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ScienceDirect
Phytochemistry 69 (2008) 1534–1547
PHYTOCHEMISTRY
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Safety, efficacy and anti-inflammatory activity of rho *iso*-alpha-acids from hops

Amy J. Hall [a,*], John G. Babish [b], Gary K. Darland [a], Brian J. Carroll [a], Veera Reedy Konda [a], Robert H. Lerman [a], Jeffery S. Bland [a], Matthew L. Tripp [a]

[a] *Metagenics/MetaProteomics Nutrigenomics Research Center, 9770 44th Avenue, Gig Harbor, WA 98332, USA*
[b] *MetaProteomics, 30 Brown Road, Ithaca, NY 14850, USA*

Received 19 November 2007; received in revised form 25 January 2008
Available online 20 March 2008

Abstract

A defined mixture of rho *iso*-alpha-acids (RIAA), a modified hop extract, was evaluated for anti-inflammatory efficacy and safety. RIAA inhibited LPS-stimulated PGE_2 formation with >200-fold selectivity of COX-2 ($IC_{50} = 1.3$ μg/ml) over COX-1 ($IC_{50} > 289$ μg/ml). This occurred only when RIAA was added prior to, but not post, LPS stimulation. Consistent with this observation, RIAA produced no physiologically relevant, direct inhibition of COX-1 or COX-2 peroxidase activity. This suggests that RIAA inhibits inducible but not constitutive COX-2. In support, we found RIAA showed minimal PGE_2 inhibition ($IC_{50} = 21$ μg/ml) relative to celecoxib ($IC_{50} = 0.024$ μg/ml), aspirin ($IC_{50} = 0.52$ μg/ml) or ibuprofen ($IC_{50} = 0.57$ μg/ml) in the AGS gastric mucosal model, where COX-1 and -2 are expressed constitutively. Taken together these results predict RIAA may have lower potential for gastrointestinal and cardiovascular toxicity observed with COX enzyme inhibitors. Following confirmation of bioavailable RIAA administered orally, gastrointestinal safety was assessed using the fecal calprotectin biomarker in a 14-day human clinical study; RIAA (900 mg/day) produced no change compared to naproxen (1000 mg/day), which increased fecal calprotectin 200%. Cardiovascular safety was addressed by PGI-M measurements where RIAA (1000 mg) did not reduce PGI-M or affect the urinary PGI-M/TXB_2 ratio. Drug interaction potential was evaluated against six major CYPs; of relevance, RIAA inhibited CYP2C9. Toxicity was assessed in a 21-day oral, mouse subchronic toxicity study where no dose dependent histopathological effects were noted. Clinically, RIAA (1000 mg/day) produced a 54% reduction in WOMAC Global scores in a 6-week, open-label trial of human subjects exhibiting knee osteoarthritis.

Keywords: *Humulus lupulus* L.; Cannabidaceae; Hops; Rho *iso*-alpha-acids; NSAID; Anti-inflammatory; Cyclooxygenase; PGE_2; Prostaglandin; Knee osteoarthritis; Calprotectin

1. Introduction

A key component of inflammation is the increase in prostaglandin (PG) biosynthesis resulting from activity of the cyclooxygenase (COX) enzymes (Prostaglandin-H-synthase EC 1.14.99.1). Three isozymes of COX have been identified: COX-1 (Holtzman et al., 1992); COX-2 (Hla and Neilson, 1992); and the splice variant COX-3 present in the brain and heart (Chandrasekharan et al., 2002). It is generally believed that COX-1 is expressed constitutively in most tissues, whereas COX-2 is an inducible isozyme triggered by pro-inflammatory stimuli such as cytokines and bacterial lipopolysaccharide (LPS) *in vitro* and at inflamed sites *in vivo*. Based primarily on such differences in expression, COX-1 is thought to be involved in maintaining physiological functions, such as cytoprotection of the gastric mucosa, regulation of renal blood flow and control of platelet aggregation (Dubois et al., 1998; Vane et al., 1998). While COX-2 induction (iCOX-2) is considered to function primarily as a mediator of inflammation in many tissues, constitutive expression (cCOX-2) is found in the

* Corresponding author. Tel.: +1 253 853 7318; fax: +1 253 851 9749.
E-mail address: amyhall@metagenics.com (A.J. Hall).

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doi:10.1016/j.phytochem.2008.02.001

brain, kidney, gastrointestinal tract and blood vessels (Topper et al., 1996; Vane et al., 1998; Vanegas and Schaible, 2001).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely prescribed medications in the world, with approximately 111 million prescriptions issued from 1999 to 2000 and more than 30 billion over-the-counter tablets sold annually in the US alone (Arroyo and Lanas, 2006; Lanas et al., 2006). Shared adverse effects of NSAIDs include (i) gastrointestinal bleeding, (ii) renal toxicity, (iii) increased risk of hemorrhage, and more recently identified (iv) increased risk of cardiovascular events such as myocardial infarction. These complications make NSAID therapy the most recognized cause of iatrogenic pathology, estimated to cause as many as 16,500 deaths/year in the US (Hernandez-Diaz et al., 2006; McGettigan and Henry, 2006). Moreover, the development of newer, COX-2 specific inhibitors has not abrogated GI toxicity associated with chronic NSAID therapy. The development of newer, COX-2 specific inhibitors requires validated approaches to diminish the signs and symptoms of inflammation with concomitant sparing of the gastric mucosa. However, the effect of COX-2 specific inhibitors on other organ systems was considered little, if at all, in the safety profile of the new generation compounds over traditional NSAIDs (James and Cleland, 2006).

Humulus lupulus L., commonly known as hops, has been employed as a medicinal plant, with a history of use dating back to Roman times. In modern herbal medicine, whole hop cones are used as a sedative to treat anxiety, restlessness, sleep disorders, menstrual symptoms and as an analgesic (Anonymous, 2003). Whole hop cones, as well as modified hop extracts, are currently used in brewing and food supplements. Numerous classes of compounds have been identified in extracts from the female flower cones of hops including alpha- and beta-acids and their oxidative degradation products. The structural and chemical properties of the alpha, *iso*-alpha and reduced *iso*-alpha-acids have been previously described (Verzele and De Keukeleire, 1991). Due to their beta tri-keto moiety these molecules are easily deprotonated and are commonly referred to as acids. The rho *iso*-alpha-acids (RIAA) can be produced via sodium borohydride reduction of the C6 carbonyl of the *iso*-alpha-acids which are produced from the isomerization of humulone. This isomerization is stereoselective for the *cis* (4*R*, 5*S*) isomer. Nord et al. (2003), have shown that the absolute configuration at C6 for the major *n*-analog of RIAA is *S* (Fig. 1). Our previous work showed that, among the modified hop extracts, RIAA was a potent and selective inhibitor of iCOX-2 driven PGE_2 biosynthesis in the LPS-stimulated RAW 264.7 macrophage (Tripp et al., 2004).

The aims of these studies were to characterize the anti-inflammatory activity and examine the safety profile of a defined mixture of RIAA, a modified hop extract. The COX inhibitory potency and selectivity of RIAA were analyzed in a cell-free system and in the RAW 264.7 murine macrophage utilizing LPS-dependent protocols. *In vitro* safety assessment included (i) the determination of relative gastrotoxicity using the RAW 264.7 macrophage and AGS gastric mucosal cell lines; and (ii) estimation of RIAA drug interaction potential through inhibition of cytochrome P-450 (CYP) catalytic activity. *In vivo* safety of RIAA was assessed in a subchronic, oral toxicity study in CD-1 mice. Clinically, RIAA potential gastrointestinal toxicity and cardiotoxicity were estimated, respectively, using fecal calprotectin excretion, and urinary prostaglandin I_2 metabolites (PGI-M) and thromboxane B_2 (TXB_2) in healthy human subjects. Blood chemistries were used as a measure of healthy kidney and liver function, as well as blood pressure for healthy cardiac function in all trial subjects administered RIAA for 2 or 6 weeks. Bioavailable RIAA administered orally in humans was examined using *ex vivo* analysis and RIAA diastereomer detection in plasma. Finally, the clinical anti-inflammatory efficacy of RIAA was evaluated in an open-label, pilot study in subjects with osteoarthritis of the knee (OA).

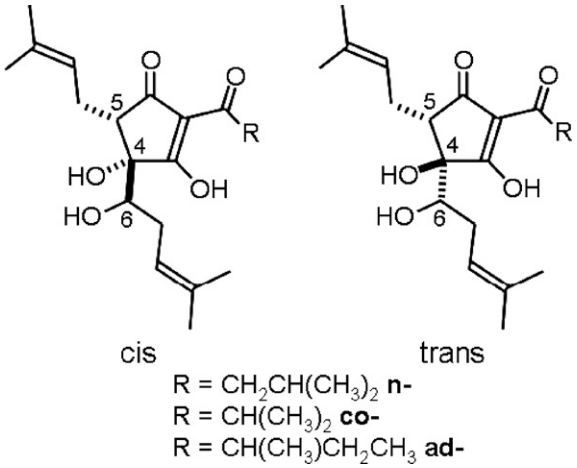

Fig. 1. The chemical composition of RIAA as determined by LC–MS and NMR analysis where *n* = isobutyl, co = isopropyl, and ad = sec-butyl.

