit COX-2, inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF)- α abundance *in vitro* (A. Hall, unpublished data; Konda et al. 2006), supporting the understanding that NG440 inhibits inflammatory signal transduction.

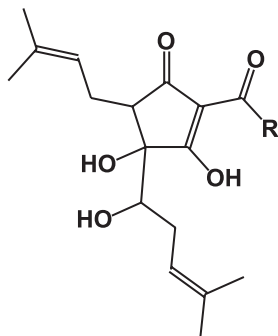
The objective of this report is to describe our work with NG440 in humans as it relates to efficacy and safety. For added flexibility, we tested 2 different formulations: the initial composition, NG440-1, which has 440 mg of active ingredients (200 mg RIAs, 200 mg rosemary extract, 40 mg oleanolic acid) per tablet, and a subsequent formula, NG440-2, with 225 mg RIAs, 112.5 mg rosemary extract, and 1 mg oleanolic acid per tablet. In addition to quantifying PGE₂ production by means of a mouse cell model and plasma from individuals taking an NG440 formula, subjective and objective data were collected in an uncontrolled, multicentre trial involving a variety of licensed healthcare practitioners prescribing NG440-2 to patients, some concurrently taking dietary supplements and pharmaceuticals.

Finally, and perhaps most importantly, since cardiovascular and gastrointestinal health parameters are of concern for COX-2 inhibitors, measures of these parameters were collected as part of our clinical trials. We describe the results of 2 clinical trials in which several measures of cardiovascular health were obtained, including *ex vivo* PGI₂ production, blood pressure, blood cell count, and blood clotting indices. Other general chemistries such as kidney and liver function markers were also evaluated. Lastly, we explored gastrotoxicity by measuring fecal calprotectin in subjects taking either the NG440 formula or naproxen.

Materials and methods

Description of the product

A version of the product used in the studies listed in this manuscript is commercially available as the nutraceutical Kaprex, which contains a proprietary blend of RIAs from

Fig. 1. Chemical structure of rho iso-alpha acids.

rho n-iso-alpha acid: R = CH₂CHC(CH₃)₂
 rho ad-iso-alpha acid: R = CH(CH₃)CH₂CH₃
 rho co-iso-alpha acid: R = CH(CH₃)₂
 rho post-iso-alpha acid: R = CH₂CH₃
 rho pre-iso-alpha acid: R = CH₂CH₂CH(CH₃)₂
 rho adpre-iso-alpha acid: R = CH₂(CH₂)₃CH₃

Table 1. Results of oral toxicity study in mice gavaged with NG440-2.

Test, M/F	NG440-2, mg·kg ⁻¹ ·day ⁻¹		
	25	75	250
Body mass	ns/ns	ns/ns	*/ns
Body mass gain	ns/ns	*/ns	**/ns
Absolute food consumption	ns/ns	ns/ns	*/ns
Organ mass to body mass	ns/ns	ns/ns	ns/ns

Note: Male (M) and female (F) mice were gavaged with control or NG440-2 for 21 days, $n = 10$ for each group. Organs measured were heart, liver, brain, kidneys, and spleen. Statistically significant at * $p < 0.05$ and ** $p < 0.01$ vs. control group; ns, not statistically significant.

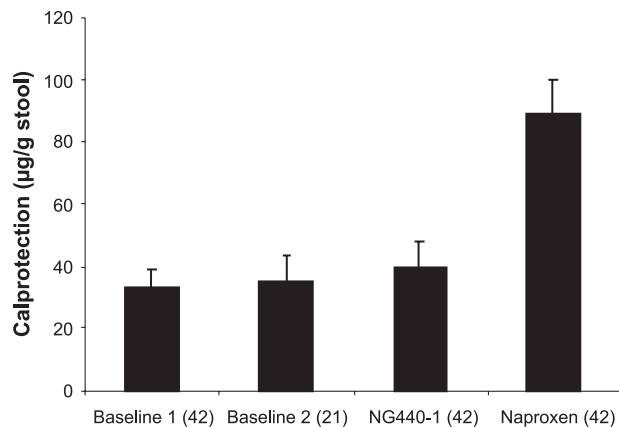
hops, rosemary, and oleanolic acid. Samples of all authenticated raw materials in NG440-1 and NG440-2, and of the commercial product, are retained for 1 year past the expiration date (2 years). Voucher specimens are kept in a secure, controlled environment by a third-party laboratory.

Rho iso-alpha acids

RIAs from hops (*Humulus lupulus* L.) are the main ingredients of NG440-1 and NG440-2 (Fig. 1). The precise amount of the total RIAs present in the extract and the finished product is accurately measured by using a well-established ultra performance liquid chromatography (UPLC)-based assay with UV detection. The extract is consistently formulated as a magnesium salt according to the method previously described in US Patent No. 5 624 701, containing approximately 60% by mass of total RIAs. The magnesium salt form of the extract is a free-flowing powder that allows for the blending and tablet manufacture of the finished product.

Rosemary extract (*Rosmarinus officinalis*)

Kaprex utilizes a commercially available extract of rosemary leaf (*rosmarini folium*), which is obtained through continuous extraction (percolation) with alcohol and water, according to the method in European Pharmacopoeia monograph *Extracta*. The identification of the material is verified

Fig. 2. Pooled calprotectin data for subjects taking either NG440-1 or naproxen. The number in parentheses indicates the number of data points per each condition. Baseline 1 is the average of all baseline values from visit 1. Baseline 2 is the average of all wash-out values. The 7-day and 14-day data for NG440-1 and for naproxen were pooled for this analysis, respectively. Data are means \pm SE. Statistically significant at * $p < 0.05$ vs. baseline 1 by Wilcoxon and Kruskal–Wallis ranked sums.**Table 2.** Fecal calprotectin levels in 8 subjects taking NG440-2 and followed for up to 3 years.

Patient	Calprotectin, µg/g						
	Initial	Follow-up, months					
		2–4	4–6	6–9	9–12	12–24	24–36
1	—	22	—	—	—	—	—
2	—	21	—	—	—	—	—
3	—	—	<16	—	—	<16	—
4	—	—	<16	—	—	—	—
5	73	—	—	25	24	62	—
6	—	—	—	—	—	24	—
7	—	—	—	—	—	37	31
8	—	—	—	—	—	<17	<16

Note: Fecal calprotectin levels were measured intermittently throughout the (uncontrolled) treatment period at the Functional Medicine Research Center. Dashes indicate no measurement was taken at specified time interval. Calprotectin reference range was <50 µg/g.

with thin-layer chromatography (TLC) by using the following marker compounds: carnosol, carnosic acid, and phenolic diterpenes.

