

# Summary of effects of a reduced iso-alpha acids (RIAA), rosemary extract, and oleanolic acid supplement on parameters of cardiovascular health and kidney function

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## ABSTRACT

Based upon in vitro data indicating that reduced iso-alpha acids from hops (RIAA) has a low potential risk of gastrointestinal (GI) toxicity, we have developed and clinically tested a formula composed of RIAA and rosemary extract with oleanolic acid (Meta050). The primary concept underlying the potential safety of this formula is that it appears to modulate the gene signaling for generation of PGE<sub>2</sub> and did not directly inhibit the cyclooxygenase (COX) enzymes themselves. This ability suggests that the formula will be active only at sites where COX-2 is induced, and not where either COX-1 or COX-2 are constitutive (housekeeping) and required for maintenance of healthy function, such as in endothelial cells, platelets, or GI mucosa. In this summary, we report data in which the two COX isozymes—COX-1 and COX-2—were assayed directly, as well as comparative activity data from cell culture that further substantiate our previous findings and indicate Meta050 does not directly inhibit constitutive COX activity, but instead modulates the induction signal from inflammatory stimuli. Furthermore, in addition to our previously reported work using the GI toxicity model to test this formula, we have also reviewed the safety data from two independent clinical trials—including blood pressure (BP), general chemistry laboratories, and complete blood counts (CBC)—and have found no clinically relevant changes with the use of Meta050 over the 6 or 8 weeks of the trials. Taken together, these data provide continuing support for the potential safety of Meta050.

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## INTRODUCTION

Modulation of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) biosynthesis is an attractive target for clinical management of inflammation.<sup>1</sup> PGE<sub>2</sub> is synthesized from arachidonic acid at the site of inflammation, and the enzymes responsible for its synthesis are commonly referred to as the COX (cyclooxygenase) isoforms: COX-1 and COX-2. Although these two isoforms are similar, they differ in cell specificity, how they are regulated, and with respect to some of the products they produce at specific sites.

COX-1 is responsible for maintaining integrity of the gastrointestinal (GI) tissue, regulating blood flow to the kidneys, and supporting normal platelet function.<sup>1,2</sup> COX-1 produces PGE<sub>2</sub> in gastric mucosa, but also produces thromboxane (TxA<sub>2</sub>) in platelets,

which is involved in promoting platelet aggregation. Since it is important for the so-called “housekeeping” functions, COX-1 is not particularly responsive to induction signals, such as those from inflammation or injury. Instead, it is produced at a continuous low level (constitutive). Therefore, COX-1 is not associated with inflammation and pain, and its activity is important, especially for integrity of the GI mucosa.<sup>3</sup>

In contrast to the constitutive production of COX-1, the COX-2 isoform is not generally expressed at appreciable levels except when induced by a stressor.<sup>1-4</sup> COX-2 is primarily responsible for production of PGE<sub>2</sub> at sites of inflammation. A variety of stressors can stimulate (induce) a cell to produce COX-2, most notably signals produced at sites of inflammation. COX-2 is also expressed in

endothelial cells at a constitutive level where it produces PGI<sub>2</sub>, which acts to inhibit platelet aggregation and promote vasodilation.<sup>2</sup> The regulation of this constitutive pathway, however, differs from the inflammation induction pathway, which is only active at specific sites of inflammation.<sup>1,3</sup>

Much recent work has indicated that the differences in regulation of the constitutive and inducible COX activities may underlie the differing safety profiles of common analgesics. Since the anti-inflammatory activity of NSAIDs is considered to be due to inhibition of inducible COX-2 activity at sites of inflammation, it is important to target that activity alone for safety. For example, it has been known for some time that the traditional NSAIDs function by directly inhibiting the COX-1 and COX-2 enzymes, and the more recent coxibs primarily inhibit the COX-2 enzyme (both constitutive and induced).<sup>4,5</sup> Therefore, since these substances target the enzymes, they inhibit both the constitutive, necessary COX activities as well as the COX-2 inflammation-induced activity, and this lack of specificity has been linked to the considerable side effects observed with these therapeutics.

We have reported that reduced iso-alpha acids from hops (RIAA) are potent inhibitors of PGE<sub>2</sub> synthesis.<sup>6,7</sup> During our observations, we noted that RIAA, as well as the RIAA-containing formula Meta050, inhibited PGE<sub>2</sub> generation from a model cell system for inflammation (LPS-induced RAW 264.7 cells), but not from a human gastric cell line that primarily produced constitutive COX-1 activity (AGS cells). These observations suggested that RIAA may differentially inhibit the inflammation-stimulated COX expression, while not affecting the constitutive (housekeeping) COX activity.<sup>6-8</sup> We also showed that Meta050 had no effect on GI inflammation using fecal calprotectin, as compared to a control substance known to result in GI inflammation.<sup>9</sup> In this report, we test this notion directly by using purified COX-1 and COX-2 enzymes.