2. Results

2.1. Cell free enzymatic assay of COX-1/2

With IC_{50}, respectively, of 15 μg/ml (95% CI = 8.8–26) and 18 μg/ml (95% CI = 15–21), RIAA exhibited only modest direct inhibition of both COX-1 and COX-2 peroxidase activity with no apparent selectivity of either isozyme.

2.2. RIAA potently inhibits PGE_2 from LPS-stimulated RAW 264.7 macrophage

Additional analyses of PGE_2 inhibition were conducted using RIAA and celecoxib, a highly selective direct inhibitor of COX-2, in the LPS-stimulated murine macrophage RAW 264.7 model. As seen in Fig. 2A and B, respectively, both RIAA and celecoxib inhibited the production of PGE_2 in a dose-dependent manner when administered 1 h prior to LPS stimulation. Alternatively, RAW 264.7 cells

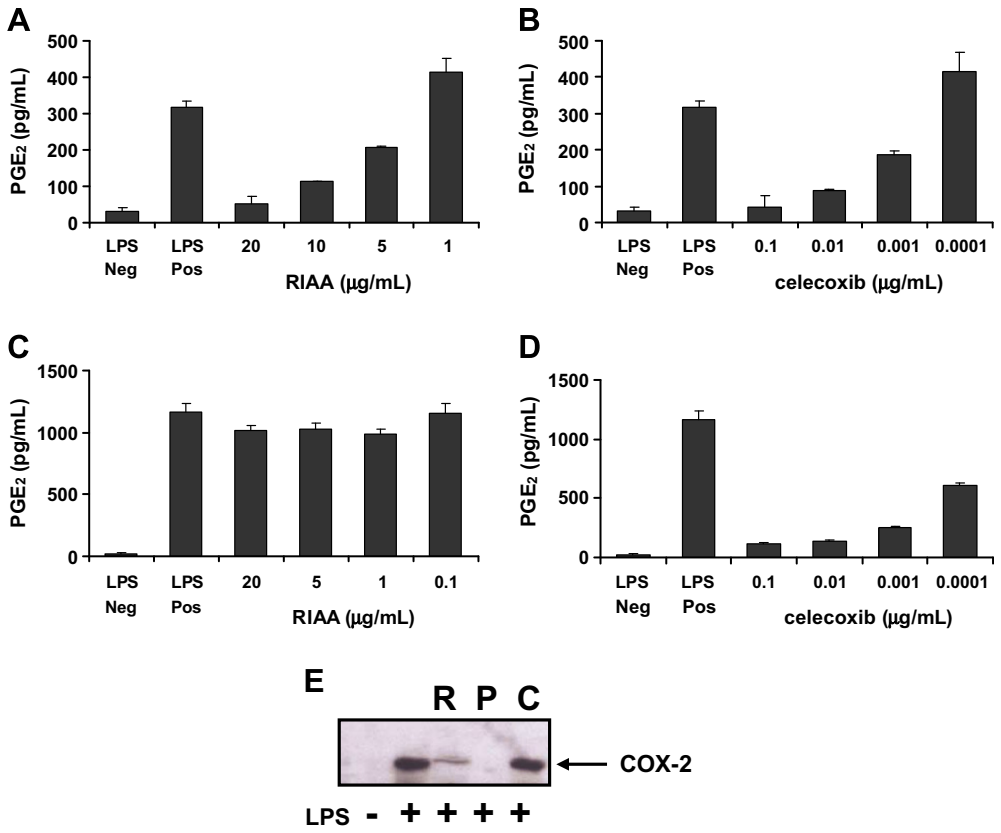

Fig. 2. Rho *iso*-alpha-acids inhibit PGE_2 production in LPS-stimulated RAW 264.7 macrophage cells without directly inhibiting COX-2. In A and B, cells were treated with RIAA (A) or celecoxib (B) in serum-free media for 1 h, stimulated with LPS for 4 h, and PGE_2 concentrations were determined. In C and D, the order of test material and LPS addition was reversed such that cells were stimulated with LPS for 12 h, washed, and RIAA (C) or celecoxib (D) was added with LPS for 1 h. Cells were washed, test substance and LPS added again for one additional hour. Media were then collected and PGE_2 concentrations determined. The graphed values represent the means ± SEM of four observations. Total cell extracts of equal protein were electrophoresed and COX-2 protein was detected by Western blot (E). Prior to lysis, RAW 264.7 cells were treated overnight with solvent control or test compound (R: RIAA 20 μg/ml; P: parthenolide 5 μg/ml; C: celecoxib 0.01 μg/ml) as indicated and subsequently stimulated with LPS (50 ng/ml) for 4 h.

were stimulated with LPS for 12 h, media removed, and the test compounds added to determine the amount of PGE_2 inhibition over 1 h. The COX-2 enzyme inhibitor, celecoxib, inhibited PGE_2 in a dose dependent manner. In sharp contrast, RIAA showed no inhibition of induced COX-2 (Fig. 2C and D). Overall, these protocol-dependent results suggest that while RIAA inhibited PGE_2 production in the LPS stimulated RAW 264.7 macrophage, it did not directly inhibit the COX-2 enzyme activity.

The abundance of the iCOX-2 protein was visualized in total cell extracts by Western blot after loading equal amounts of protein (10 μg) in each lane (Fig. 2E). Upon stimulation with LPS, the iCOX-2 protein was expressed in 4 h as expected. As a known inhibitor of NF-κB, parthenolide (5 μg/ml) (Hehner et al., 1999) attenuated iCOX-2 expression, whereas celecoxib (0.01 μg/ml) did not. The abundance of iCOX-2 protein following treatment with RIAA (20 μg/ml) was markedly diminished.

Further characterization of the RIAA inhibition of COX-1/2 mediated PGE_2 biosynthesis in the RAW 264.7 macrophage model used exogenous arachidonic acid (AA) at 5 or 50 μM and two LPS-stimulation protocols. At concentrations greater than 10 μM AA, PGE_2 biosynthesis is mediated primarily through COX-1 and at AA concentrations less than 10 μM the COX-2 isozyme is the major source of PGE_2 (Murakami et al., 1999). Therefore, high (50 μM) and low (5 μM) exogenous AA concentrations were used to examine inhibition of COX-1 and COX-2 biosynthesis of PGE_2, respectively. RIAA produced only minimal inhibition of COX-1 mediated PGE_2 production using non-stimulated RAW 264.7 cells and 50 μM AA; the extrapolated estimate of the IC_{50} was 289 μg/ml RIAA (Table 1). When RIAA or NSAIDs were added 1 h prior to LPS stimulation followed by the addition of 5 μM AA for 30 min, RIAA displayed striking iCOX-2 mediated PGE_2 inhibition with an IC_{50} of 1.3 μg/ml, similar to the IC_{50} of aspirin (1.1 μg/ml) and ibuprofen (0.85 μg/ml).

2.3. Low relative gastropathy predicted for RIAA with AGS gastric mucosal model

Examination of RIAA effects on PGE_2 biosynthesis in the AGS mucosal cell model in combination with inhibition of LPS-stimulated PGE_2 in the RAW 264.7

Table 1
PGE_2 median inhibitory concentrations (IC_{50}) mediated by COX-1 and COX-2 in RAW 264.7 and AGS cell models

Compound	RAW 264.7 cells		AGS cells	Ratio	
	$COX\text{-}1_{(50\,\mu M\ AA)}$ PGE_2 IC_{50} (μg/ml)	$COX\text{-}2_{(LPS/5\,\mu M\ AA)}$ PGE_2 IC_{50} (μg/ml)	$AGS_{(A23187)}$ PGE_2 IC_{50} (μg/ml)	IC_{50} ratio, COX-1/COX-2	IC_{50} ratio, $AGS/RAW_{COX\text{-}2}$
RIAA	289[a] (119–697)	1.3 (0.65–2.6)	21 (3.0–145)	222	16
Celecoxib	6.4 (2.6–16)	0.015 (0.008–0.31)	0.024 (0.007–0.081)	427	1.6
Aspirin	0.095 (0.03–0.35)	1.1 (0.48–2.6)	0.52 (0.26–1.1)	0.086	0.47
Ibuprofen	16 (3.1–83)	0.85 (0.48–1.49)	0.57 (0.27–1.2)	19	0.67

Median inhibitory concentrations were computed from a minimum of four concentrations over at least two independent experiments. Parenthetic values represent the 95% confidence intervals. For estimating COX-1 mediated PGE_2 inhibition, non-stimulated RAW 264.7 cells were incubated with test compounds overnight then treated with 50 μM AA for 15 min, after which the media was collected and PGE_2 concentrations determined. For determining COX-2 mediated PGE_2 inhibition, RAW 264.7 cells were incubated with test compounds for one h, then stimulated with LPS (1 μg/ml) for 4 h, the media was removed and 5 μM AA was added for an additional 15 min. For determining PGE_2 inhibition in the AGS cells, 60 min following addition of the test materials, 50 μM A23187 was added for 30 min and PGE_2 concentrations determined.