Oleanolic acid (*Olea europaea*)

The third ingredient of Kaprex is a commercially available oleanolic acid that is made from the leaf of *Olea europaea* (olive). The final material is tested for oleanolic acid content (80%) by using high performance liquid chromatography (HPLC).

Safety

Animal oral toxicology

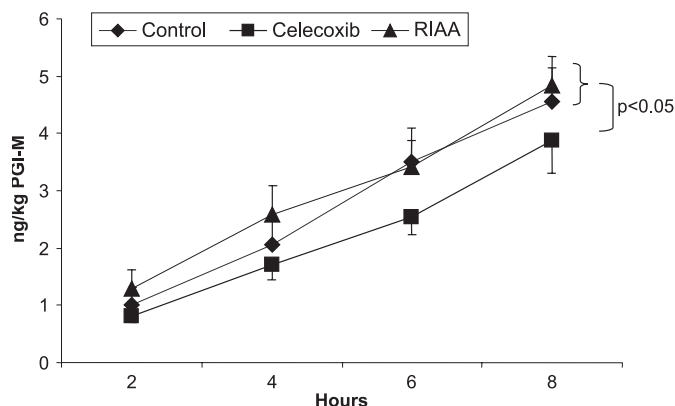
A total of 40 male and female CD-1 mice approximately 7–8 weeks of age were assigned to either a control or NG440-1 at 25, 75, or 250 mg·kg⁻¹·day⁻¹ ($n = 10$ mice per group, each 5 male and 5 female). The treatment of animals was in accordance with regulations outlined in the USDA

Table 3. The effect of rho iso-alpha acids (RIAs) and celecoxib on the urinary recovery of PGI-M and TxB₂.

Subject	Age, years	PGI-M, ng			TxB ₂ , ng		
		Control	Celecoxib	RIAs	Control	Celecoxib	RIAs
F1	43	299	245	244	448	930	329
F2	60	349	224	235	288	244	196
F3	59	330	449	398	355	261	310
F4	45	698	388	742	570	616	585
F5	46	374	448	452	436	418	275
F6	63	188	148	219	221	433	343
M7	47	377	283	448	768	470	778
M8	63	206	187	374	244	335	246
Geometric mean		327	276	360	384	424	347
95% CI		233–459	196–389	254–510	269–549	293–613	238–507

Note: PGI-M, sum of prostaglandins 6-keto-PGF_{1 α} and 2,3-dinor-6-keto-PGF_{1 α} ; TxB₂, thromboxane B₂; M, male; F, female. One gram of test agent was administered to each subject. Total urine was collected at 2-hour intervals. The total amounts of PGI-M and TxB₂ excreted over an 8-hour period were determined by immunoassay.

Fig. 3. The effect of rho iso-alpha acids (RIAs) and celecoxib on the rate of PGI-M excretion in urine of healthy individuals. One gram of test agent was administered to each subject. Total urine was collected at 2-hour intervals. The total amounts of PGI-M excreted over an 8-hour period were determined by immunoassay. PGI-M represents the sum of 2,3-dinor-6-keto-PGF_{1 α} and 6-keto-PGF_{1 α} . Data are means \pm SE, $n = 8$.

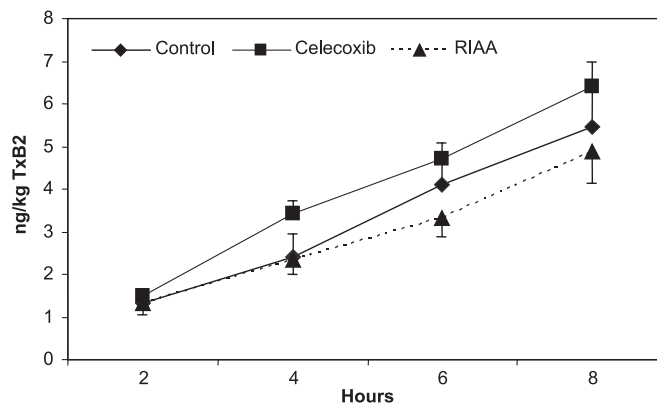


Animal Welfare Act (9 CFR Parts 1, 2, and 3) and the conditions specified in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996). Chow and tap water were supplied ad libitum during the course of the study. Each animal received either the test (NG440-1) formulated in Labrasol or the control article (Labrasol) orally by gavage for a minimum of 21 consecutive days. The animals were evaluated on a weekly basis for changes in clinical signs, food consumption, body mass, and other parameters. All surviving mice were euthanized on the day after the last dose. A full necropsy was conducted on all animals, and tissues were collected, preserved, processed, and examined.

Clinical studies to assess cardiovascular health parameters

Four separate clinical trials were executed to determine the safety of the NG440 constituents (RIAs, rosemary, and oleanolic acid) as determined by PGI₂ production, blood pressure, and clinical chemistries. Study protocols were sub-

Fig. 4. The effect of rho iso-alpha acids (RIAs) and celecoxib on the rate of TxB₂ excretion in urine of healthy individuals. One gram of test agent was administered to each subject. Total urine was collected at 2-hour intervals. The total amounts of TxB₂ excreted over an 8-hour period were determined by immunoassay. No statistically significant differences were noted between groups. Data are means \pm SE, $n = 8$.



ject to internal review, and subjects gave informed consent before participating. In all studies, complete blood count (CBC), and comprehensive metabolic panel (CMP) were performed by Northwest Laboratories (Tacoma, Wash.).

Trial A

Two men and 6 women with no history of NSAID use for at least 1 week were treated in random order with a single dose of control (placebo), RIAs (one of the unique ingredients of NG440-1 and NG440-2) at 600 mg daily, or celecoxib (Celebrex 200 mg tablets obtained from a local pharmacy) at 400 mg daily. A minimum of 2 days elapsed between treatments. After an initial voiding, subjects were administered test agent along with 4 oz (118 mL) of water. Urine samples were collected at 2-hour intervals for the next 8 h. Urine volumes were determined and aliquots of approximately 20 mL were immediately stored at -20°C until assayed. Subjects were allowed free access to food and water during the study. Three to 4 weeks after the final treatment,

Fig. 5. Absence of an effect of NG440-1 on blood pressure in 50 subjects during an 8-week, open-label observational clinical trial (Trial B). In this trial, rheumatology patients (30 with osteoarthritis, 17 with fibromyalgia, and 3 with rheumatoid arthritis) consumed either 2 tablets twice daily or 1 tablet 3 times daily over the 8 weeks. Initial (black bars) and final (grey bars) values are shown. Data are means \pm SE.