Since we have also tested Meta050 in several clinical trials in humans, as part of our in vivo review of safety, we summarize the relevant data from two of the clinical trials: Trial A, an open-label 8-week clinical trial with rheumatology patients; and Trial B, a 6-week randomized, placebo-controlled, double-blind clinical trial with osteoarthritis (OA) patients. In particular, we report findings on blood pressure (BP) and kidney function markers. A subsequent report summarizes a recent clinical trial investigating the effect of Meta050 on blood clotting and platelet function.

## RESULTS AND SUMMARY

### *RIAA INHIBITS INDUCTION, NOT DIRECT ENZYME ACTIVITY*

In a series of experiments, we addressed the question of whether the inhibition of PGE<sub>2</sub> production by RIAA was due to interference with induction of gene expression—which would mean the activated COX activity would be inhibited, but not the constitutive (housekeeping) activity—or, alternatively, whether RIAA could directly inhibit the COX-1 and/or COX-2 enzymes themselves. First, we asked whether the RIAA needed to be present before the inflammation signal in order to function. If so, this would suggest RIAA modulates the signaling of the induction, and may not interfere with the enzymes' functions.

For this exploration, we used the LPS-stimulated RAW 264.7 cell culture assay and measured PGE<sub>2</sub> production. RIAA was added either before the LPS stimulation or afterwards. As shown in Table 1, we found that RIAA inhibits PGE<sub>2</sub> production only when it is present before the LPS-stimulation. Once the cells are stimulated to generate the COX enzyme it is not an effective inhibitor. This type of experiment indicates RIAA inhibits the formation of the COX enzymes after an inflammation-like stimulus, but does not inhibit the enzymes themselves once they are already formed.

**Table 1.** Inhibition of cellular PGE<sub>2</sub> biosynthesis by RIAA.\*

	IC <sub>50</sub> (µg/mL)
Pre-incubation with RIAA prior to LPS stimulation	0.08 (0.03-0.23)
RIAA added after LPS stimulation	>50

\*Experiments performed essentially as described in Tripp et al, 2003.<sup>8</sup>

In order to directly assess the ability of RIAA to interact with the COX enzymes, commercially-available, purified COX-1 and COX-2 enzymes were assayed in an in vitro assay using added arachidonic acid. Known inhibitors of COX-1 and COX-2 were included as controls to assure integrity of the assay. Indomethacin is a well-known inhibitor of both COX-1 and COX-2 enzymes and is used clinically, whereas NS 398 is a COX-2-specific inhibitor that is used for research studies only.

**Table 2.** The effect of standardized RIAA on COX-1 and COX-2 enzymes. Shown is the IC<sub>50</sub> (µg/mL) in the cell-free arachidonic acid assay (± 95% CI)

	IC <sub>50</sub> (µg/mL)	
	COX-1*	COX-2*
Indomethacin	0.013 (0.011-0.015)	0.00032 (0.0002-0.00046)
NS 398	>16	0.126 (0.063-0.346)
RIAA	12 (8-18)	17 (14-21)

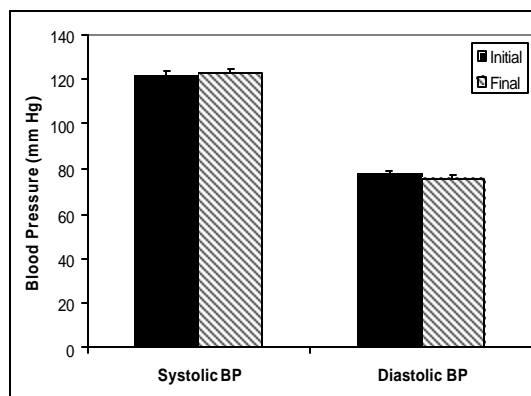
\*Ovine COX-1 and baculovirus expressed human COX-2 were obtained from Cayman Chemical, Ann Arbor, MI. The assays were performed by published protocols.<sup>10</sup> Briefly, test and control substances were pre-incubated with the enzymes for 15 min at 25°C, 1 mM arachidonic acid was added, and the reaction was monitored spectrophotometrically at 610 nm. Rate of reaction over 2 min was determined, and the estimated rate was calculated by subtracting background (rate in the absence of added enzyme). The IC<sub>50</sub> was calculated using the median effects methodology on CalcuSyn software (Biosoft, Ferguson, MO).<sup>11</sup>

Table 2 shows the results of direct inhibition experiments. As shown, indomethacin is an excellent inhibitor of both COX-1 and COX-2, whereas the NS 398 control substance is a good COX-2 inhibitor, but does not inhibit COX-1. The IC<sub>50</sub> for RIAA to inhibit both COX-1 and COX-2 activities is at least 2-orders of magnitude higher than indomethacin and NS 398. Since the physiological concentration of RIAA is calculated to be ~0.5 µg/mL, the levels necessary for 50% inhibition of the enzymes, at 12 µg/mL and 17 µg/mL, respectively, are not within, or even near, physiological concentrations.