[a] Extrapolated IC_{50} values are stated for the purpose of calculating ratios.

macrophage cell model allowed for the determination of relative gastrotoxic potential. RIAA, celecoxib, aspirin and ibuprofen were tested for their ability to inhibit PGE_2 production in the AGS model using A23187 as a stimulator of AA release. Our earlier work showed this methodology provided a significant ($p < 0.05$) correlation to clinical observations of NSAID gastropathy (Hall et al., 2006). As seen in Table 1, celecoxib (IC_{50} 0.024 μg/ml), aspirin (IC_{50} 0.52 μg/ml) and ibuprofen (IC_{50} 0.57 μg/ml) were potent inhibitors of PGE_2 production in the AGS model, whereas RIAA (IC_{50} 21 μg/ml) proved to be a weak inhibitor.

Selectivity of COX-1/2 inhibition in RAW 264.7 cells by RIAA, celecoxib, aspirin and ibuprofen is illustrated by graphing the log IC_{50} ratio of COX-1/COX-2 (Fig. 3A). Further, by plotting the log of the IC_{50} ratio AGS/RAW 264.7, a scale of relative gastrotoxic potential can be constructed (Fig. 3B). These graphs indicate that RIAA exhibited COX-2 selectivity similar to that of celecoxib. However, RIAA exhibited remarkably lower relative gastrotoxic potential compared to celecoxib, ibuprofen or aspirin.

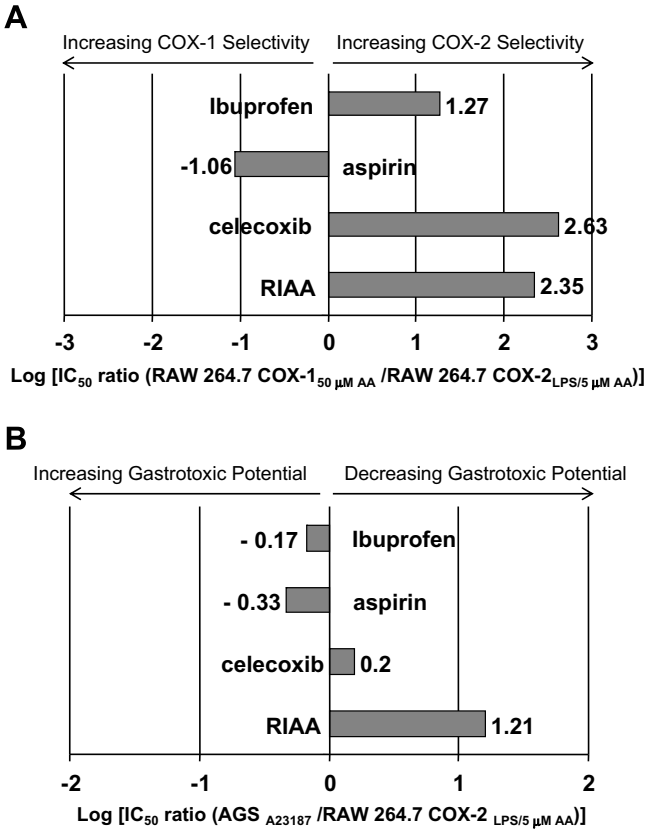

Fig. 3. COX-1/COX-2 selectivity and gastrotoxic potential of rho *iso*-alpha-acids. (A) Graphic representation of log IC_{50} ratios for COX-1 and COX-2 inhibition in RAW 264.7 cells. Positive log IC_{50} ratio values to the right indicate increasing COX-2 selectivity, while negative log IC_{50} ratio values to the left indicate increasing COX-1 selectivity. (B) Graphic representation of log IC_{50} ratios for PGE_2 inhibition in AGS and RAW 264.7. Positive log IC_{50} ratio values to the right indicate decreasing gastrotoxic potential, while negative log IC_{50} ratio values to the left indicate increasing gastrotoxic potential.

2.4. RIAA inhibition of CYP isozymes

Six CYP isozymes 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4, collectively involved in the metabolism of approximately 85% of all phase I metabolized drugs and other xenobiotics (Guengerich, 2003), were tested for inhibition by RIAA. With an IC_{50} of 0.30 μg/ml RIAA, 2C9 was the most strongly inhibited CYP isozyme, while 2C19 was moderately inhibited with an IC_{50} of 6.3 μg/ml RIAA. 3A4 was weakly inhibited by RIAA with IC_{50} of 12 and 14 μg/ml, respectively, for dibenzyl fluorescein and 7-benzyloxy-4-trifluoromethylcoumarin substrates. No inhibition was noted at the highest concentrations of RIAA tested for 1A2 (>100 μg/ml), 2D6 (>100 μg/ml) and 2E1 (>50 μg/ml).

2.5. Subchronic toxicity of RIAA in mice

Forty CD-1 mice were assigned to receive either the control or RIAA by gavage at 25, 75 or 250 mg/kg day (five male and five female mice per group). Table 2 details the body weights, organ weights and relative organ weights of the vehicle control and high dose of 250 mg RIAA/kg day, as the intermediate doses showed no effects of

Table 2
Body weights, organ weights and relative organ weights of CD-1 mice receiving 250 mg RIAA/kg day by gavage for 21 days ($n = 5$ in each group; mean ± SD)

Day	Control		RIAA (250 mg/kg day)	
	Male	Female	Male	Female
	Food consumption (g/animal day)			
−6	5.7 ± 0.26	4.8 ± 0.22	5.7 ± 0.16	5.0 ± 0.37
6	5.8 ± 0.16	4.8 ± 0.24	5.8 ± 0.27	5.0 ± 0.28
13	5.4 ± 0.37	4.8 ± 0.29	5.8 ± 0.46	5.0 ± 0.35
20	5.3 ± 0.42	4.9 ± 0.58	5.9 ± 0.68	5.2 ± 0.28
Day	Body weight (g)			
−6	30 ± 0.34	26.3 ± 0.92	29.9 ± 1.78	26.0 ± 0.68
−1	32 ± 1.0	26.8 ± 0.89	32.0 ± 1.16	26.7 ± 0.81
6	32 ± 1.1	26.0 ± 1.53	32.3 ± 1.36	26.0 ± 0.82
13	32 ± 1.8	26.2 ± 1.47	33.5 ± 1.71	26.6 ± 1.03
20	33 ± 2.0	27.5 ± 1.28	35.4 ± 2.12	27.8 ± 1.19
Organ	Organ weight (g)			
Fasting	29 ± 1.7	24.2 ± 1.50	30.8 ± 2.09	24.7 ± 1.19
Brain	0.45 ± 0.059	0.47 ± 0.040	0.44 ± 0.041	0.45 ± 0.050
Liver	1.3 ± 0.091	1.01 ± 0.050	1.52 ± 0.174[a]	1.10 ± 0.056[a]
Heart	0.15 ± 0.027	0.13 ± 0.011	0.16 ± 0.012	0.12 ± 0.004
Kidneys	0.47 ± 0.059	0.36 ± 0.029	0.51 ± 0.035	0.33 ± 0.015
Spleen	0.08 ± 0.007	0.09 ± 0.008	0.08 ± 0.02	0.09 ± 0.01
Organ	Organ weight relative to body weight			
Brain	15.5 ± 1.56	19.4 ± 1.29	14.2 ± 1.08	18.4 ± 2.02
Liver	44.7 ± 2.25	41.5 ± 1.45	49.5 ± 4.59	44.7 ± 1.31[a]
Heart	5.3 ± 0.67	5.3 ± 0.53	5.1 ± 0.36	5.0 ± 0.33
Kidneys	16.4 ± 1.13	14.7 ± 0.52	16.6 ± 0.87	13.4 ± 0.63
Spleen	2.6 ± 0.34	3.7 ± 0.26	2.6 ± 0.58	3.8 ± 0.50
Organ	Organ weight relative to brain weight			
Liver	2.92 ± 0.411	2.15 ± 0.095	3.48 ± 0.172[a]	2.45 ± 0.320
Heart	0.35 ± 0.065	0.27 ± 0.028	0.36 ± 0.030	0.28 ± 0.037
Kidneys	1.07 ± 0.126	0.76 ± 0.072	1.17 ± 0.108	0.74 ± 0.06
Spleen	0.17 ± 0.033	0.19 ± 0.009	0.18 ± 0.042	0.21 ± 0.031

[a] Indicated statistically significant from control group ($p \leqslant 0.05$).