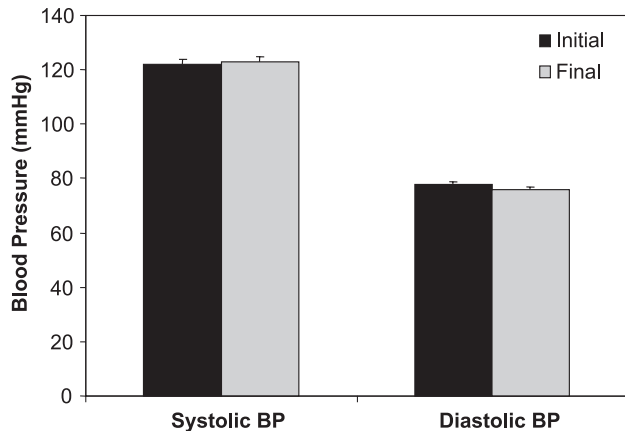
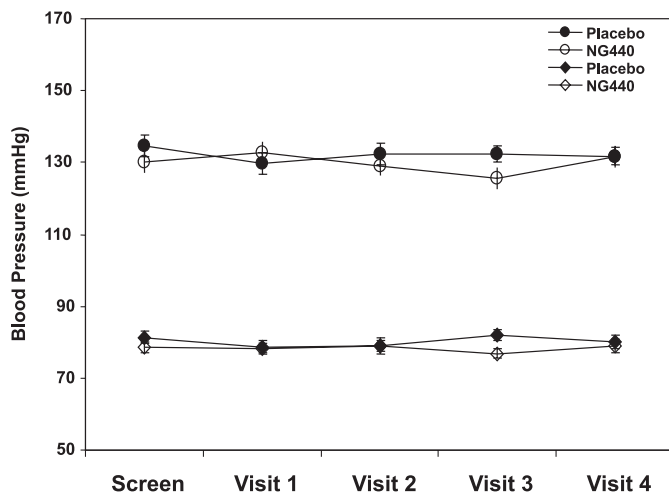
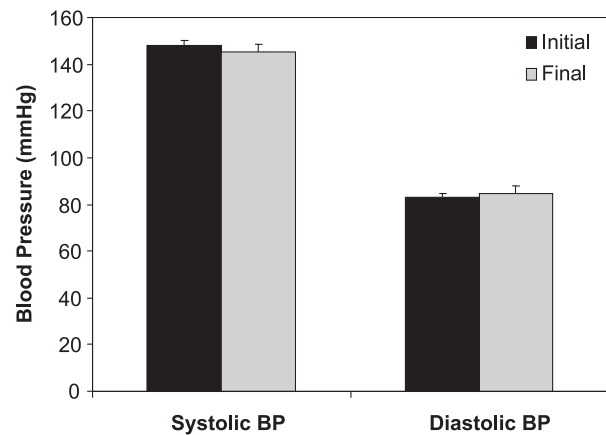


Fig. 6. Blood pressure measurements from a randomized, double-blind, placebo-controlled 6-week clinical trial with NG440-2 at 1 tablet 3 times daily (Trial C). Systolic (circles) and diastolic (triangles) data are shown for the placebo (closed symbols) and NG440-2 (open symbols) groups. The screen, visit 1, and visit 2 measurements occurred before administration of NG440-2. Visit 3 occurred after 2 weeks of treatment with either NG440 ($n = 39$) or placebo ($n = 29$) and visit 4 occurred at 6 weeks of treatment. Data are means \pm SE.



control urine samples were collected as above. Thromboxane (Tx) B₂ was analyzed by using a TxB₂ immunoassay kit (R&D Systems, Minneapolis, Minn.). PGI₂ spontaneously decomposes to 6-keto-PGF_{1 α} , which is further metabolized by β -oxidation to 2,3-dinor-6-keto-PGF_{1 α} , both of which are chemically stable. In the discussion that follows, they are referred to collectively as PGI-M since both are detected by the kit. Both assays utilize a competitive-binding format. Samples were thawed at room temperature and diluted into assay buffer. A maximum of 4 freeze-thaw cycles were involved. The manufacturer's protocol was used with-

Fig. 7. Absence of an effect of NG440 formulas on blood pressure in 14 hypertensive subjects. Data were pooled from 2 independent trials, an open-label, 8-week observational clinical trial (Trial B) ($n = 5$) and a randomized, double-blind, placebo-controlled 6-week trial (Trial C) ($n = 9$). Data are means \pm SE.



out modification. In the case of the treatment groups, 2 analyses were performed, each comprising duplicate, but independent, dilutions. The baseline samples were analyzed once but involved 3 independent dilutions.

Trial B

An institutional review board (IRB)-approved human study was conducted with 50 volunteers, ages 18 to 65 years, with a diagnosis of osteoarthritis, rheumatoid arthritis, or fibromyalgia. Dosage was 1 tablet 3 times daily of NG440-1 for 4 weeks and increased to 2 tablets twice daily for the ensuing 4 weeks for the majority of subjects. Symptoms were monitored before the start of treatment and at 4 and 8 weeks posttreatment by using VAS (visual analog scale) methodology. Blood pressure, general chemistries for liver and kidney function, and CBC were obtained for these subjects at the start and end of the trial.

Trial C

This 6-week, IRB-approved, randomized, double-blind, placebo-controlled trial in patients with knee osteoarthritis was conducted with 39 subjects in the treatment arm (1 tablet of NG440-2 three times daily) and 29 subjects on placebo. Similar to Trial B, blood pressure, general chemistries, and CBC were measured at baseline and at the end of the trial.

Trial D

An open-label trial of platelet function and blood coagulation after 7 days of NG440-2 treatment was executed in 6 healthy participants not taking NSAIDs or other (botanical or pharmaceutical) antiinflammatory agents. Subjects were initially screened at the first visit and a blood draw was obtained for screening laboratories (CBC and CMP). After acceptance into the study, a baseline blood draw was obtained at the second visit and subjects were assigned treatment of 2 tablets of NG440-2 twice daily for 7 days. On the 8th day, subjects returned for the third visit, at which time they consumed the morning dose of NG440-2 and blood was drawn 4 h later. A washout period of approximately 7 days occurred, after which blood was drawn for assessment of pla-

Table 4. Absence of an effect of NG440 formulas on kidney function markers and electrolytes during 2 independent trials.