## CLINICAL OBSERVATIONS WITH META050

### BLOOD PRESSURE

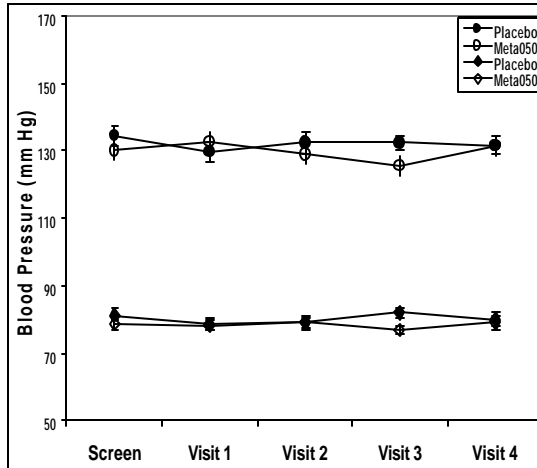
Two intervention trials were performed with patients having conditions characterized by chronic pain. In the first of these trials, data were obtained for 30 osteoarthritis (OA), 17 fibromyalgia (FM), and 3 rheumatoid arthritis (RA) patients after 8 weeks on either 880 mg bid or 440 mg tid Meta050.<sup>12</sup> As shown in Figure 1, no significant changes were noted for systolic or diastolic BP.



**Figure 1.** Absence of an effect of Meta050 on blood pressure in 50 subjects during an 8-week, open-label observational clinical trial. In this trial, rheumatology patients (30 OA, 17 FM, and 3 RA) consumed Meta050 at either 2 bid or 1 tid over the 8 weeks. Initial (closed boxes) and final (slashed boxes) data are shown.

The second trial was a randomized, double-blind, placebo-controlled trial with OA patients lasting 6 weeks. Thirty-two subjects completed the treatment arm of 1 tablet three

times daily Meta050 (440 mg tid) and 23 subjects completed the placebo arm in compliance and were included in this analysis. Figure 2 shows the BP measurements for each trial visit and, consistent with the earlier results, no significant changes in systolic or diastolic BP were observed with Meta050.

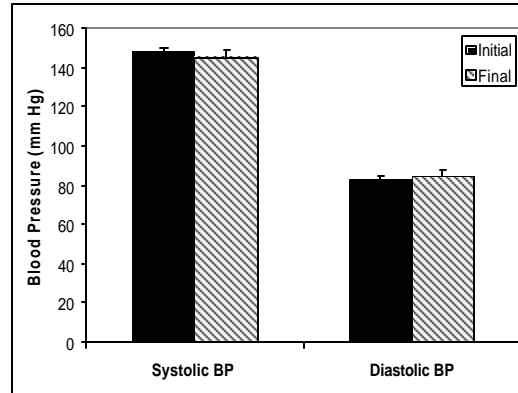


**Figure 2.** Blood pressure measurements from a randomized, double-blind, placebo-controlled 6-week clinical trial with Meta050 at 1 tid (Trial B). Systolic (circles) and diastolic (triangles) data are shown for the placebo (closed symbols) and Meta050 (open symbols) groups. The screen, visit 1 and visit 2 measurements occurred before administration of Meta050. Visit 3 occurred after 2 weeks of treatment with either Meta050 (N=32) or placebo (N=23), and Visit 4 occurred at 6 weeks of treatment.

Data from these two trials were pooled for greater statistical power (N=81). In this analysis, no change was observed between the baseline systolic BP of 126.3 mmHg (SD, 15.1; 95% CI, 122.7 to 129.9) and the final reading of 126.4 mmHg (SD, 15.6; 95% CI, 123.0 to 129.8). Similarly, no significant difference was noted between the baseline diastolic BP of 78.2 mmHg (SD, 7.4; 95% CI, 76.6 to 79.8) and the final reading of 77.5 mmHg (SD, 8.6; 95% CI, 75.6 to 79.4).