RIAA in any variables examined. Five mice, one male and two females in the 25 mg RIAA/kg day, and one male and one female in the mid-dose 75 mg RIAA/kg day group died during the course of the study. However, since no cause of death could be determined from the tissues examined at necropsy and there were no deaths at the highest dose, it appeared the deaths were unrelated to RIAA and likely to physical manipulations (i.e. gavage). The only statistically significant ($p < 0.05$) change noted was an increase in absolute and relative liver weights in both males and females. Histological examination noted changes only in the liver and spleen after 21 days. Liver alterations included minimal centrilobular hepatocellular hypertrophy, indicative of a normal adaptive response usually associated with enzyme induction and likely the cause of increased liver weight. Spleen changes consisted of an increased incidence of extramedullary hematopoiesis and was noted in both control and treatment groups. All noted changes were evaluated as minor histological changes during the course of the study.

2.6. RIAA effect on fecal calprotectin and urinary prostanoid excretion in humans

Potential clinical gastrointestinal toxicity of RIAA was evaluated using fecal calprotectin, a biomarker of gastrointestinal mucosal inflammation (Konikoff and Denson, 2006). In combined observational studies, fecal calprotectin was measured at baseline, 7, and 14 days following dosing with 450 mg RIAA b.i.d. Fecal calprotectin excretion was compared to dosing with 500 mg naproxen b.i.d. (Fig. 4A) to illustrate the power of the fecal calprotectin biomarker to identify gastrointestinal inflammation during the observational time frame as previously reported (Meling et al., 1996). As expected, naproxen increased fecal calprotectin 241% and 158% over baseline in the first and second weeks, respectively. RIAA administration, however, did not increase fecal calprotectin excretion over baseline in the first- or second-week.

Urinary prostanoid excretion of PGI_2 and TXA_2 are indicators of cardiovascular homeostasis as they function, respectively, as a potent vasodilator and vasoconstrictor (Grosser et al., 2006). However, neither is stable for detection and the metabolic products PGI-M and TXB_2 are measured to reflect levels, respectively, of PGI_2 and TXA_2 *in vivo* (Fig. 4B). Samples were corrected for recovery which averaged 108% for PGI-M and 85% for TXB_2. The ratio of PGI-M to TXB_2 increased in the RIAA study to 1.07 (95% CI = 1.38–0.80), while the ratio decreased in the celecoxib study to 0.71 (95% CI = 1.04–0.44). This change in the ratio of PGI-M to TXB_2 was statistically significant ($p = 0.035$).

2.7. RIAA effect on blood chemistries and blood pressure in humans

Potential adverse clinical effects of RIAA were assessed by blood pressure measurement and clinical chemistries at the initial and final visit of two trials. No subjects exhibited changes of any variables outside the normal range during the trial. Values for all 22 subjects were averaged (± SEM) to note any directional changes or trends in measured biomarkers. No clinical significant changes ($p > 0.05$) between the initial and final visit were observed for systolic blood pressure, 133 ± 17.1 vs. 130 ± 16.1, or diastolic blood pressure, 80 ± 9.0 vs. 78 ± 10.9. Similarly no changes were observed for WBC ($10^3/mm^3$), 6.6 ± 1.98 vs. 6.2 ± 1.79; RBC ($10^6/\mu l$), 4.77 ± 0.427 vs. 4.74 ± 0.373; HGB (g/dl), 14.3 ± 1.18 vs. 14.2 ± 0.88; HCT (%), 42.1 ± 3.41 vs. 41.8 ± 2.49; PLT ($10^3/mm^3$), 256.5 ± 57.39 vs. 260.4 ± 58.56. No change was observed for electrolytes and the kidney function markers Na (mequiv./l), 142 ± 1.8 vs. 141 ± 1.3; K (mequiv./l), 4.4 ± .0.36 vs. 4.3 ± 0.42; Cl (mequiv./l), 104 ± 1.9 vs. 103 ± 2.2; CO_2, 31 ± 1.6 vs. 30 ± 1.9; BUN (mg/dl), 16 ± 4.7 vs. 16 ± 4.1; creatinine (mg/dl), 0.93 ± 0.177 vs. 0.92 ± 0.169.

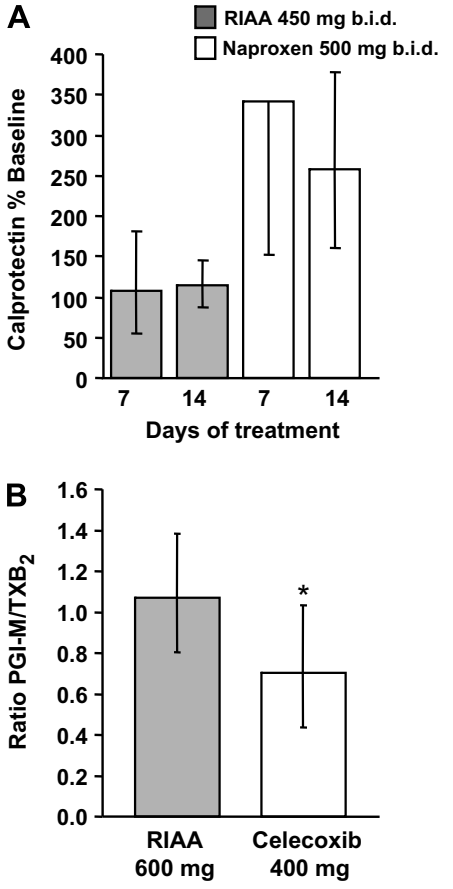

Fig. 4. Excretion of fecal calprotectin and urinary prostanoids in healthy human subjects administered RIAA. (A) Fecal calprotectin measurements are shown of nine subjects following the administration of either 450 mg RIAA b.i.d. (*gray bars*) or 500 mg naproxen b.i.d. (*white bars*) in separate studies. Samples were taken at the start of the trial (baseline) and at 7 and 14 days during administration of the compound. The 7- or 14-day calprotectin (μg/g stool) value was divided by the baseline value to determine the percent baseline calprotectin as graphed. The average baseline was 22.6 ± 7.6 (SEM) μg/g stool in the RIAA study and 27.2 ± 3.6 (SEM) μg/g stool in the naproxen study. The error bars represent the 95% upper and lower confidence limits. (B) Urinary prostanoid ratios are shown for eight subjects following the administration of 600 mg RIAA or 400 mg celecoxib in a single dose. Urinary metabolites of PGI-M and TXB_2 were assayed as described in Section 4. Control urine was taken several weeks later for comparison. The average ratio of PGI-M to TXB_2 was 1.07 (range = 1.38–0.80) in the RIAA study and 0.71 (range = 1.04–0.44) in the celecoxib study. The error bars represent the 95% upper and lower confidence limits with * indicating $p = 0.035$ statistically significant between treatment groups.

2.8. Bioavailable RIAA

To determine the bioavailable RIAA following oral administration, a single dose of RIAA was administered to healthy human volunteers and blood was drawn at regular intervals. The collected plasma was applied to RAW 264.7 cells followed by LPS stimulation and the inhibition of PGE_2 biosynthesis by the plasma was determined by *ex vivo* analysis. The production of PGE_2 was inhibited by plasma collected from subjects administered RIAA (600 mg) and celecoxib (200 mg), but not placebo (Fig. 5A). Inhibition of PGE_2 biosynthesis rose sharply at 2 h post-administration followed by a gradual increase over the 6-h period of observation. The $AUC_{ex\,vivo}$ for celecoxib over this 6-h period was approximately 50% greater than that of RIAA. In a parallel study, following oral administration, bioavailable RIAA was determined by the measurement of two separate diastereomers of the *n*-analog of RIAA using a LC–MS method operating in mass reaction monitoring (MRM) scanning mode. This method is capable of detecting and quantifying both the major *cis* diastereomer of the *n*-analog, 6*S* (Nord et al., 2003), and the major *trans* diastereomer of the *n*-analog, tentatively assigned as 6*R*. Both the *cis* 6*S* and *trans* 6*R* *n*-diastereomers were present in the plasma following oral administration and reached a maximum concentration at 4 h (Fig. 5B). The AUC_{LC-MS} for the *trans* 6*R* *n*- and *cis* 6*S*

