2-Hydroxybenzylamine acetate (2-HOBA) Phase 2 Clinical Trial in Rheumatoid Arthritis

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Summary of Changes from Previous Version:

Affected Section(s)	Summary of Revisions Made	Rationale
Presections Inclusion/Exclusion Study procedures AE reporting	Added amendment log, page numbers, abbreviations. Added exclusion for liver disease/LFT abn. NSAID d/c as inclusion criteria Change corticosteroid to glucocorticoid Statement of compliance. Extend table 3 With or without food. Treatment duration cannot exceed 28 days Added CDAI as exploratory outcome Ask subjects which treatment arm they suspect they were on. Clarified AE/SAE/unanticipated problem reporting	Request of FDA or NIAMS/NCR prior to dual safety officer meeting Addition of CDAI per request of PI

ABBREVIATIONS

2-HOBA = 2- hydroxybenzylamine
ACR= American College of Rheumatology
AE= Adverse event
ALT= alanine transaminase
AST= aspartate transaminase
BW= body weight
CDAI = clinical disease activity index
CI = confidence interval
CRC = clinical research center
CVD = cardiovascular disease
DAS28 = disease activity score based on 28 joint count.
dsDNA= double stranded deoxyribonucleic acid
DSMB= data safety monitoring board
ESR = Erythrocyte sedimentation rate
EULAR= European League Against Rheumatism
GHS = Global Harmonized System
HDL= high density lipoprotein
HED = human equivalent dose
hERG = human ether a-go-go
IDS= Investigational Drug Service
IRB = Institutional review board
isoLG = isolevuglandin
kg= kilogram
Ldlr= low density lipoprotein receptor
MAO-I = monoamine oxidase inhibitor
MDA = malondialdehyde
mg= milligram
mHAQ = Modified health assessment questionnaire
NET = neutrophil extracellular trap
NIAMS = National Institute of Arthritis and Musculoskeletal and Skin Diseases
ng= nanogram
NOAEL = no-observed-adverse-effect-levels
RA = rheumatoid arthritis
REDCap = Research electronic data capture
ROS= reactive oxygen species
SAE= serious adverse event
SBP = systolic blood pressure
SD = standard deviation
SLE= Systemic Lupus Erythematosus
SO = safety officer
ULN= upper limit of normal
VAS = visual analog scale

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1.0 BACKGROUND

Rheumatoid arthritis (RA): challenges achieving adequate disease control

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease affecting nearly 1.5 million adults in the US[1,2]. Early and aggressive treatment of RA improves likelihood of remission and reduces disability[3]. Despite use of aggressive "treat to target" approaches[4] as few as 7% of patients achieved remission after 52 weeks of therapy[5,6]. Moreover, RA patients have two-fold increased risk of ischemic heart disease, which is a main contributor to early mortality in RA[7]. Excess inflammation increases ischemic heart disease in RA, but some highly effective RA drugs that lower inflammation, like tofacitinib, may increase risk of serious heart-related problems (FDA drug safety communication 2-4-2021). Thus, therapeutics for RA targeting novel pathways that treat RA and reduce cardiovascular risk are needed. A potential target is blocking the proinflammatory, immunogenic, and proatherogenic effects of oxidative stress.

Need ROS but not the damaging effect of lipid peroxidation

Oxidative stress occurs when reactive oxygen species (ROS) are generated in excess of normal antioxidants. ROS are needed during bacterial killing, mitochondrial oxidative metabolism, and other cellular activities critical for cell proliferation and survival[8]; thus, high dose antioxidants can cause harm. Indeed, high dose antioxidants increased mortality in trials[9], and their use in RA is discouraged due to adverse effects on T cell function[10]. Targeting downstream damaging effects of ROS is an attractive alternative.

Identification of isolevuglandins (isoLGs) and effective scavengers

ROS causes lipid peroxidation of polyunsaturated fatty acids yielding highly reactive dicarbonyls such as malondialdehyde (MDA) and isolevuglandins (isoLGs). IsoLGs, the most reactive of these substances identified to date, bind covalently to proteins and DNA nearly instantaneously, which leads to protein misfolding, crosslinking, and DNA damage[11]. These modified proteins can become immunogenic and proinflammatory[12-14]. Through efforts over 20 years, our Vanderbilt colleagues, Drs L Jackson Roberts and Sean Davies, have identified and characterized 2-hydroxybenzylamine (2-HOBA), a derivative of buckwheat, that scavenges isoLGs nearly 1000 times faster than isoLGs can bind to proteins and also scavenges other less reactive dicarbonyls like MDA[15] (**Figure 1**). We and our colleagues have used 2-HOBA to demonstrate the effect of scavenging reactive dicarbonyls on disease models, as shown below, and also that 2-HOBA is safe in humans[16,17].

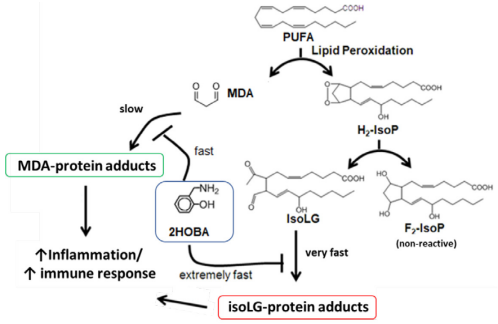

Figure 1. 2-HOBA scavenges highly reactive products of lipid peroxidation, decreasing inflammation amplifying protein adducts.