	Trial B, 8 weeks, <i>n</i> = 45		Trial C, 6 weeks, <i>n</i> = 34	
	Initial	Final	Initial	Final
BUN, mg/dL	15.04±0.52	14.82±0.60	16.94±0.83	16.44±0.70
Creatinine, mg/dL	0.838±0.021	0.816±0.020	0.903±0.028	0.916±0.028
Na, mEq/L	139.6±0.3	139.3±0.3	139.3±0.3	139.6±0.2
Cl, mEq/L	103.8±0.3	103.9±0.3	103.5±0.4	104.0±0.3
K, mEq/L	4.19±0.04	4.15±0.04	4.37±0.06	4.19±0.05
CO ₂ , mEq/L	27.7±0.4	28.5±0.3	29.0±0.4	28.6±0.3

Note: In the 8-week trial (Trial B), rheumatology patients consumed NG440-1 at either 2 tablets twice daily or 1 tablet 3 times daily. In the 6-week trial (Trial C), subjects with knee osteoarthritis consumed NG440-2 at 1 tablet 3 times daily. Reference ranges: blood urea nitrogen (BUN), 8.0–24.0 mg/dL; creatinine, 0.8–1.5 mg/dL; Na, 135–148 mEq/L; Cl, 97–107 mEq/L; K, 3.6–5.3 mEq/L; CO₂, 24–33 mEq/L. Data are means ± SE.

Table 5. Blood values for white and red blood cells, platelets, hematocrit, and hemoglobin, initially, after 1 week of NG440-2 at 2 tablets twice daily, and after a 1-week washout (Trial D).

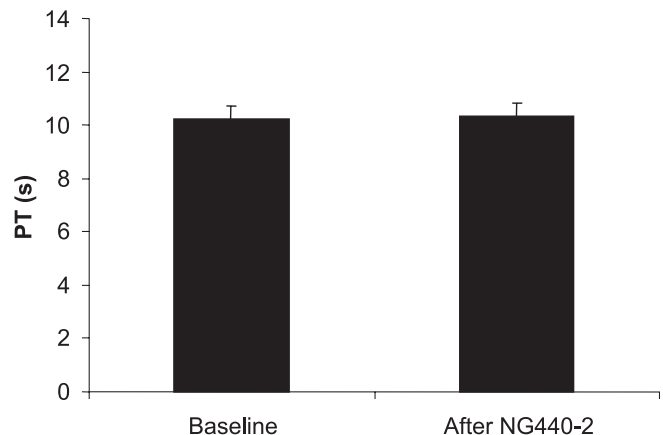
	Baseline	After NG440-2	After washout
WBC, × 10 ³ /mm ³	7.4±0.6	5.9±0.5	5.9±0.8
RBC, × 10 ⁶ /μL	4.5±0.1	4.6±0.2	4.5±0.2
PLT, × 10 ³ /mm ³	256±14	260±23	247±17
HCT, %	41±1	42±2	41±2
HGB, g/dL	14.1±0.4	14.2±0.6	14.0±0.6

Note: Reference ranges: WBC, white blood cells, 4.0–12.0 × 10³/mm³; RBC, red blood cells, 4.0–6.0 × 10⁶/μL; PLT, platelets, 150–450 × 10³/mm³; HCT, hematocrit, 37%–54%; and HGB, hemoglobin, 12.0–18.0 g/dL. Data are means ± SE, *n* = 6.

telet function. Subjects were then administered 325 mg of aspirin and blood was again drawn 4 h later for control assessment of platelet function. Platelet closure times were assessed by using the PFA-100 system, which assesses the time to develop a platelet plug from platelet attachment, activation, and aggregation to full occlusion of an aperture on a membrane. The platelet function is reported as “closure time.” Platelet closure times were obtained from Puget Sound Blood Center (Seattle, Wash.).

Clinical studies to assess gastrointestinal health parameters

The effects of NG440-1 (2 tablets twice daily) and naproxen (500 mg twice daily) on gastrointestinal health, specifically on the production of fecal calprotectin, were studied in an IRB-approved, randomized, 14-day crossover study with 21 healthy men ages 18 to 45 years. A 21-day washout occurred between the crossover treatments. Fecal calprotectin assays were performed by using the PhiCal enzyme immunoassay (Great Smokies Diagnostic Laboratory, Asheville, N.C.). The test sensitivity is 15 μg/g stool (equivalent to 6.25 ng/mL) and the test is linear to 250 μg/g stool (equivalent to 100.00 ng/mL). Within-run variation was 6% CV, and day-to-day variation was 14% CV. Subjects were provided kits to obtain stool samples at home and bring them to each visit. Collection of stool samples was performed as described in literature provided by the laboratory. Two stool samples were collected from the subjects at baseline for intraindividual variation and baseline determination.

Fig. 8. Prothrombin time (PT) for 6 subjects at baseline and after NG440-2 two tablets twice daily for 1 week (Trial D). The laboratory reference range for PT was 9.2–12.8 s. Data are means ± SE.

Additionally, stool samples were collected at every visit (6 biweekly visits) until the end of the study.

Separate from this study, 8 subjects taking NG440-2, along with medications (e.g., antibiotics, ibuprofen, and acetaminophen) and (or) other botanical or nutritional supplements (e.g., multivitamin and vitamin C) as prescribed by their healthcare practitioner, were followed for a longer time period under the supervision of a healthcare professional at the Functional Medicine Research Center (Gig Harbor, Wash.). Fecal calprotectin levels were measured at random intervals for up to 3 years.

Efficacy

Clinical multicentre trial with NG440-2 to determine relief of pain symptoms

VAS and health history intake were collected by licensed healthcare practitioners using NG440-2 as part of their clinical practice. Data were obtained for 60 subjects of unspecified sex with a median age of 56 (range 28–85) years. Four subjects were omitted from analysis because 3 had incomplete data collection and 1 had a low initial VAS score of 0.6 cm (on a scale of 10 cm). Therefore, a total of 56 subjects were included in the analysis. Data were collected at the initial visit (day 0) and 2 subsequent visits at 2 (visit 2)

Table 6. Assessment of platelet function with NG440-2.

	Closure times, s	
	Epi/Col	ADP/Col
Baseline	107.0±4.53	80.7±6.25
After NG440-2	104.8±7.8	77.3±7.2
After washout	114.3±6.4	76.2±4.2
Aspirin control	283.7±17.3	81.7±6.8

Note: The mean (\pm SE) closure times are shown for 6 subjects after 1 week of NG440-2 twice daily. Platelets were activated with collagen and then stimulated to produce a plug by either epinephrine (Epi/Col) or ADP (ADP/Col). Reference ranges are 83–107 s for Epi/Col closure time and 62–104 for ADP/Col closure time.