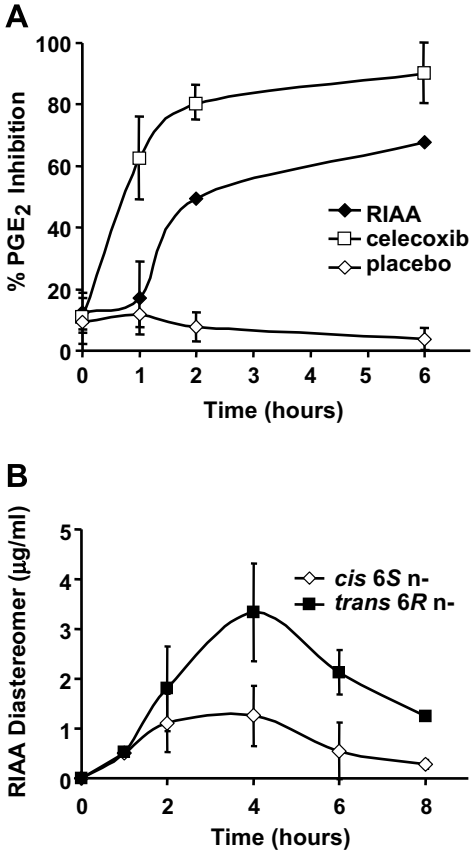

Fig. 5. Bioavailable RIAA following oral administration. By *ex vivo* analysis, the inhibition of PGE_2 in LPS-stimulated RAW 264.7 macrophages treated with subject's plasma was demonstrated (A). Fasting subjects (two) were administered 600 mg RIAA, 200 mg celecoxib, or placebo and blood was drawn pre-dose and at times indicated. Percent PGE_2 inhibition was determined using the PGE_2 stimulation of each subject's plasma at pre-dose (time zero) with and without LPS-stimulation for 4 h as maximum stimulation. Each sampling period on the graph represents the average of two observations with eight replicates each (mean ± SD). By LC–MS analysis, the presence of the RIAA diastereomers was measured in subject's plasma (B). Following oral administration of 1000 mg RIAA, blood was drawn pre-dose and at times indicated. The graph represents the average *cis* 6*S* and *trans* 6*R* *n*-diastereomers of RIAA (mean ± SD) of a representative subject.

n-diastereomers over this 8-h period was 18.8 and 7.15 μg h/ml, respectively.

2.9. Pilot trial of RIAA clinical efficacy for knee OA

The anti-inflammatory activity of RIAA was assessed clinically in subjects with knee OA in an open-label, single-arm study. Thirteen subjects were given 500 mg RIAA b.i.d. for 6 weeks, and the effect of RIAA supplementation was assessed using the validated WOMAC and VAS questionnaires. In all of the questionnaires used, a reduction in score was due to a reduction in symptoms, such as pain and stiffness. After 6 weeks, RIAA administration led to a 54% reduction ($p < 0.001$) in the WOMAC Global score (Fig. 6A). The mean global WOMAC score (each visit was treated as a group for the analysis) were significantly reduced ($p < 0.001$) at V3 and V4. The WOMAC sub-scales of pain, stiffness and physical function assessment (Fig. 6B–D) all showed an average 45% reduction in scores at V4. RIAA administration also led to a reduction in VAS scores for pain relative to V1 (Fig. 6E), indicating alleviation in pain in response to therapy with statistically significant differences from V1 to V3 (1.89 cm; $p < 0.05$) and V4 (2.50 cm; $p < 0.01$).

3. Discussion

Our cell free studies with COX-1/2, whole cell experiments with LPS-stimulated RAW 264.7 macrophages and AGS gastric mucosal cells, and Western blotting results strongly suggest that RIAA selectively inhibits iCOX-2 stimulated PGE_2 production with minimal effect on COX-1 or cCOX-2. These results are consistent with earlier

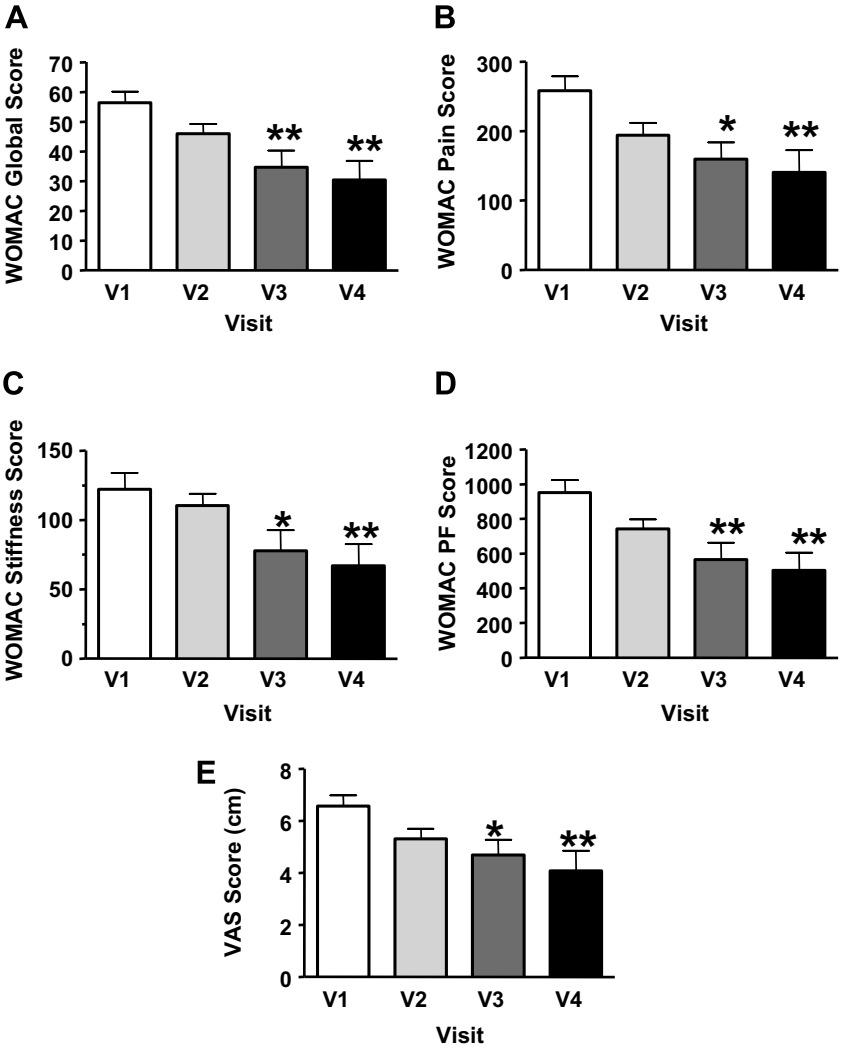

Fig. 6. Rho *iso*-alpha-acids decreased WOMAC and VAS Scores in subjects with knee osteoarthritis. Thirteen subjects began this open-label, uncontrolled, single-arm trial. At Visit 1 (V1), RIAA was administered 500 mg b.i.d. Subjects returned at 1 week (V2), 2 weeks (V3) and 6 weeks (V4; final visit) for physical exams and review of symptoms. Shown is the individual WOMAC global score (A), as the primary endpoint, with secondary endpoints of assessment of WOMAC sub-scores of pain (B), stiffness (C), physical function (D) and VAS for pain (E). The error bars are SEM with ** indicating $p < 0.001$ and * indicating $p < 0.01$ significantly different from visit 1 (V1).

studies utilizing the hops alpha-acid humulone that demonstrated an inhibition of iCOX-2 transcription and expression resulting in a high selectivity for iCOX-2 synthesis of PGE_2 in TNFα-stimulated MC3T3-E1 osteoblasts (Yamamoto et al., 2000). Later, Nozawa et al. (2005), using a protocol of *iso*-alpha-acid (IAA) addition 1 h prior to LPS/interferon-γ stimulation, demonstrated inhibition of PGE_2 biosynthesis in RAW 264.7 cells. A possible mechanism for the anti-inflammatory effects of RIAA may be inferred from the decreased iCOX-2 protein abundance observed in the LPS-stimulated RAW 264.7 cell following treatment with RIAA. In addition, lack of PGE_2 inhibition in AGS gastric mucosal cells would be consistent with little or no effect on cCOX-2 in these cells.

Another *in vitro* technique employed to assess the relative safety of RIAA was the inhibition of specific CYP isozymes. Inhibition of CYP biotransformation of drugs may lead to adverse drug interactions with potential toxicity or the loss of drug efficacy. RIAA was found to inhibit CYP isozymes 2C9 and 2C19 with IC_{50}, respectively, of 0.25 μg/ml (~0.69 μM) and 6.1 μg/ml (~17 μM). While clinical relevance is difficult to predict from single probe *in vitro* assays, potent inhibitors, defined as compounds with $IC_{50} < 1$ μM, demonstrate greater potential than weak inhibitors with $IC_{50} > 10$ μM to be associated with clinically significant drug interactions (Friden et al., 2006). Our findings on the ability of RIAA to inhibit six CYP isozymes predict low clinically relevant potential drug interactions for 1A2, 2C19, 2D6, 2E1 and 3A4. Isozyme 2C9 is involved in the metabolism of several important groups of drugs including coumarin anticoagulants, sulfonylureas, angiotensin II blockers, many NSAIDs, phenytoin and rosiglitazone. NSAID substrates include celecoxib, diclofenac, flurbiprofen, ibuprofen, indomethacin, meloxicam, naproxen and piroxicam. Numerous *in vitro* and individual case studies have reported that natural products including boldo (*Peumus boldus* Molina), cranberry juice, garlic, grape juice, ginkgo biloba, coenzyme Q, danshen (*Salvia miltiorrhiza*), Devil's claw, dong quai (*Angelica sinensis*), fenugreek, fish oil, ginseng, mango, tea and vitamin E may inhibit 2C9 and increase INR (International Normalized Ratio) (Greenblatt et al., 2006; Holbrook et al., 2005). To date, clinical information on herbal medicine interactions with warfarin consists almost entirely of single case reports that have proven unreliable in terms of deducing a cause-and-effect relationship. Inferences regarding the likelihood of clinical drug interactions based only on *in vitro* data involving natural products should be made with caution. Putative CYP targets for inhibitors may be verified for clinical relevance using pharmacokinetic studies.