IsoLGs as drivers of autoimmunity and inflammation

IsoLGs may drive inflammatory autoimmune disease. For example in systemic lupus erythematosus (SLE) patients we found that isoLG-adduct positive (isoLG$^+$) dendritic cells were significantly increased in 11 SLE versus 10 control subjects[13]. In the *B6.SLE123* murine model of SLE, scavenging isoLGs with

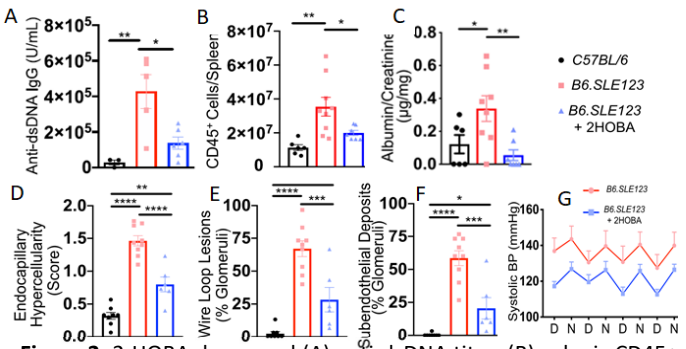

Figure 2. 2-HOBA decreased (A) anti-dsDNA titer, (B) splenic CD45+ cells, (C) proteinuria, and renal (D) endocapillary hypercellularity, (E) wire loop lesions, and (F) subendothelial deposits. (G) 2-HOBA decreased systolic blood pressure. (n = 6-8, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).[13]

2-HOBA from 7 to 32 weeks of age, significantly decreased isoLG^{+} dendritic cells, anti-dsDNA titer, splenic cellular expansion, renal disease, and blood pressure (**Figure 2**)[13].

Scavenging isoLGs reduces hypertension, which is increased in RA patients

The observation that 2-HOBA reduced blood pressure in an SLE mouse model is important given that presence of hypertension is increased 1.9-fold patients with SLE[18] as well as 1.8-fold in established RA[19] and contributes to increased cardiovascular risk in these diseases[18-23]. IsoLGs may drive hypertension through several mechanisms. IsoLGs increase dendritic cell IL-1β, IL-6, IL-23 production and costimulatory proteins CD80 and CD86, which promote T cell proliferation and generation of IL-17 and IFN-γ, all contributing to hypertension[24]. In a murine hypertension model 2-HOBA prevented these effects and decreased cytokine production, T cell proliferation and ultimately blood pressure[24].

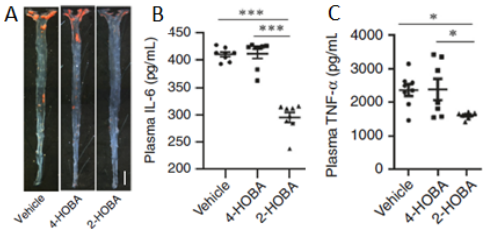

Figure 3. In Ldlr$^{-/-}$ mice 2-HOBA reduced (A) atherosclerosis, (B) plasma IL-6 and (C) TNFα. 8 mice/ group. *p<0.05, ***p<0.0001.[14]

Scavenging isoLGs and MDA reduces atherosclerosis

Reactive dicarbonyls also accelerate atherosclerosis, likely through effects on inflammation, lipoprotein modification, and potentially other mechanisms. Our colleagues used 2-HOBA to scavenge reactive dicarbonyls in the Ldlr$^{-/-}$ mouse model of familial hypercholesterolemia. 2-HOBA decreased aorta MDA and IsoLG adduct content by 59% and 23%, respectively, decreased atherosclerosis 60% (**Figure 3A**), plasma IL-6 (**Figure 3B**) and TNFα (**Figure 3C**) concentrations, and improved plaque stability features versus vehicle or nonreactive analogue (4-HOBA) without changing cholesterol concentrations[14]. IsoLG adducts increase cytokine production by inducing endoplasmic reticulum stress[12], and scavenging them with 2-HOBA reduces macrophage inflammatory cytokines *in vitro* (**Figure 4**)[14]. In addition to 2-HOBA's effect on inflammation, other factors such as decreased HDL-adducts leading to improved HDL function may have contributed to 2-HOBA's impact on atherosclerosis. Such effects would likely be highly beneficial in RA, a disease with increased systemic inflammation, lipoprotein dysfunction, hypertension and atherosclerosis[25-27].

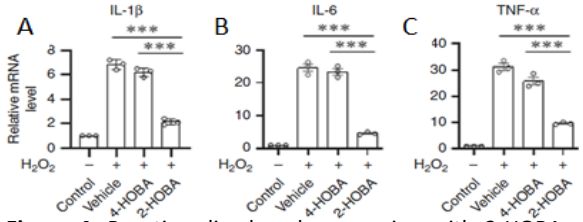

Figure 4. Reactive dicarbonyl scavenging with 2-HOBA reduces macrophage inflammatory cytokines versus vehicle or non-reactive analog 4-HOBA. (A-C: IL-1β, IL-6, TNFα). N = 3 independent experiments (mean ± SEM). ***p<0.0001.[14]

IsoLGs are enriched in RA patients

RA patients have increased oxidative stress[28,29]. In preliminary work using flow cytometry, peripheral blood isoLG^{+} dendritic cells (CD45^{+}/HLA-DR^{+}/CD11c^{+}) are increased 2.7-fold in 7 RA patients versus 3 controls (P=0.03) (**Figure 5**). In a larger study, a marker of isoLG production, urinary F_2-isoprostane concentration (non-reactive stable lipid peroxidation product formed in parallel with isoLGs and reliably detected in urine, see **Specific Aims Figure**) was increased ~50% in 169 RA versus 92 control subjects[29]. Moreover, urinary F2-isoprostane concentration was associated with RA joint damage assessed by Larsen score independent of age, race, sex, hypertension, BMI and smoking[29]. Given increased isoLG-adducts in RA, isoLG association with joint damage, and isoLG increasing