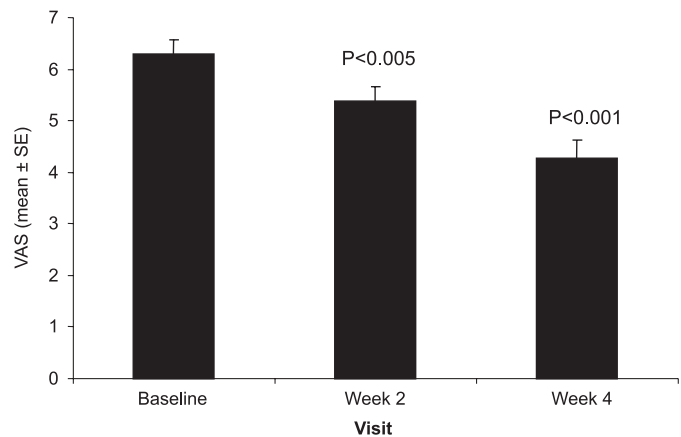
and 4 weeks (visit 3). Additionally, there was considerable variation in the timing of the 2 follow-up visits (median, 14 days for visit 2 and 29 days for visit 3). In cases in which uncertainty existed, an attempt was made to estimate the visit to which the data applied.

Ex vivo studies with NG440-1 to determine PGE₂ production

The trial was a 3-arm, open-label clinical study with 6 subjects in a Latin square design to assess the effects of 2 different individual doses of NG440-1 versus a standard dose of 200 mg celecoxib on the inhibition of PGE₂ synthesis. Baseline fasting blood was obtained, a bolus dose of either 1 tablet or 3 tablets of NG440-1 or 200 mg celecoxib was given orally, and then blood was drawn at 1, 2, 4, 6, and 8 h postdosing. Subjects remained fasting until after the 2-hour blood collection. A minimum washout of 24 h occurred between each dose. Inhibition of PGE₂ synthesis was determined by ex vivo analysis in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage cells. Briefly, RAW 264.7 cells (ATCC TIB-71, Manassas, Va.) were maintained in log phase growth in Dulbecco's modified Eagle's medium (DMEM) with 10% fasting blood sugar (FBS), supplemented with penicillin (50 units/mL) and streptomycin (50 µg/mL) until initiation of the assay. Cells were plated in 96-well microtiter plates at 10⁵ cells/well with 200 µL growth medium and grown to approximately 80% confluence. Cells were incubated in serum-free media for 30 h, after which the media were removed and replaced by 200 µL test plasma, and the cells were shaken gently for 60 min at 37 °C in 5% CO₂ air. Cells were stimulated by addition of LPS (10 ng/mL) and incubated for 24 h at 37 °C in 5% CO₂ air. After incubation, 50 µL of supernatant culture media were assayed for PGE₂. Four columns (32 wells) containing the untreated (time 0) plasma as well as 8 wells with commercial plasma were also assayed as controls. A commercial EIA kit (Cayman Chemical, Ann Arbor, Mich.) was employed for determination of PGE₂ and the recommended procedure of the manufacturer was used with-out modification.

Statistical methods

Statistical analyses were performed by using standard procedures with a SAS statistical package (JMP, SAS Institute Inc.) or with Microsoft Excel (Microsoft, Redmond, Wash.).

Fig. 9. The effect of NG440-2 on joint symptom relief, as measured by visual analog scale (VAS) scores. All subjects ($n = 56$ for baseline, $n = 53$ for 2 weeks) showed statistically significant improvement in pain scores between visit 1 (baseline) and visit 3 ($p < 0.001$, 2-tailed paired t test). Data are means \pm SE.

Significance was predetermined as $p < 0.05$. Data are presented as means \pm SE.

The intra-assay coefficient of variation for the PGE₂ analysis, provided by the manufacturer, was <10%, with cross-reactivity with PGD₂ and PGF_{2 α} of less than 1%, and linearity over the range of 10 to 1000 pg/mL. PGE₂ concentration in plasma was computed as picograms of PGE₂ per millilitre. Percentage inhibition of PGE₂-biosynthesis was computed as follows:

$$\frac{[\text{PGE}_2](\text{control}) - [\text{PGE}_2](\text{postdosing})}{[\text{PGE}_2](\text{control})} \times 100$$

Control plasma corresponds to predosing activity (0 time), which was obtained from a fasting blood sample at the initiation of the experiment. Postdosing was obtained for each time point after consumption of the test or control substance at the indicated time points.

For the multicentre trial results, a variety of statistical methods was used to stratify the data.

These included the following:

1. A total of 7 groups were defined as follows: (i) all subjects, (ii) subjects who used pharmaceutical agents concurrently, (iii) subjects who used other dietary supplements during the study, (iv) subjects who used both pharmaceuticals and dietary supplements, (v) subjects not on pharmaceuticals or dietary supplements, (vi) subjects in whom therapeutic changes were made during the course of the case series, and (vii) subjects using narcotics during the study. Groups (i) and (iii) and groups (ii) and (vii) are not mutually exclusive.
2. Several of the large clinical trials using NSAIDs have reported "responder" rates of approximately 30%. In those studies, statistical analyses have included subjects defined as responders. We have submitted our data to a similar analysis and have chosen to define a <30% reduction in VAS as a non-response.

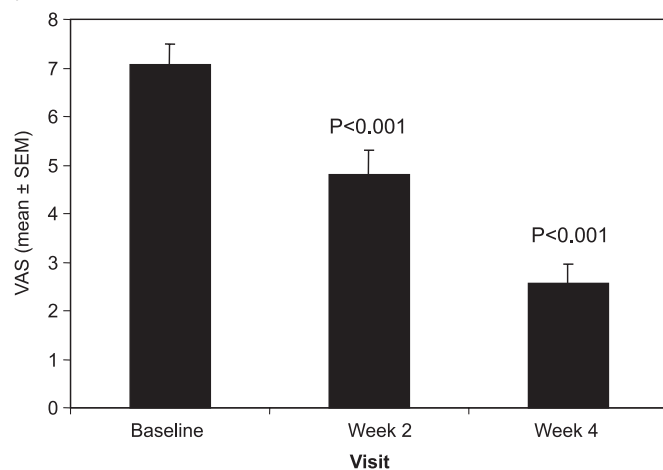
Within-group comparisons were made with an unpaired t test against the null hypothesis that percentage change was equal to 0 (Excel, Microsoft, Redmond, Wash.). A two-way

Table 7. Subgroup analysis of the multicentre trial using NG440-2 in a population of patients with joint discomfort, showing initial and final VAS scores.

Group	Description	<i>n</i>	VAS initial	VAS final	Reduction, %	<i>p</i>
<i>i</i>	All subjects	56	6.29	4.28	27.9	≤0.0001
<i>ii</i>	On medication	33	6.22	4.32	29.7	≤0.0005
<i>iii</i>	On supplements	25	6.03	4.00	32.1	≤0.0005
<i>iv</i>	On both	16	5.96	3.98	34.5	≤0.005
<i>v</i>	On neither	9	6.26	3.78	26.4	ns
<i>vi</i>	Additions	5	7.02	5.34	18.6	ns
<i>vii</i>	Narcotics	9	6.57	5.27	12.1	ns

Note: VAS, visual analog scale; ns, not significant.