Our *in vitro* results with the AGS gastric mucosal cell line and clinical fecal calprotectin study indicate RIAA possess low potential gastrointestinal toxicity relative to acidic NSAIDs such as aspirin, ibuprofen and naproxen. Gastropathy is the most common adverse event with NSAID administration, producing symptoms of dyspepsia and peptic ulcer disease in up to 50% and 20%, respectively, of individuals taking them at therapeutic dosages (Peura, 2004). The prevalence of NSAID-associated serious outcome events in the lower gastrointestinal tract has been estimated at 40% (Laine et al., 2003). Inhibition of iCOX-2 mediated PGE_2 biosynthesis as demonstrated in the RAW 264.7 macrophage with little inhibition of cCOX-2 as seen in AGS gastric mucosal cells indicates that RIAA has the potential for reduced gastrointestinal toxicity relative to that seen with traditional NSAIDs. Clinical support for low gastrointestinal toxicity of RIAA was further provided by the lack of increased fecal calprotectin excretion at therapeutically relevant doses.

In addition to GI toxicity, NSAIDs can affect vascular function through both COX-1 and COX-2 mechanisms (Davidge, 2001). Recent safety concerns have arisen with evidence of increased risk of cardiovascular events with selective COX-2 inhibitors. Previously published findings that three selective COX-2 inhibitors, rofecoxib, celecoxib and valdecoxib, are associated with increased risk of myocardial infarction in placebo controlled clinical trials (Bombardier et al., 2000; Bresalier et al., 2005; Nussmeier et al., 2005; Solomon et al., 2006) strongly implies that selective inhibition of cCOX-2-dependent PGI_2 unaccompanied by inhibition of platelet COX-1 translates into an increased risk of myocardial infarction and stroke (Capone et al., 2007; Grosser et al., 2006). The clinical information is compatible with evidence that inhibition of cCOX-2-derived PGI_2 removes a protective constraint on thrombogenesis, hypertension and atherogenesis *in vivo*. While celecoxib decreased the urinary PGI-M/TXB_2 ratio as expected in our clinical study, RIAA did not. Using GC–MS, McAdam et al. (1999) observed a ~43% reduction in the ratio of urinary 2,3-dinor-6-keto-PGF1α to TXB_2 during the 4–6 h interval in subjects treated with 400 mg celecoxib. The ratio returned to the baseline as the collection interval was increased. These results are consistent with our hypothesis that inhibition of iCOX-2 mediated prostaglandin biosynthesis rather than enzyme activity will avoid the imbalance of PGI_2 and TXA_2 synthesis attributed to highly selective cCOX-2 inhibitors. Additionally, no change in blood pressure was observed in a 2- or 6-week study with RIAA. Thus, RIAA would likely represent a lower cardiovascular risk relative to direct COX-2 inhibitors.

The mouse subchronic oral toxicity study reported here supports a lowest observed adverse effect level (LOAEL) of 250 mg RIAA/kg day and a no observable adverse effect level (NOAEL) of 75 mg RIAA/kg day. Based upon a 70 kg human receiving 500 mg RIAA b.i.d. or 14 mg RIAA/kg day for knee OA, the 75 mg/kg day NOAEL inferred from the 21-day mouse gavage study support a conservative safety factor of 5.3-fold for RIAA. Further, no identified histological changes in the mouse were observed in the kidney, such as papillary necrosis and interstitial nephritis, indicative of NSAID-associated renal toxicity (Kramer, 1986), respectively, at the 250 or 500 mg RIAA/kg day doses. Thus, RIAA

exhibits an 18- to 36-fold safety factor for nephrotoxicity.

On a molecular level, NSAID nephrotoxicity may be associated with COX inhibition. Recent studies have demonstrated that cCOX-2 is expressed in renal tissues of all species. Drugs that selectively inhibit cCOX-2 and iCOX-2 might, therefore, be expected to produce effects on renal function similar to nonselective NSAIDs (Brater et al., 2001). As an inhibitor of iCOX-2 with little effect on COX-1 or cCOX-2, RIAA would not be expected to affect prostaglandin-dependent renal homeostatic processes. Indeed, no change was observed in kidney or liver biomarkers measured in a 2- or 6-week study with RIAA.

We previously reported a pilot trial evaluating patients with osteoarthritis, rheumatoid arthritis and fibromyalgia and found that 600 mg RIAA/day, administered as a component in a proprietary combination that included a rosemary extract and oleanolic acid, decreased pain and condition specific symptoms in osteoarthritis subjects. A decreasing trend of high sensitivity C-reactive protein (hsCRP) was also observed in all subjects who presented with elevated ($\geqslant$7.0 mg/l) hsCRP (Lukaczer et al., 2005). Additionally, this proprietary combination demonstrated to be safe in mouse oral toxicity studies and did not negatively impact cardiovascular and GI biomarkers (Minich et al., 2007). These reports substantiate our initial findings and further characterize the efficacy of RIAA as a single agent when administered at a dose of 1000 mg/day.

The efficacy of RIAA as an anti-inflammatory was evidenced in a reduction in clinical symptoms in subjects with knee OA. After 6 weeks of RIAA administration, pain, stiffness and physical function all showed marked improvement as assessed by WOMAC and VAS questionnaires. Results from the *ex vivo* analysis provided an indirect measurement of bioavailable RIAA. More conclusive evidence indicating oral bioavailable RIAA was obtained by LC–MS measurement of the RIAA diastereomers. Taken together, the results corroborate the presence and activity of RIAA in plasma following an oral dose. The concentration of each of the predominant *cis* and *trans* *n*-diastereomers in human plasma after 4 h (1.3 μg/ml of *cis* 6*S* *n*- and 3.3 μg/ml of *trans* 6*R* *n*-) is sufficient to reduce PGE_2 biosynthesis as measured by the RAW 264.7 cell assay (RIAA IC_{50} 1.3 μg/ml). Given that these two diastereomers comprise ~40% of the total RIAA administered, and the similarity of the RIAA analogs, the total amount of RIAA species in the blood is likely to be higher than the combined amount measured for these two diastereomers. It is therefore reasonable to infer that the *ex vivo* activity observed in the plasma following an oral dose of RIAA is most likely due to the presence of the RIAA in the blood. Given that the ratio of the *cis* 6*S* *n*-diastereomer to the *trans* 6*R* *n*-diastereomer in RIAA is 3.6:1 (data not shown), it is apparent from the LC–MS analysis that the relative bioavailability of the tentatively assigned *trans* 6*R* *n*-diastereomer is approximately nine times that of the of the *cis* 6*S* *n*-diastereomer. Further investigation of the differences in activity and bioavailability between the various RIAA analogs and diastereomers is currently on going and will be reported in due course.

The goal of this research was to characterize the hops derived RIAA phytochemical with respect to its anti-inflammatory activity and relative safety *in vitro* and *in vivo*. These results indicate that RIAA selectively inhibits iCOX-2 mediated PGE_2 biosynthesis by a mechanism different than that of traditional NSAIDs. It is likely that for this reason RIAA does not negatively impact gastrointestinal and cardiovascular biomarkers commonly associated with NSAIDs. Furthermore, clinical efficacy and bioavailability imply that RIAA is efficacious for pain relief associated with inflammatory conditions.

4. Experimental

4.1. Chemicals

Heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin solution, Ham's F12K medium and Dulbecco's Modification of Eagle's Medium (DMEM) were purchased from Mediatech (Herndon, VA). The commercial formulation of celecoxib (Celebrex®, G.D. Searle & Co., Chicago, IL) was used and all concentrations were based on the active material although excipients were included. LPS, ibuprofen, aspirin, naproxen, parthenolide and A23187 were obtained from Sigma (St. Louis, MO). Ovine COX-1, baculovirus expressed human COX-2 (rhCOX-2), and an ethanolic solution of arachidonic acid (AA) were obtained from Cayman Chemical (Ann Arbor, MI). A commercial preparation of the dried RIAA magnesium salt (Mg-RIAA) provided by John I. Haas (Yakima, WA) was used for this study. This material has a high degree of consistency and is shown to contain approximately 25% inorganic salts, e.g., Mg^{2+}, K^+ and SO_4^{2-}, 5% low-molecular resin and 68% total RIAA. RIAA is a well defined mixture of related analogs and diastereomers (Fig. 1). Within the RIAA portion, the ratio of *cis* to *trans* is 3:1 and the ratio of *co*- to *n*- is 1:2.2 as determined by HPLC, respectively (data not shown). Mobile phases used for all HPLC analyses were supplied by either Burdick & Jackson or EMD and used directly. All other reagents were obtained from Sigma.