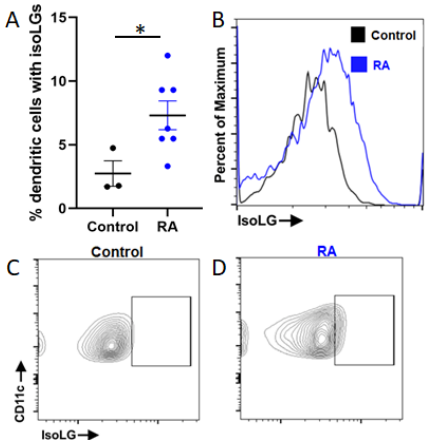

Figure 5. Isolevuglandin (isoLG) positive dendritic cells (DC) are increased 2.7-fold in RA vs controls (p=0.03) (A). Representative histogram of DC isoLG signal, (B). Example DC isoLG gating for control (C) and RA (D). *Unpublished*

proinflammatory cytokines (**Figures 3&4**)[12,14], *reactive dicarbonyls like isoLGs may contribute to disease in RA.*

Reactive dicarbonyls may contribute to RA pathogenesis

While isoLGs may be the most reactive products of oxidative stress and increase inflammation and autoimmunity, MDA also contributes to disease. In excess, MDA can break down to acetaldehyde and combine with other MDA molecules to form MDA-acetaldehyde (MAA). MAA adducts are enriched in RA versus osteoarthritis synovial tissue, colocalize with citrullinated protein[30], and induce antibody formation without adjuvant[31]. Anti-MAA antibodies increase significantly 2-3 years prior to RA onset[32]. MAA-adduction increased anti-citrullinated protein antibody concentration in an RA mouse model, and increased *in vitro* T cell response to citrullinated protein and monocyte cytokines[33]. *These data suggest that reactive dicarbonyls may be co-factors in RA pathogenesis and amplify existing disease.*

Strength of scientific premise

This trial is based on: 1) an *unmet medical need* for therapeutics using novel pathways that treat RA and reduce its associated cardiovascular risk, 2) a *plausible biologic mechanism* that scavenging reactive dicarbonyls such as isoLGs will reduce their damaging effects leading to decreased RA disease activity and 3) *compelling preclinical data* that 2-HOBA decrease inflammation, autoantibodies, aberrant T cell function, hypertension, and atherosclerosis.

2.0 RATIONALE AND SPECIFIC AIMS

We hypothesize that 2-HOBA, which has proven safe in animal models and two phase one human clinical trials, will reduce RA disease activity and inflammation and reduce associated cardiovascular risk. Thus, **the purpose of this pilot study is to determine 2-HOBA's tolerability, safety, and effect on isoLG-adducts, inflammation and blood pressure in RA patients.** We will perform a randomized, double blind, parallel group trial of 2-HOBA 750mg or placebo three times a day in up to 32 patients with RA. Patients will receive 2-HOBA or placebo for 4 weeks.

Specific Aim 1: Determine safety and tolerability of 2-HOBA in patients with rheumatoid arthritis. Patients with RA with at least 4 tender or swollen joints will be randomized to placebo or 750mg 2-HOBA (a dose that achieves plasma concentrations in excess of those effective in vitro and in animal studies and previously well tolerated in humans) three times a day for 4 weeks in a double-blind study. Adverse events will be monitored to determine safety and tolerability of 2-HOBA compared to placebo. Treatment compliance by pill count will be assessed.

Specific Aim 2: Determine if 2-HOBA treatment reduces isoLG-adducts, inflammation and blood pressure in patients with rheumatoid arthritis. Change in cellular and plasma isoLG-adducts, C-reactive protein, erythrocyte sedimentation rate, DAS28 score and 24-hour blood pressure will be measured comparing baseline to week 4 across treatment groups.

The proposed pilot clinical trial will inform the feasibility and design of future studies to examine the efficacy of 2-HOBA in patients with RA.

3.0 ANIMAL STUDIES AND PREVIOUS HUMAN STUDIES

Acute Oral Toxicity in Rats

A study was performed to evaluate the acute oral toxicity level of 2-HOBA acetate and to classify the compound into the appropriate toxicity class as defined by fixed LD_{50} cut-off values when administered as single oral dose to Wistar rats followed by a 14-day observation period. 2-HOBA acetate was administered in 0.5% carboxy-methyl-cellulose (CMC) at a dose of 2000 mg/kg body weight. In the first group of three

rats, the rats exhibited mild clinical signs, but fully recovered by day 3. In the second group of three rats, two animals exhibited no clinical symptoms by day 2, but one rat died on day 1 approximately 5 hours after 2-HOBA acetate administration. Gross pathological findings observed in dead animal were attributed to the test item, but no external gross abnormalities or pathological findings were detected in any of the other 5 animals. Based on the above results, 2-HOBA acetate was classified, per Global Harmonized System (GHS), as category 5 and the LD_{50} cut-off value was determined to be 2,500 mg/kg body weight when administered orally[34].

Short-term (28-day) Toxicity in Mice

A total of 40 mice were randomized to 0, 0.156, 0.469, or 1.56% 2-HOBA acetate mixed in the diet by weight (5 male and 5 female per treatment group). After one week of feeding, mice fed the highest level of 2-HOBA acetate (1.56%) had reduced food intakes (36% and 25% lower intakes in male and female mice, respectively) when compared with control feed intakes. Due to the decreased body weight in the high 2-HOBA acetate (1.56%) treatment group, the mice were euthanized. Because organ and tissue histopathology from the high dose mice did not indicate any adverse treatment effect, the loss of body weight was likely due to decreased feed intake. All mice in the lower treatment dose groups survived until scheduled euthanasia. There were no differences in body weight, feed intake, organ weights, histopathology, or blood chemistry were observed between the 0.156% and 0.469% 2-HOBA acetate groups and the 0% control group. Based on the highest level of 2-HOBA acetate fed for the full 28-day study (0.469%), the no-observed-adverse-effect-levels (NOAEL) determined by this short-term study were 610 and 721 mg/kg BW/d in male and female mice, respectively. These values correspond to human equivalent dosages of 49.5 and 58.5 mg/kg BW/d, respectively.[34]