Fig. 10. The effect of NG440-2 on “responders,” i.e., those subjects with >30% improvement in VAS (visual analog scale) at 4 weeks ($n = 26$ of 56 total subjects, or 46%). Each visit indicated statistically significant improvement in pain scores between baseline and week 4 ($p < 0.001$). At 2 weeks, 24 subjects were analyzed. Data are means \pm SE.



ANOVA (GraphPad Prism, Version 4.0, San Diego, Calif.) was used to compare differences between groups.

For Trial A, standard curves were constructed by fitting the logarithm of the concentration to B/B_0 with a 4-parameter logistic equation and forcing the maximum value for B/B_0 through 1.0, where B_0 represents the background corrected absorbance of the blank standard ($C = 0$ pg/mL). Rate determinations were made by linear regression on the means and forcing the origin through 0.

Calprotectin data were analyzed as follows: entries that were below the limit of detection (<15 μ g/g stool) were converted to 15. Data were analyzed by ANOVA (Wilcoxon and Kruskal–Wallis ranked sums test). Data were also transformed logarithmically to insure homoscedasticity and all statistical tests were confirmed on the transformed data. Grubb test for outliers was performed. Tukey HSD test was used for post hoc comparisons.

Results

Safety

Animal oral toxicity study

There were no adverse effects on body mass, food con-

sumption, organ mass, ratio of organ mass to body mass, or gross or microscopic pathology associated with the administration of NG440-1 for 21 consecutive days at dosages ≤ 250 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (Table 1).

Clinical observations of NG440-1 and NG440-2 on parameters of gastrointestinal health

In the crossover study, no difference was noted between baseline measurements of fecal calprotectin in the 2 study arms (Fig. 2). As expected, fecal calprotectin after the naproxen (7- and 14-day) treatment was significantly elevated to 154% above baseline, whereas the fecal calprotectin values after NG440-1 did not differ from baseline ($p > 0.05$) after 14 days of treatment. Subjects taking NG440-2 who were followed for up to 3 years experienced no clinically significant increase in fecal calprotectin (Table 2).

Clinical observations of NG440 on parameters of cardiovascular health

PGI_2 , TxB_2 , and blood pressure

Table 3 summarizes the result of prostanoid recovery in the 8 subjects in Trial A. Intrasubject variability is high, ranging in control samples from approximately 190 to 700 ng for PGI_2 and from 220 to 770 ng for TxB_2 during the 8-hour collection period. Treatment did not significantly affect total recovery for either analyte. There is a suggestion, however, that celecoxib slightly inhibits (approximately 15%) PGI_2 , whereas RIAAs were without effect. Celecoxib decreased the rate of PGI_2 excretion by 19% ($p < 0.05$) (Fig. 3) without affecting TxB_2 excretion (Fig. 4). RIAAs had no effect on the excretion of either prostanoid. The results are consistent with our hypothesis that inhibition of the COX-2 mediated prostaglandin biosynthesis by interfering with enzyme induction rather than enzyme activity will avoid the imbalance of PGI_2 and TxA_2 synthesis that has been attributed to the coxibs.

No significant changes were noted for systolic or diastolic blood pressure in Trial B (Fig. 5) or Trial C (Fig. 6). Data from these 2 trials were pooled for greater statistical power ($n = 81$). In this analysis, no change was observed between the baseline systolic blood pressure of 126.3 mmHg (SD, 15.1; 95% CI, 122.7–129.9) and the final reading of 126.4 mmHg (SD, 15.6; 95% CI, 123.0–129.8). Similarly, no significant difference was noted between the baseline diastolic blood pressure of 78.2 mmHg (SD, 7.4; 95% CI, 76.6–79.8) and the final reading of 77.5 mmHg (SD, 8.6; 95% CI, 75.6–79.4).

Table 8. Subgroup analysis of the multicentre trial using NG440-2 in a population of “responders,” and their initial and final VAS scores.

Group	Description	Response, %	n	VAS initial	VAS final	Reduction, %	p
<i>i</i>	All subjects	48.1	26	7.05	2.55	66.2	≤0.0001
<i>ii</i>	On meds	48.5	26	6.54	2.46	65.5	≤0.0001
<i>iii</i>	On supplements	48	12	6.68	2.53	64.9	≤0.0001
<i>iv</i>	On both	56	9	6.09	2.5	63.2	≤0.0001
<i>v</i>	On neither	55.6	5	6.86	1.48	76.9	≤0.0005
<i>vi</i>	Additions	40	5	9.6	5.85	39.3	ns
<i>vii</i>	Narcotics	33.3	3	8.33	4.70	44.2	≤0.0005

Note: VAS, visual analog scale; ns, not significant.

Thirteen subjects had initial readings of systolic blood pressure ≥140 mmHg and 1 subject had a diastolic blood pressure ≥90 mmHg. Again, as shown in Fig. 7, no significant difference was observed between the initial and final systolic blood pressures of 148.2 mmHg (SD, 7.9; 95% CI, 144.0–152.3) and 145.3 mmHg (SD, 12.4; 95% CI, 138.8–152.4), nor between the initial and final diastolic blood pressures of 82.8 mmHg (SD, 7.0; 95% CI, 79.1–86.4) and 84.7 mmHg (SD, 11.1; 95% CI, 78.9–90.5).

General Chemistries

No clinically significant changes in general chemistries and CBC were noted in either Trial B or Trial C. As shown in Table 4, kidney function markers remained well within the reference range after intervention with either NG440-1 or NG440-2. Electrolytes also remained within established reference range limits, further indicating no clinical relevant effects on kidney function. Similar results were obtained for liver function markers and other chemistry values (data not shown). In addition, Table 5 shows control values from the CBCs analyzed during Trial D.

Blood clotting measures

The prothrombin time (PT) values for the subjects before and after NG440-2 are shown in Fig. 8. No clinically relevant change was noted between the baseline of 10.2 s (95% CI, 9.8–10.6) and the value after NG440-2 of 10.33 s (95% CI, 9.9–10.7), which were both within the laboratory reference range of 9.2–12.8 s. The actual change in individual PT values ranged from –0.5 s to 0.4 s, with an average change of 0.1 s.

Table 6 shows the closure times obtained after 1 week of continuous NG440-2 intake and 4 h after the day 8 morning dose of NG440-2. As shown, no significant change was observed with either the epinephrine or the adenosine diphosphate (ADP) stimuli ($p = 0.7$ and 0.55 , respectively) after 1 week of NG440-2. In contrast, the control tests with the 4-hour aspirin challenge resulted in a significant increase in closure time with epinephrine ($p = 0.0002$), but, as expected, the ADP closure time was not significantly changed ($p = 0.15$). The data with the aspirin challenge and the epinephrine and ADP control indicate integrity of the assay.