4.2. Cell free, enzymatic assay of COX-1/2

The peroxidase activity of purified COX was measured based upon the oxidation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2 as described by Ouellet et al. (2004). The reaction contained test compounds (50 μg/ml) with a final 1% DMSO concentration. The reaction was monitored at 610 nm for 2 min and the initial rate was estimated by fitting data collected over the first 36 s to a second degree polynomial. The mean inhibitory concentration (IC_{50}) with

95% confidence limits were estimated by the CalcuSyn software package (Biosoft, Ferguson, MO), which utilizes median effects methodology. The positive control NS-389 exhibited an IC_{50} for COX-1 and COX-2, respectively, of >50 and 0.14 μg/ml, while the IC_{50} for indomethacin were 0.72 and 0.31 μg/ml; IC_{50} for both positive controls were in agreement with published results (Ouellet et al., 2004).

4.3. Cell culture and treatment with RIAA

RAW 264.7 cells (TIB-71) were obtained from the American Type Culture Collection (Manassas, VA) and sub-cultured in high glucose DMEM supplemented with 10% FBS according to the instructions of the supplier. Test compounds were dissolved in DMSO, then diluted in serum-free media and added in a final concentration of 0.1% DMSO. For estimating COX-1 mediated PGE_2 formation, non-stimulated cells were incubated with test compounds overnight. The cells were washed and treated with 50 μM AA and test material containing media for 15 min, the media was collected and PGE_2 concentrations determined. For determining iCOX-2 inhibition, cells were incubated with test compounds for 1 h, then stimulated with LPS (1 μg/ml) for 4 h or overnight as indicated and PGE_2 concentrations determined. In a second protocol for iCOX-2 inhibition, the order of test material and LPS addition was reversed. Cells were stimulated with LPS (12 h), washed, test material and LPS were added for 1 h, then washed again and test substance and LPS were added for a subsequent 1-h incubation. Media were then collected and PGE_2 concentrations determined.

The AGS human gastric mucosal cell line (CRL-1739) was obtained from the American Type Culture Collection and sub-cultured in Ham's F12K supplemented with 10% FBS according to the instructions of the supplier. Test compounds were added in serum-free media at a final concentration of 0.1% DMSO. Sixty minutes following addition of the test materials, calcium ionophore A23187 was added (50 μM final concentration) for an additional 30 min and PGE_2 derived from both COX-1 and cCOX-2 determined.

4.4. Determination of PGE_2 and calculation of median inhibitory concentrations

A commercial, non-radioactive procedure for quantification of PGE_2 was employed (Cayman Chemical, Ann Arbor, MI) and the recommended procedure of the manufacturer was used. IC_{50} and 95% confidence intervals were calculated using CalcuSyn.

4.5. Western blot detection

The RAW 264.7 macrophage cell line was cultured as above and test compounds were added in a final concentration of 0.1% DMSO. The following day, LPS (50 ng/ml) was added for an additional 4 h, after which the cells were lysed on ice in buffer containing 1% Triton X-100. Total protein concentration was determined using the Bicinchoninic Acid Kit (Sigma). Total cell lysates (10 μg total protein) were electrophoresed and detection of COX-2 was performed using primary antibody incubation for 2 h (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Secondary antibody linked to horseradish peroxidase was incubated for 1 h (GE Healthcare, Piscataway, NJ), after which proteins were visualized using the enhanced chemiluminescence (ECL) system.

4.6. Inhibition of cytochrome P-450 (CYP)

RIAA was tested for its ability to inhibit recombinant human CYP isozymes 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4. Substrates used included 3-cyano-7-ethoxycoumarin for 1A2 and 2C19; 7-methoxy-4-trifluoromethylcoumarin for 2C9 and 2E1; 3-[2-(*N*,*N*-diethyl-*N*-methylamino)-ethyl]-7-methoxy-4-methylcoumarin for 2D6; and dibenzyl fluorescein and 7-benzyloxy-4-trifluoromethylcoumarin for 3A4. Positive control inhibitors included furafylline (1A2), sulfaphenazole (2C9), diethyldithiocarbamic acid (2C19), quinidine (2D6), tranylcypromine (2E1) and ketoconazole (3A4). All assays and IC_{50} calculation were performed by BD Gentest Contract Research Services (BD Biosciences, Woburn, MA).

4.7. Subchronic toxicity of RIAA in mice

To assess the potential subchronic toxicity of RIAA, 40 male and female CD-1 mice (Charles River Laboratory, Portage, MI) approximately 7–8 weeks of age were randomly assigned to receive either the Labrasol® vehicle control or RIAA by gavage at 25, 75 or 250 mg/kg day (five male and five female mice per group). Female mice used in the study were nulliparous and nonpregnant. The treatment of animals was in accordance with regulations outlined in the USDA 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* (Institute of Laboratory Animal Research, 1996). PMI Feeds Inc. Certified Rodent Diet #5002 and tap water were supplied *ad libitum* during the course of the study. Each animal received RIAA formulated in Labrasol® or Labrasol® alone by gavage for a minimum of 21 consecutive days. Dose suspensions were prepared fresh weekly and stored refrigerated when not in use. Stability determinations for RIAA in Labrasol were conducted prior to the study, at week 1 and again at week 3.

The mice were observed upon receipt and each morning and afternoon until completion of the study for evidence of mortality or morbidity. All mice received an overall health examination once weekly during the study. This visual exam included observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia, as well as evaluations of respiration, behavior and excretions. All observations were recorded. Body weights

were recorded one week prior to the initial dosing on the day prior to the initial dosing and weekly for the duration of the study. Food consumption was measured on all animals weekly beginning the week prior to dosing and continuing weekly throughout the study.

In-life data, such as body weights, observations, group means and standard deviations were calculated for all numerical data including food consumption, body weights and organ weights. The following tissues and organs were collected from all mice that died or were terminated at the completion of the study and preserved in 10% neutral buffered formalin: heart, stomach, esophagus, sections of duodenum jejunum ileum of the small intestine, sections of cecum, colon and rectum from the large intestine, kidneys, spleen, mesenteric lymph nodes, brain, sections of throracic, cervical and lumbar regions of the spinal cord, and sections of any gross lesions. The Dunnett test was used to identify differences between treatment and control groups with the probability of a type I error set at the nominal 5% level. All animal testing, gross necropsies and data analysis were performed by Ricerca Biosciences, LLC (Concord, OH).

Histological preparation was performed for all mice from the control and 250 mg RIAA/kg day groups and all mice that died spontaneously during the study. All tissues collected at necropsy were embedded in paraffin, sectioned and stained with hematoxylin and eosin. These tissues were then examined by light microscopy. In addition to the above, all gross lesions noted at necropsy were also examined. Histological preparation and evaluation of tissues were conducted at Experimental Pathology Laboratories Inc. (Research Triangle Park, NC).

4.8. Human clinical trials

All protocols complied with the Helsinki Declaration as revised in 1983 and Informed Consents were signed by subjects and investigators prior to start of the trial. The trial protocols were approved by the Bastyr University Institutional Review Board.

4.9. RIAA effect on fecal calprotectin excretion

Calprotectin is a surrogate marker of neutrophil turnover and is elevated in a number of inflammatory conditions. Fecal calprotectin is emerging as a valuable biomarker in the diagnosis and monitoring of commonly encountered gastroenterological conditions (Poullis et al., 2003). NSAIDs such as diclofenac, naproxen, indomethacin and ibuprofen have been demonstrated to increase fecal calprotectin excretion within seven days of treatment as well as during chronic administration (Meling et al., 1996; Tibble et al., 1999). In a *post hoc* analysis, nine subjects from two studies assessing fecal calprotectin excretion in response to hops derivatives or naproxen were evaluated. Both were controlled, randomized studies occurring at different times. All subjects were healthy males between 20 and 47 years of age (mean age 30 years). Subjects were administered 450 mg RIAA or 500 mg naproxen b.i.d. in the separate studies. Fecal samples were taken pre-dosing (baseline) and at 1 and 2 weeks following treatment. Fecal calprotectin excretion was determined by Genova Diagnostics (Asheville, NC) and values recorded as below the limit of detection of 16 μg/g stool were assigned the value 16 for data analysis. The primary analysis variable was the percent change from baseline at 7 and 14 days. Confidence intervals (95%) were computed for mean percent baseline calprotectin excretion at 7 and 14 days utilizing the square root transformation to normalize the data.