Short-term (28-day) Toxicity in Rats

This study was performed to evaluate the toxicity of 2-HOBA acetate after 28 days of repeated oral administration (gavage) in Wistar rats and to characterize any observed toxicity of 2-HOBA acetate for indication of a dose-response relationship and determination or a NOAEL. Doses of 100, 500, and 1000 mg 2-HOBA acetate/kg BW/day were studied and compared to a vehicle (1% carboxymethyl cellulose) control group; each group contained 10 male and 10 female Wistar rats. Two additional recovery (vehicle control and 1000 mg/kg BW/day 2-HOBA) groups were also studied; these groups were administered vehicle or 2-HOBA acetate for 28 days and then allowed to recover for 8 days before sacrifice. No morbidity or mortality was observed in any group. Rats receiving the highest dose (1000 mg/kg BW/day) showed slight abdominal breathing after administration but recovered within 10-15 minutes. There were no remarkable clinical signs or symptoms, ophthalmological findings, or behavioral findings. Some small statistically significant differences in organ weights and blood chemistry/hematology were observed between the vehicle control and dose groups, but none of the changes were determined to be of any toxicological significance. No toxic indications were observed on gross pathology in histopathology. The NOAEL based on this study was determined to be 1000 mg/kg BW.[35]

Sub-chronic (90-day) Toxicity in Rats

In this study, 2-HOBA acetate was orally administered to male and female rats for 90 consecutive days at doses of 100, 500, and 1000 mg/kg BW/d (n = 20 per sex/group). Subchronic administration of 2-HOBA was well tolerated at all dose levels. 2-HOBA-treated male rats were slightly heavier in the last weeks of the study, but this difference was very small (<5%), did not show a dose–response relationship, and was not observed in female rats. Similarly, some statistically significant changes in serum biochemistry and hematology parameters were noted, but these were not considered to be of biological or toxicological significance. Sporadic differences in organ weights were observed between groups, but all were small (<10%) and unlikely to indicate toxicity. The incidence of histopathological lesions was similar between

treated and control groups across all organs. Based upon these findings, the NOAEL was determined to be ≥ 1000 mg/kg BW/d, which was the highest dose tested.[36]

Sub-chronic (90-day) Toxicity in Rabbits

2-HOBA acetate was orally administered to male and female New Zealand White Rabbits for 90 days at doses of 100, 500, and 1000 mg/kg BW/d (n = 5 per sex/group). As previously observed in rodents, 2-HOBA acetate administration was well tolerated. No toxic effects of 2-HOBA acetate were detected in body weight, feed consumption, hematology, blood chemistry, urine chemistry, organ weights, gross pathology or histopathology. Based on these findings, the NOAEL of 2-HOBA acetate in rabbits was determined to be 1000 mg/kg BW/d, which was the highest dose tested.[37]

Long-term Administration in Mice

A long-term study was conducted to determine if 2-HOBA would alter changes in memory and neurodegeneration observed in a transgenic model of Alzheimer's disease. Wild-type C57BL/6 mice and mice with targeted replacement of the human ApoE4 gene were given either normal drinking water or water supplemented with 1 g/L 2-HOBA acetate beginning at 4 months of age and continuing for the life of the animal. Supplementation with 2-HOBA did not result in significant changes either in the body weight of aged animals (14 months) or in survival compared with mice receiving normal drinking water.[38]

Mammalian Bone Marrow Chromosome Aberration in Mice

This test, conducted in accordance with OECD Guideline No. 475, "Mammalian Bone Marrow Chromosomal Aberration Test", examined the mutagenic potential of 2-HOBA acetate at doses up to 1000 mg/kg in mice. Mice were administered 2-HOBA acetate (250, 500, or 1000 mg/kg), vehicle, or cyclophosphamide monohydrate (positive control) via oral gavage for two consecutive days. Colchicine (10 mL/kg) was injected intraperitoneally 3 to 5 hours prior to sacrifice to arrest cells in metaphase. Twenty-four hours after the final dosing, mice were sacrificed, and bone marrow cells were collected and stained with Giemsa stain to examine the incidence of structural aberration. The percent reduction in mitotic index was ≤ 4.57% at 2-HOBA doses up to 1000 mg/kg dose compared to the vehicle control, whereas in the positive control group, the percent reduction in mitotic index observed was 8.3-8.6%. Further, there were no significant increases in aberrant cells in 2-HOBA treated groups in comparison with the vehicle control group. The observed mean percent aberrated cells was ≤ 0.70 in all dose groups, compared with 5-5.6% in the positive controls. These results support that 2-HOBA is non-mutagenic at doses up to 1000 mg/kg (unpublished data, see investigators brochure).

Prenatal Development Toxicity

This study was conducted in accordance with OECD Guideline No. 414, "Prenatal Development Toxicity Study". 2-HOBA acetate was administered to pregnant female rats from gestation day 5 through gestation day 20 at 0 (vehicle control), 100, 500, or 1000 mg/kg. Dams were sacrificed and fetuses excised on gestation day 20, and dams and fetuses were examined for signs of prenatal development toxicity. No signs of prenatal development toxicity were observed in dams or offspring at the 100 mg/kg dose level. However, significantly lower weight, weight gain, and feed consumption were noted in dams treated with 500 or 1000 mg/kg 2-HOBA. The lower confidence limit of the BMD modeling (BMDL) for a 10% difference (BMDL10) in weight gain during 2-HOBA treatment (GD 5-20) was 169.3 (absolute weight gain) or 178.8 (relative weight gain) mg/kg, while the BMDL for feed intake over the same time period was 287.5 mg/kg. Gravid uterus weight was reduced in the 1000 mg/kg dose group. Relative gravid uterus weight (% of body weight) was 19.2±2.6, 19.5±2.1, 17.1 ± 3.9, and 16.7 ± 4.1% in the 0, 100, 500, and 1000 mg/kg groups, respectively. The BMDL for a 5% difference (BMDL5) in relative gravid uterus weight was 187.7 mg/kg. There were no differences between groups in corpora lutea, implantations, early or late resorptions, pre-