Efficacy

Multicentre trial with NG440-2 and symptomatic pain relief

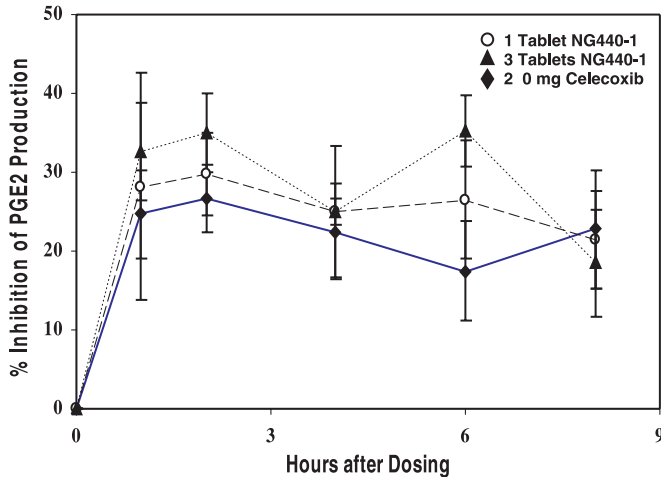
These data represent the clinical experience of licensed healthcare practitioners using NG440-2 in patients with joint

discomfort, as an adjunct for the relief of joint symptoms and mild pain. As is commonly seen in clinical practice, many of the subjects were on additional medications for pain relief. Evaluation of all 56 subjects as an intention-to-treat group demonstrated a significant 27.9% reduction in VAS scores from 6.29 cm to 4.28 cm (Fig. 9). Given the fluidity of certain data points and the possible confounding influences of other therapies, several subgroups were evaluated (Table 7). Subgroup analysis revealed that an approximate 30% reduction in clinical symptoms of joint distress was shown consistently across groups (*i*) to (*v*). Statistical significance was demonstrated for groups (*i*) to (*iv*), and although group (*v*) did not achieve clinical significance, this small group, not on other agents, showed a 26.4% reduction.

Several large clinical trials evaluating the efficacy of NSAIDs have defined the concept of differentiating between “responders” and “nonresponders” in assessing the clinical response to the intervention. Both Case et al. (2003) and Pincus et al. (2004) have suggested a reduction of 20% to discriminate between responders and nonresponders. On the other hand, Miceli-Richard et al. (2004) defined response more conservatively as more than 30% reduction in symptoms. In general, NSAID studies have reported that approximately 30% to 50% of subjects on active intervention were responders and that subjects on placebos generally had a response rate of less than 30%.

Within our data, we selected for subjects who had a ≥30% reduction in symptoms (which we defined as “responders”). Based on this classification, 26 out of 56 subjects (46%) responded to NG440 therapy. These data are consistent with the response to the active intervention in prior NSAID studies. The effect of NG440-2 on responders on VAS scores at 2 and 4 weeks is demonstrated in Fig. 10. As early as 2 weeks after NG440 therapy, responders experienced a statistically significant improvement in VAS scores ($p < 0.001$). At the final visit (4 weeks), VAS scores were 66.2% lower compared with baseline (7.05 cm to 2.55 cm, $p < 0.001$). Subgroup analysis of the responders demonstrated that highly significant clinical significance was achieved for all groups (44.2% to 76.9% reduction in symptoms) except group (*vi*), which represented the small number of subjects ($n = 5$) who required additional therapies during the course of the study (Table 8). The subgroup that experienced the greatest percentage reduction in VAS scores (76.9%) was group (*v*), the group not previously treated for joint relief.

Fig. 11. Analysis of inhibition of PGE₂ production by NG440-1 in 6 subjects randomly assigned to 1 of 3 dosing schedules. Ability of NG440-1 to inhibit PGE₂ production in vivo was found in each of the 6 subjects using 1 tablet NG440-1 (open circles), 3 tablets NG440-1 (closed triangle), and the standard of 200 mg celecoxib (closed diamond). Data are means \pm SE.



Ex vivo studies with NG440-1 and PGE₂ production

By use of the murine macrophage cells, inhibition of PGE₂ production was found in each of the 6 subjects using either 1 or 3 tablets of NG440-1 (Fig. 11) or celecoxib. Average time to peak inhibiting activity was 2.8 h and 3.2 h for 1 and 3 tablets of NG440, respectively, and 4.8 h for celecoxib. Average peak inhibition was 29.8% and 35.2% for 1 and 3 tablets of NG440-1, respectively, and 26.7% for celecoxib. Area under the curve (AUC) for inhibition of PGE₂ for NG440-1 at 3 tablets daily was 49% greater than NG440-1 at 1 tablet and 17% greater than celecoxib (Fig. 12).

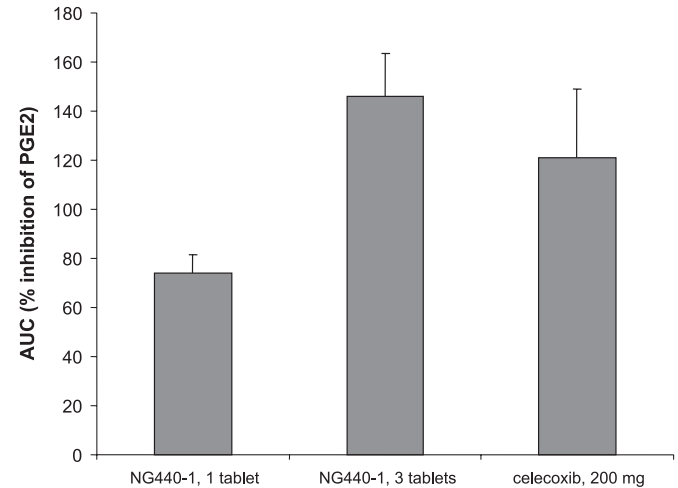
Discussion

Patients with chronic inflammatory conditions such as osteoarthritis, rheumatoid arthritis, or other autoimmune conditions require long-term treatment to manage their illnesses. Because of the lack of long-term safety of NSAIDs, including coxibs, as evidenced by the recent removal of some prescription NSAIDs from the market, novel anti-inflammatory alternatives are under development.

In this paper, we examined the safety and efficacy in humans of NG440, a phytochemical-based antiinflammatory formula consisting of a combination of RIAAs, rosemary, and oleanolic acid. Lukaczer et al. (2005) reported that this formulation was effective at significantly decreasing pain by 50% in subjects with osteoarthritis with no serious side effects.