4.10. Urinary PGI-M and TXB_2 excretion

A second *post hoc* analysis was conducted from an open label, randomized, parallel cross-over study. Six females with no history of NSAID use for at least 1 week were administered 600 mg RIAA or 400 mg celecoxib. Subjects were between 43 and 64 years of age (mean age 54 years). Two male subjects were also included in data analysis from a later single arm study. Subjects were allowed free access to food and water while on the study. There was a 48-h washout period between treatments. Following initial urine voiding, subjects were administered test agent along with 120 ml of water. Urine samples were collected at 2-h intervals for 8 h. Total urine volumes were determined and aliquots of 20 ml were immediately stored at −20 °C until assayed. Three to four weeks following the final treatment, control urine samples were collected as above.

Both thromboxane (TXB_2) and PGI_2 metabolites were analyzed by immunoassay kits from R&D Systems (Minneapolis, MN). PGI_2 spontaneously decomposes to 6-keto-$PGF_{1\alpha}$, and further metabolized by β-oxidation to 2,3-dinor-6-keto-$PGF_{1\alpha}$. Both isomers of $PGF_{1\alpha}$ are chemically stable metabolites referred to collectively as PGI-M. The manufacturer's protocol was used without modification. For post-dosing samples, two analyses were performed each comprising duplicate independent dilutions, while baseline samples were analyzed once with three independent dilutions. Rate determinations were made by linear regression on the means and forcing the origin through zero. Confidence intervals (95%) for PGI-M/TXB_2 ratios were computed utilizing the square root transformation to normalize the data (Excel Spreadsheet, Microsoft, Redmond, WA).

4.11. General blood chemistries and blood pressure

In all studies, complete blood count (CBC) and comprehensive metabolic panel (CMP) were performed by Laboratories Northwest (Tacoma, WA). In all, 13 variables were measured to assess the potential adverse effect of RIAA including: systolic and diastolic blood pressure (BP); blood values including white blood cells (WBC), red blood cells (RC), platelets (PLT), hematocrit (HCT) and hemoglobin (HGB); and kidney function including

BUN, creatinine, sodium (Na), chloride (Cl), potassium (K) and carbon dioxide (CO_2). The references ranges were: WBC, 4.0–12.0 (10^3/mm^3); RBC, 4.0–5.5 (10^3/μl); PLT, 150–450 (10^3/mm^3); HCT, 37–47 (%); HGB, 12.0–16.0 (g/dl); BUN 8.0–24.0 (mg/dl); creatinine 0.8–1.5 (mg/dl); Na 135–148 (mequiv./l); Cl 97–107 (mequiv./l); K 3.6–5.3 (mequiv./l); CO_2 24–33 (mequiv./l). Values were averaged for all subjects in two trials at the initial visit (V1) and the last visit. Subjects ($n = 9$) in the fecal calprotectin study were evaluated after 2 weeks of RIAA administration. In the knee osteoarthritis trial ($n = 13$) the response to RIAA was evaluated after 6 weeks.

4.12. Ex vivo RAW 264.7 cell assay

In a single blind, placebo-controlled cross-over study, two normal healthy subjects were administered 600 mg RIAA, 200 mg celecoxib, or placebo after fasting 10 h. There was a 48-h washout period between treatments. Blood was drawn into sodium heparin tubes at pre-dose and at 1, 2 and 6 h post-dosing. The day following plasma collection, the test material (human plasma) was diluted in equal volume DMEM media and overlaid on confluent RAW 264.7 cells in a 96-well plate for 60 min prior to stimulation with 1 μg/ml LPS. After a 4-h stimulation with LPS, the supernatant media were collected for PGE_2 determination. The inhibition of PGE_2 production was determined by comparing the PGE_2 generated in the presence of each subject's plasma drawn pre-dosage (time zero) with and without LPS stimulation for 4 h. The data were clipped such that values greater than or equal to pre-dose stimulated were considered 0% inhibition and values less than or equal to pre-dose non-stimulated were considered 100% inhibition. Area-under-the-curve was calculated as the sum of the products of the mean % PGE_2 inhibition for the segment × hour for each time-period.

4.13. LC–MS detection of RIAA in human plasma

Two normal healthy subjects were administered 1000 mg RIAA and blood was drawn into sodium heparin tubes at pre-dose and at 1, 2, 4, 6 and 8 h post-dosing. Following centrifugation, 300 μl of plasma was lyophilized until dry. The remaining solids were taken up in MeOH (600 μl) and sonicated for 20 min followed by centrifugation. The MeOH supernatant and three 100 μl MeOH washings were collected, filtered and concentrated using heat (50 °C) followed by high vacuum. The samples were transferred to vial inserts in HPLC sample vials in a final volume of 150 μl. LC–MS analyses were performed using a Shimadzu Prominence HPLC system (Shimadzu Scientific Instruments Inc., Columbia, MD) consisting of a DGU-20A5 solvent degasser, LC-20AD solvent pumps (2), SIL-20A auto injector, CTO-20AC column oven operating at 40 °C and a CBM 20A system controller. The mass analyses were performed using an API-2000 (Applied Biosystems, Foster City, CA) mass spectrometer equipped with an electro-spray source and switching valve. The mobile phase, following passage through the HPLC column, was infused directly into the mass-spectrometer. A Gemini C18 3 μm 1 × 100 mm (Phenomenex, Torrance, CA) was used for analysis. The HPLC buffers consisted of (A) 5 mM NH_4OAc, pH 10 and (B) 60:40 MeCN:MeOH. A sample (20 μl) was injected onto the column with a constant flow-rate of 190 μl/min using the following gradient elution: 0–3 min: 25% B isocratic; 3–5 min: 25–40% B; 5–7 min: 40–62% B; 7–8 min: 62% B isocratic; 8–9 min: 62–95% B; 9–12 min: 95% B isocratic; 12–12.5 min: 95–28% B; 12.5–16 min: 28% B isocratic. The mass analysis was conducted using electrospray ionization in negative mode using multiple reaction monitoring (MRM) scanning of the 363/196 ion pair with a dwell time of 150 ms; the mass spectrometer was optimized for MRM scanning of 363/196 prior to analysis. A standard sample of the RIAA DCHA salt (American Society of Brewing Chemists, St. Paul, MN) was utilized for construction of calibration curves. Based on a validated HPLC-UV method this standard consisted of 46.1% of the predominant *cis* 6*S n*-RIAA isomer. The LC–MS–MRM quantification was conducted using an external calibration of the predominant *cis* 6*S n*-RIAA isomer (retention time of 7.26 min). The RIAA isomer provided a molecular ion $[M-H]^-$ at 363 m/z and a characteristic product ion of 196 m/z which is attributed to the loss of both iso-prenyl groups with a concomitant proton abstraction. The LC–MS–MRM quantification was performed in triplicate, with a CV < 10%. The recovery of spiked *cis* 6*S n*-diastereomer in plasma was 88.7%, 95.1% and 96.0% for 1.5, 2.25 and 3.0 μg/ml, respectively. At these same concentrations the whole blood to plasma ratio was 75.3, 65.3 and 62.0.

4.14. Open label knee osteoarthritis trial of RIAA

An open-label, single-arm, 6-week study was conducted in 13 subjects with knee OA. Male and female volunteers between 42 and 74 years of age (mean age 56 years) with symptomatic knee OA for at least 6 months, not taking glucosamine and with a WOMAC (Western Ontario and McMaster Universities Arthritis Index) Global score >9 cm and visual analog scale (VAS) pain scores of >4 cm at Visit 1 (V1) were included in the study. Developed in 1982, the WOMAC Index has been considered one of the leading health status measures for hip and knee arthritis (Bellamy et al., 1988). The global WOMAC score was the primary endpoint in this study. Secondary endpoints included the assessment of WOMAC sub-scores of pain, stiffness and physical function as well as pain assessment using the 10 cm VAS, an extensively validated questionnaire for OA. All 13 subjects were diagnosed with OA of the knee using the American College of Rheumatology criteria for the classification of OA and X-ray confirmation of knee OA. Each patient was administered 500 mg RIAA b.i.d. for a period of 6 weeks. For each of the questionnaires administered (WOMAC and VAS), the mean scores

for all subjects at each visit were determined. The changes in scores in response to RIAA were analyzed by one-way analysis of variance (ANOVA). To determine significant differences among visits, Tukey's multiple comparison test was performed and the nominal 5% probability of a type I error was assumed.

Acknowledgements

The authors would like to acknowledge Dr. Dave Gray for his statistical analysis, Dr. DeAnn Liska and Dr. Bhaskar Shenai for their editorial support, and Dr. Daniel Lukaczer, Julie Triggs, Scott Stockmyer, and Mabel Lorenzi-Albe for their clinical support.

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