or post-implantation loss, litter size, litter survival, or sex ratio. Significant reductions in fetal weight and length were observed in the 500 and 1000 mg/kg dose groups. BMDL5 values for fetal weight were 221.7 and 207.2 mg/kg for males and females, respectively, and BMDL5 values for fetal length were 438.4 and 395.4 for males and females, respectively. External examination revealed a significantly greater number of fetuses that were considered small in size as well as a significantly greater number of fetuses with hemorrhagic spots or pale color in the 1000 mg/kg dose group, but no differences between groups were noted on visceral or skeletal examination. These data support the prenatal safety of 2-HOBA at doses up to 169 mg/kg in rats (human equivalent dose [HED] = 27 mg/kg), though equivocal observations (see One-Generation Reproductive Toxicity) suggest the actual threshold dose may be higher (unpublished data, see investigators brochure).

One-Generation Reproductive Toxicity

This study was conducted in accordance with OECD Guideline No. 415, "One-Generation Reproductive Toxicity Study". Male rats were dosed with 0 (vehicle control), 250, 500, or 1000 mg/kg 2-HOBA acetate beginning 10 weeks prior to mating. The 1000 mg/kg dose group was adjusted to 700 mg/kg on day 54 of dosing due to the emergence of clinical signs of toxicity and mortalities. Female rats were dosed with 0 (vehicle control), 250, 500, or 700 mg/kg 2-HOBA acetate once daily for 2 weeks prior to mating and through gestation and lactation. Parental animals and pups were monitored for signs of reproductive toxicity through lactation day 21 (weaning). Males receiving 1000 mg/kg 2-HOBA began exhibiting clinical signs on Day 5 of treatment, and five males were found dead between days 50 and 56 of treatment. Due to these mortalities, the dose of 1000 mg/kg body weight group was reduced to 700 mg/kg body weight from Day 54 onward. All males treated with this reduced dose recovered. In females treated with 700 mg/kg 2-HOBA, 10 out of 25 females experienced clinical signs of toxicity, but all females recovered. Lower body weight, weight gain, and feed consumption were observed in males treated with 500 or 1000/700 mg/kg 2-HOBA. There were no meaningful differences in body weight, weight gain, and feed consumption in females prior to gestation in any dose group. Estrus cycle parameters were normal in females receiving 250 or 500 mg/kg 2-HOBA, but 11 out of 25 females treated with 700 mg/kg 2-HOBA exhibited irregularities. Male fertility index was similar between the vehicle control, 250 mg/kg, and 500 mg/kg groups (80-88%) but was significantly lower in the 1000/700 mg/kg group (40%). Similarly, female fertility index was identical in the vehicle control, 250 mg/kg, and 500 mg/kg groups (92%) but was significantly lower in the 700 mg/kg group (36%). Significant reductions in reproductive organ weight were observed in the 1000/700 mg/kg groups when compared with the corresponding vehicle control groups. Gestation and parturition indices were significantly reduced (78%) only in the 700 mg/kg group. Body weight, weight gain, and feed consumption during gestation and lactation were significantly lower in females receiving 700 mg/kg 2-HOBA. Gestation length was similar in all dose groups (22.3 - 22.7 days). There were no treatment-related adverse effects on delivery outcomes or litter observations in the 250 or 500 mg/kg groups, but a number of adverse effects were noted in the 700 mg/kg dose group, including greater pre- and postnatal losses, significantly reduced live birth index, significantly more dead pups, greater pup loss from LD 1-4, and reduced live pup numbers and pup survival index through LD 14. Due to pup mortality, only 3 dams with live pups remained during LD 4-17, and only 2 dams with live pups remained from LD 14-20. Among surviving pups in the 700 mg/kg group, weight was significantly reduced (11-45%, depending on sex and age) when compared with the vehicle control group at all postnatal time points. Some developmental markers (pinna unfolding, incisor eruption, eye opening, and air righting reflex development) were also delayed in F1 pups from the 700 mg/kg dose group. Together, these data support the prenatal safety of 2-HOBA at doses up to 500 mg/kg in rats (HED = 81 mg/kg) (unpublished data, see investigators brochure).

Safety of 2-HOBA – *in vivo* human studies

A first-in-humans Phase I clinical trial using oral 2-HOBA was recently completed to assess its safety and pharmacologic profile. A single dose study (NCT-3176940) and a multidose study (NCT-03555682) were conducted, and there were no serious side effects observed in two human clinical trials[16,17]. In the single dose escalation study, 6 ascending doses of 2-HOBA were studied (50mg to 825mg as single dose). Three participants were enrolled at each dose and 18 participants successfully completed the study, and the highest planned dose was achieved (825mg)[16]. 5 participants reported mild adverse events. These

Table 1. Phase 1 dose escalation study adverse events[16]

	2-Hydroxybenzylamine acetate dose						Total ($n = 18$)
	50 mg ($n = 3$)	100 mg ($n = 3$)	200 mg ($n = 3$)	330 mg ($n = 3$)	550 mg ($n = 3$)	825 mg ($n = 3$)	
Any event, n (%)	3 (100)	0	1 (33)	1 (33)	0	0	5 (28)
Frequent urination	2 (67)	0	0	0	0	0	2 (11)
Headache	0	0	1 (33)	0	0	0	1 (5.5)
Itchy throat	1 (33)	0	0	0	0	0	1 (5.5)
Rash	1 (33)	0	0	0	0	0	1 (5.5)
Sleepiness	1 (33)	0	0	0	0	0	1 (5.5)
Abdominal bloating	0	0	0	1 (33)	0	0	1 (5.5)

included frequent urination (N=2), headache (N=1), itchy throat (N=1), rash (N=1), sleepiness (N=1), and abdominal bloating (N=1), none of which were dose dependent[16] (**Table 1**). No clinically significant changes in ECG recordings, vital signs, or laboratory parameters were observed.