Finally, since some studies have suggested that the effect of NSAIDs on BP is most apparent on hypertensive patients, data were stratified and reviewed by BP at presentation. Thirteen subjects had initial readings of systolic BP = 140 mmHg and 1 subject had a

diastolic BP = 90 mmHg. Again, as shown in Figure 3, no significant difference was observed between the initial systolic BP of 148.2 mmHg (SD, 7.9; 95% CI, 144.0 to 152.3) and diastolic BP of 82.8 mmHg (SD, 7.0; 95% CI, 9.1 to 86.4) and the final readings of 145.3 mmHg (SD, 12.4; 95% CI, 138.8 to 152.4) and 84.7 mmHg (SD, 11.1; 95% CI, 78.9 to 90.5), respectively.



**Figure 3.** Absence of an effect of Meta050 on blood pressure in 14 hypertensive subjects. Data were pooled from two independent trials, an open-label, 8-week observational clinical trial (5 subjects) and a randomized, double-blind, placebo-controlled 6-week trial (9 subjects). Meta050 was administered at either 2 bid or 1 tid during these trials.

#### **KIDNEY FUNCTION MARKERS**

General chemistries and CBC were monitored in the clinical trials as well. No clinically significant changes were noted in either trial. As shown in Table 3, kidney function markers remained well within the reference range after intervention with Meta050 in the two independent clinical trials. Electrolytes also remained within established reference range limits, further indicating no clinically relevant effects on kidney function. Similar results were obtained for liver function markers, and other CBC and chemistry values (data not shown).

**Table 3.** Absence of an effect of Meta050 on kidney function markers and electrolytes (mean ± sem) during two independent trials. In the 8-week trial (Trial A), rheumatology patients consumed Meta050 at either 2 bid or 1 tid. In the 6-week trial (Trial B), OA subjects consumed Meta050 at 1 tid.

	Trial A		Trial B	
	8 Weeks, N=45		6 Weeks, N=34	
	<i>Initial</i>	<i>Final</i>	<i>Initial</i>	<i>Final</i>
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 <sub>2</sub> (mEq/L)	27.7 (±0.4)	28.5 (±0.3)	29.0 (±0.4)	28.6 (±0.3)

\*Creatinine reference range is 0.8 - 1.5 mg/dL; BUN reference range is 8.0 - 24.0 mg/dL; Na reference range is 135 - 148 mEq/L; Cl reference range is 97 - 107 mEq/L; K reference range is 3.6 - 5.3 mEq/L; CO<sub>2</sub> reference range is 24 - 33 mEq/L

## CONCLUSION

In conclusion, we have shown that RIAA and an RIAA-containing formula (Meta050) do not inhibit COX-1 and COX-2 enzymes directly. As such, RIAA does not perform similarly to traditional NSAIDs and cannot be directly compared to these substances. Several lines of evidence support the mechanism of action of RIAA as inhibiting the inflammation-induced induction signal for COX-2 expression, and not inhibition of the COX-1 or COX-2 enzymes themselves. Most notably, RIAA does not inhibit COX activity in human AGS cells, a model of gastric mucosa, and it does not inhibit purified COX-1 or COX-2 at physiologically relevant levels. Furthermore, it does not inhibit the COX activity in murine macrophage cells if it is added after the cells are stimulated, but does inhibit the signal when added before the stimulus. Taken together, these findings support a mechanism of RIAA in inhibiting induction of COX-2 only at sites where it is stimulated by inflammatory signals.

As part of our initial safety studies, we contracted with a third-party laboratory for a sub-chronic animal toxicity study of Meta050 at dosages up to 250 mg/kg/day for 21 days. Analysis of tissues that were collected and examined (e.g., GI tract, kidneys, heart) showed no evidence of toxicity of Meta050 (data not shown). These data support no in vivo effect of Meta050 on constitutive housekeeping functions of COX-1 or COX-2.

Clinical trial data also support this model of RIAA action. COX-1 and COX-2 are known to be important in maintenance of healthy BP by their abilities to promote vasoconstriction and vasodilation, respectively. Furthermore, both COX-1 and COX-2 are found in the kidneys and contribute to managing healthy renal flow.<sup>13,14</sup> Several clinical trials have found an effect of BP elevation after NSAID use, which averages about 5 mmHg.<sup>15-17</sup> 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 two independent clinical trials, we found no evidence of an effect of Meta050 on BP. Furthermore, when data were compiled and stratified by BP at presentation, those subjects with hypertension did not show an effect of elevation of either systolic or diastolic BP after Meta050. Taken with the in vitro mechanistic studies, these data continue to support the potential safety of Meta050. It should be noted that the role of the COX enzymes in BP is complex, and particularly sensitive or susceptible patients should be closely monitored with any substances known to affect the COX enzymes.

## NOTE

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