NSAIDs can induce gastrointestinal damage, and long-term use causes inflammatory changes of the gastroduodenal region in a high percentage of patients, ultimately leading to significant morbidity and mortality (Adebayo and Bjarnason 2006). Some studies suggest that up to 65% of patients taking NSAIDs regularly for more than 6 months will develop enteropathy (Tibble and Bjarnason 2001). Damage to the gastrointestinal tract appears to occur rapidly with NSAID use. For example, in one study, 19% of subjects developed

Fig. 12. Area under the curve (AUC) for inhibition of PGE₂ production by NG440-1. The individual AUC (means \pm SE) after dosing with 1 tablet or 3 tablets of NG440-1 or the control substance of 200 mg celecoxib is shown for the 6 subjects in the study. AUC is lowest for the 1-tablet dose of NG440-1 and increases slightly less than 3-fold for the 3-tablet dose of NG440-1.



gastroduodenal ulcers within 4 weeks of naproxen treatment (500 mg twice daily) and 41% of patients developed ulcers after 12 weeks of treatment (Goldstein et al. 2001).

Tripp et al. (2005) used the AGS human gastric mucosal cell line to test both NSAIDs and select compounds derived from hops, including RIAAs, the major component of NG440. They reported that celecoxib strongly inhibited PGE₂ production (IC₅₀ = 0.024 μ g/mL), a finding consistent with reports that the majority of the PGE₂ from AGS cells is produced by COX-2. In contrast, very little inhibitory activity was demonstrated by hop-derived RIAAs (IC₅₀ > 20 μ g/mL). Based on these preliminary results, we had not expected significant clinical gastrotoxic effects of NG440. Confirming our in vitro results, NG440 had no significant effect on fecal calprotectin, a sensitive, early-stage marker of gastroduodenal damage from inflammation, in healthy human volunteers in the acute clinical study or in random subjects during 3 years of use of NG440-2. Consistent with published literature, fecal calprotectin increased in individuals during short-term treatment with naproxen. These results are similar to those of Meling et al. (1996) who showed that naproxen treatment led to an increase of over 2-fold after 7 days. Fecal calprotectin increased in 44% of subjects on chronic NSAID use, and this increase correlated significantly with 4-day excretion of indium-111-labeled white cells (Tibble et al. 1999).

In addition to the toxic gastrointestinal effects of NSAIDs, it has been reported that they cause adverse effects on the cardiovascular function. For example, aspirin is known to lengthen platelet closure times and naproxen has been shown to reduce platelet response to various stimuli (Jagroop et al. 2004). The accepted mechanism underlying these alterations is the inhibition of platelet COX-1 activity, which then results in alteration of TxA₂ levels, and thus affects platelet function (Gross and Moore 2004). Moreover, PGI₂, a key vasodilator and anticlotting compound produced as a result of the COX-2 cascade, is decreased in healthy

volunteers taking COX-2 inhibitors, celecoxib, and rofecoxib (Belton et al. 2000; Catella-Lawson et al. 1999; FitzGerald 2002; McAdam et al. 1999). The increased incidence of cardiovascular events in coxib users may be related, in part, to this untoward effect of COX-2 inhibitors (FitzGerald 2002). Consequently, we assessed the effect of one of the key constituents of NG440, RIAAs, on ex vivo PGI₂ production in healthy volunteers. No inhibition of PGI₂ or TxB₂ was noted, indicating that the production of these compounds is unaffected by NG440 and thereby consistent with inhibition of inflammatory signal transduction as the mode of action.

Because of their influence on prostaglandin production, COX-1 and COX-2 are important in maintenance of healthy blood pressure by their abilities to promote vasoconstriction and vasodilation, respectively. Both COX-1 and COX-2 are found in the kidneys and contribute to managing healthy renal flow (Davidge 2001; Krum et al. 2004). Several clinical trials have found an effect of blood pressure elevation after NSAID use, which averaged 5 mmHg (Cheng and Harris 2004; Cho et al. 2003; Johnson et al. 2003). Most studies have noted an increase within 6 weeks, and data suggest that hypertensive patients are more susceptible to this effect. In our review of 2 independent clinical trials, we found no evidence of an effect of NG440 formulas on blood pressure. Furthermore, when data were compiled and stratified by blood pressure at presentation, those subjects with hypertension did not show an effect of elevation of either systolic or diastolic blood pressure after taking NG440 for several weeks. The results are consistent with our hypothesis that inhibiting the COX-2 mediated prostaglandin biosynthesis by interfering with enzyme induction rather than enzyme activity will avoid the imbalance of PGI₂ and TxA₂ synthesis that has been attributed to the coxibs.

Therefore, we have found that NG440 does not appear to have gastrointestinal or cardiovascular side effects like those seen during NSAIDs use. Moreover, animal toxicity data revealed that NG440 at $\leq 250 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ for 21 days led to no adverse effects on body mass, food consumption, organ mass, organ-to-body mass ratios or gross or microscopic pathology. These data are supported by in vitro mechanistic studies, which have indicated that NG440 does not directly inhibit the COX enzymes, but may modulate the signal transduction at sites of inflammation (Tripp et al. 2003, 2005). Specifically, the ex vivo data suggest that NG440 leads to decreased PGE₂ production, a mechanism that may be attributed to its efficacy in reducing pain from inflammation. Data on patients collected from a variety of independent licensed healthcare practitioners indicate that the NG440 formula is efficacious for pain relief in a number of clinical scenarios, ranging from using the formula alone to taking it in conjunction with other dietary supplements and pharmaceuticals. Interestingly, all subjects from the multicentre trial appeared to receive statistically significant benefit from NG440 (27.8% reduction in symptoms). Twenty-six of the 56 subjects (46%) were categorized as responders (i.e., having $\geq 30\%$ reduction in VAS scores). Overall, the subgrouped responders experienced approximately 60% reduction in their symptoms. Our response rates of approximately 50% reveal a therapeutic equivalency to traditional NSAIDs. Rowbotham (2001) has reported that, in chronic conditions,

patients have reported that an approximately 30% reduction in clinical symptoms is meaningful. By this measure, groups (i) to (v) had a meaningful reduction in clinical symptoms. In summary, this collection of newer data when added to our existing clinical work with NG440 indicates that it results in pain relief in subjects with inflammation, most likely due to its effect on inhibiting inflammatory signal transduction leading to lowering of inflammatory cytokines and prostaglandins such as PGE₂.

In conclusion, NG440 appears to be a safe phytochemical combination that does not impact cardiovascular and gastrointestinal clinical markers of toxicity commonly affected by NSAIDs. Moreover, clinical data suggest that NG440, which may work through its effects on inflammatory cytokine and PGE₂ production, is efficacious for pain relief when taken alone or in combination with dietary supplements and pharmaceuticals. Given its different mechanism of action from traditional NSAIDs and COX-2 inhibitors and consequently its reduced frequency of expected adverse events, we consider that NG440 presents an alternative choice to NSAIDs.

Acknowledgements

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