In a multiple dose randomized double-blind clinical trial placebo, 500mg or 750mg were given 3 times a day to 18 healthy subjects over two weeks to assess safety, tolerability, and pharmacokinetics of 2-HOBA. 2-HOBA dosages were chosen to achieve similar plasma levels at steady state compared with the single dose study. No serious AEs were observed. 14 patients reported at least 1 adverse event (**Table 2**). Reported AEs included headache (N=6), GI distress (N=3), rash/itching (N=3), urine odor (N=2), dry mouth (N=2), nasal congestion (N=2), lethargy/sleepiness (N=2), hypertension (N=1), and eye irritation (N=1) in all groups combined. One volunteer experienced a rash of moderate intensity, and was withdrawn from the study, though this AE was not determined to be study-related or dose-dependent. None of these were considered drug-related or dose dependent[17]. A 2-HOBA metabolite, salicylic acid, had similar pharmacokinetic values as single doses of 40.5–324 mg of acetylsalicylic acid (aspirin)—i.e., 2-HOBA doses tested gave a similar salicylic acid exposure as low doses of aspirin[17]. Vital signs, ECGs, and laboratory parameters did not significantly change[17]. Thus, 2-HOBA is considered safe in healthy humans.

Table 2. Phase 1 repeated dose randomized controlled trial adverse events[17]

	2-Hydroxybenzylamine acetate dose			Total ($n = 18$)
	Placebo ($n = 6$)	500 mg ($n = 6$)	750 mg ($n = 6$)	
Any event, n (%)	4 (67)	6 (100)	4 (67)	14 (78)
Headache	2 (33)	2 (33)	2 (33)	6 (33)
GI distress (nausea, bloating, constipation)	2 (33)	1 (17)	0 (0)	3 (17)
Rash/itching	1 (17)	1 (17)	1 (17)	3 (17)
Urine odor	0 (0)	2 (33)	0 (0)	2 (11)
Dry mouth	1 (17)	1 (17)	0 (0)	2 (11)
Nasal congestion	0 (0)	2 (33)	0 (0)	2 (11)
Lethargy/sleepiness	0 (0)	1 (17)	1 (17)	2 (11)
Hypertension	0 (0)	1 (17)	0 (0)	1 (6)
Eye irritation	0 (0)	1 (17)	0 (0)	1 (6)

Rationale for 2-HOBA dose selection

SLE, hypertension, and Ldlr$^{-/-}$ murine studies using 2-HOBA supplemented water at 1g/L significantly reduced isoLG and modified disease[13,14,24]. In the Ldlr$^{-/-}$ study after 16 weeks of 2-HOBA, plasma level of 2-HOBA was 469 ± 38 ng/mL[14]. Human studies using 2-HOBA surpassed the observed mouse plasma concentration needed to reduce isoLG and MDA adducts and see effects; in humans after 15 days of 750mg TID dosing average 24-hour concentration was 1190 ± 455 ng/ml[17] (**Figure 6**). Thus, we choose 750mg TID, because it was safe and well tolerated in initial phase one studies and the highest dose tested in repeated dosing.

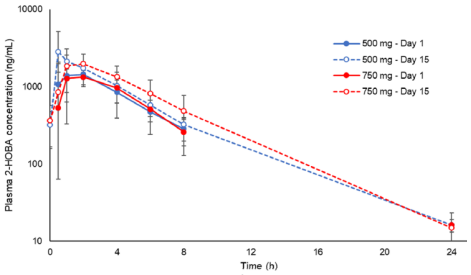

Figure 6. 2-HOBA plasma concentrations measured for 8 hours (Day 1) or 24 hours (Day 15) after initial and repeated oral administration in humans.[17]

4.0 INCLUSION/EXCLUSION CRITERIA

Inclusion Criteria:

- Written informed consent
- Age ≥18 years
- Meets 2010 American College of Rheumatology/European League Against Rheumatism Rheumatoid Arthritis classification criteria
- ≥ 4 tender or swollen joints
- No change in disease-modifying antirheumatic drugs (DMARDs), glucocorticoids in ≥ 4 weeks
- If of childbearing potential, willingness to use effective birth control throughout the study and 4 weeks after completion of the study (examples: condom, diaphragm, oral contraceptive pill, intrauterine device)
- If using non-steroidal anti-inflammatory drugs (NSAIDs), willingness to discontinue use of NSAIDs for 2 weeks prior to the study and throughout the study

Exclusion criteria:

- Pregnant or breastfeeding
- Active cancer except for non-melanoma skin cancer
- Active infection requiring medical intervention
- Concomitant inflammatory autoimmune disease
- Major surgery in ≤ 3 months
- Aspirin allergy
- Use of MAO-I
- Estimated creatinine clearance <30 ml/min
- Prior diagnosis of liver cirrhosis or the following abnormal liver function studies: AST or ALT >1.5x the upper limit of normal or total bilirubin ≥1.5 mg/dl

Rationale for inclusion and exclusion criteria:

We study adults to better exclude juvenile idiopathic arthritis, which is a separate disease. Subjects will have no significant change in DMARDs or glucocorticoid dose in the past 4 weeks to avoid possible confounding due to drugs other than 2-HOBA. Subjects will have active disease evidenced by at least 4 tender or swollen joints.

Subjects will be excluded if they are pregnant or breastfeeding or unwilling to use required birth control if the female subject has childbearing potential because 2-HOBA has not previously been studied in pregnant or breastfeeding women. Prenatal development toxicity studies have not yet been published, but per report (see investigator's brochure and **3.0 Animal Studies and Previous Human Studies**) human equivalent doses of up to 27 mg/kg in one study and 81 mg/kg in another were safe. The 81mg/kg human equivalent dosing may be more accurate because in that animal study more doses were tested, giving a better estimation of a precise safe dose.

Subjects with active cancer other than non-melanoma skin cancer, concomitant inflammatory autoimmune disease, active infection, or major surgery in the past 3 months will be excluded due to potential confounding effects on systemic inflammation. Patients with severe co-morbid conditions likely to compromise study participation will be excluded. Those with an aspirin allergy will be excluded because salicylic acid is a metabolite of 2-HOBA. Those using monoamine oxidase inhibitors (MAO-I) will be excluded due to some inhibition of MAO-A in the anticipated therapeutic range of 2-HOBA (See Safety of 2-HOBA – in vitro), and estimated creatinine clearance <30 ml/min due to the likelihood of renal clearance of 2-HOBA[35]. In healthy control subjects 2-HOBA did not affect liver function studies; however, the effect of hepatic impairment on the pharmacokinetics of 2-HOBA has not been evaluated in humans. Thus, subjects with known liver cirrhosis or those with AST or ALT greater than 1.5x the upper limit of normal or with total bilirubin ≥1.5mg/dl will be excluded.

5.0 ENROLLMENT/RANDOMIZATION

Recruitment and informed consent

Patients with RA will be recruited from subjects who have already taken part in our studies, from the Vanderbilt Rheumatology Clinic by word of mouth or identification in the Vanderbilt electronic medical record, by advertising, and through ResearchMatch (a database of individuals willing to participate in clinical studies that is maintained by the Vanderbilt Clinical Research Center), as we have done in the past[39,40]. Consent will be obtained by the Principal Investigator or a trained study coordinator/research fellow. Consent will be documented by keeping a copy of the signed consent form and will also be documented in the medical record. Subjects willing to participate who provide written consent will be screened for eligibility. Those fulfilling inclusion and exclusion criteria will be enrolled.

Randomization

Enrolled subjects fulfilling inclusion and exclusion criteria will be randomized 1:1 to 2-HOBA 750mg, or placebo by a permuted block randomization with block sizes of 2 and 4, stratified by age (>50 versus <=50), sex, and swollen/tender joint count (>8 versus <=8) to minimize the potential confounding effects from these factors. The study statistician, Dr Fei Ye, who has extensive expertise in clinical trials, will design the randomization tool within REDCap database. After an eligible patient enrolls, the study coordinator/ research fellow/ PI will notify the Vanderbilt Investigational Drug Service (IDS) to randomize the patient based on the REDCap randomization tool, and the IDS will dispense the study drug or placebo to the research coordinator/ research fellow/ PI in a blinded fashion.

6.0 STUDY PROCEDURES

The study will be conducted in compliance with the protocol, International Council for Harmonization/Good Clinical Practice requirements (ICH/GCP), and applicable state, local and federal regulatory requirements.

Source of 2-HOBA and placebo

MTI BioTech (MTI) will provide 2-HOBA acetate (the same formulation as previously used[16,17,34-37], but referred as 2-HOBA throughout) and matching placebo capsules for the study (see letter), and ship to the Vanderbilt Investigational Drug Service (IDS). 2-HOBA acetate and matching placebo are manufactured in a good manufacturing practice (GMP) providing >98% purity and encapsulated in gelatin capsule shells by TSI group LTD (Missoula, MT) for MTI.

Screening

Subjects providing written informed consent will undergo screening for eligibility based on inclusion and exclusion criteria (**4.0 Inclusion/Exclusion Criteria**). During the screening a history and physical will be performed and joints will be assessed. We will measure a complete metabolic panel, rheumatoid factor, and anti-CCP antibody, if not available at that time (**Table 3**). Urine pregnancy test will be performed on subjects with childbearing capacity.

Study protocol overview

We anticipate screening 64 subjects to randomize up to 32 subjects with the goal of 24 patients completing the study (allowing for up to 25% dropout) for the 4-week study. At week 0 subjects will be randomized 1:1 to receive either 2-HOBA 750mg, or placebo three times a day for 4 weeks in combination with their baseline DMARD therapy for RA. Subjects may take 2-HOBA fasting or with food. History and physical will be performed and venous blood will be drawn at each study visit. A window of up to 10 days will be permitted to for scheduling study visit dates to permit flexibility for patient convenience; however, the treatment duration cannot exceed 4 weeks (28 days). The effect of varying time within the 10-day window will be examined.

Concurrent medication use during study

Subjects will be asked to discontinue use of non-steroidal anti-inflammatory drugs for 2 weeks prior to the baseline visit and throughout the study, because the cyclo-oxygenase (COX) enzymatic pathway can generate certain isoLG adducts[41,42]; however, 2-HOBA does not inhibit cyclooxygenases[17]. Subjects and their physicians will be instructed to maintain DMARD and glucocorticoids at baseline dosages during protocol participation. If the subject's physician feels that a change in disease status warrants a significant change in therapy, the patient will be brought in for an exit visit and withdrawn from the study. Patients will be asked to avoid injections of glucocorticoids if possible. However, if a joint is injected, that joint will be excluded from the joint count analysis. Women of childbearing potential will be asked to use effective birth control throughout the study and for 4 weeks after completion of the study. At each visit we will ask each patient about medications, doses, any new prescription medications, and use of over the counter or herbal medications.

Monitoring parameters

At each study visit a physical examination and RA disease activity indices including tender and swollen joint counts and modified health assessment questionnaire will be collected. Clinical details, including,

current and past smoking history, and a cumulative medication history with attention to cumulative glucocorticoids, DMARDs, and cardiovascular therapies will be obtained from the patient and medical record at baseline (**Figure 7**). At the week 0 visit a urine pregnancy test will be obtained on subjects with childbearing capacity prior to receiving drug.

At each visit traditional office blood pressure will be measured. The average of two blood pressure readings separated by 1-2 minutes will be obtained in the following validated[43] manner: subjects will be in the seated position at rest for at least 5 minutes prior to measurement. The arm will be supported at the level of the right atrium (midpoint of the sternum), and the same arm will be used for all blood pressure measurements. Correct cuff size so that the bladder excircles 75-100% of the arm will be used.

At week 0 and 4 subjects will be fitted with a 24-hour blood pressure monitor to measure ambulatory blood pressure. The device will be programmed to measure blood pressure at 15-minute intervals during the day (6am-10pm) and 30-minute intervals during the night (10pm-6am). Subjects will be asked to wear the monitor for 24-hours total. Subjects will be asked to record activities such as exercise during this time. Subjects may temporarily remove the monitor for bathing, as needed. Subjects will not start 2-HOBA until after completing 24-hour blood pressure measurement at week 0.

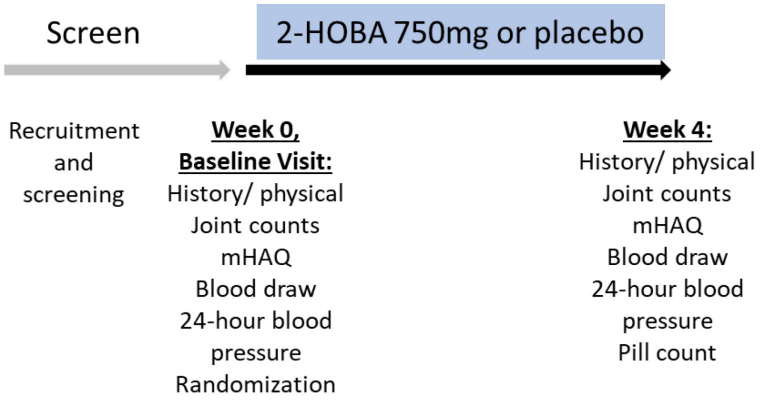

Figure 7. Clinical trial protocol

Venous blood will be collected and immediately processed for studies or stored (**Table 3**). Although there is no evidence that 2-HOBA alters blood counts, hepatic or renal function, we will obtain safety labs including complete blood count at study visits Week 0 and 4 and complete metabolic panel at screening and study visits Week 0 and 4. Additionally, we will measure hs-CRP and ESR at study visits Week 0 and 4. These will be measured in the hospital clinical laboratory.

Subjects will be asked about side effects in a standardized manner at each visit. Pill counts will be performed at the week 4 study visit. Subjects will also be asked at study completion which treatment arm they believe they were assigned.

The study coordinator will contact the patient between study visits to inquire about potential side effects and ensure compliance with protocol.

Primary outcome measures:

1) Safety and tolerability
   We will determine safety and tolerability as a primary outcome through adverse event reporting of the 2-HOBA arm compared to the placebo arm. At each study visit, patients will be directly questioned on potential side effects or other adverse events. Rates of adverse events will be compared between active and placebo arms and presented as summary statistics.
2) Cellular isoLG-adducts

PBMCs collected at weeks 0 and 4 will be isolated by Ficoll separation. Change in cellular isoLG adducts between week 0 and week 4 comparing active and placebo arms will be a primary outcome.

Other prespecified exploratory outcome measures:

1) Compliance
   Treatment compliance will be assessed through pill counts at week 4.
2) Plasma isoLG-adducts
   Total plasma isoLG-adducts will be measured from week 0 and 4 stored plasma by Dr. Sean Davies' lab, who developed the technique[44] using liquid chromatography tandem mass spectrometry (LC/MS)[14,44,45]. Change in total plasma isoLG-adducts between week 0 and week 4 comparing active and placebo arms will be measured.
3) Disease activity score based on 28 joints (DAS28 score) and the Clinical Disease Activity Index (CDAI)
   Disease activity will be measured as the DAS28 score and CDAI at weeks 0 and 4. Change in DAS28 score and CDAI between week 0 and week 4 comparing active and placebo arms will be measured.
4) High-sensitivity C-reactive protein (CRP)
   Hs-CRP will be measured at weeks 0 and 4 in the hospital clinical laboratory. Change in hs-CRP between week 0 and week 4 comparing active and placebo arms will be measured.
5) Erythrocyte sedimentation rate (ESR)
   ESR will be measured at weeks 0 and 4 in the hospital clinical laboratory. Change in ESR between week 0 and week 4 comparing active and placebo arms will be measured.
6) Plasma interleukin 6 (IL-6)
   The Olink Inflammation proteomics panel will be used to measure plasma concentration of IL-6. Change in IL-6 between week 0 and week 4 comparing active and placebo arms will be measured.
7) Plasma tumor necrosis factor α (TNFα)
   The Olink Inflammation proteomics panel will be used to measure plasma concentration of TNFα. Change in TNFα between week 0 and week 4 comparing active and placebo arms will be measured.
8) 24-hour systolic and diastolic blood pressure
   The average systolic and diastolic blood pressure over 24-hours of ambulatory monitoring will be calculated at weeks 0 and 4. The change in 24-hour systolic and diastolic blood pressure between week 0 and week 4 comparing active and placebo arms will be measured.

Table 3. Study events and measures						
Procedure/Lab	Screen *~Day -90 to Day -4*	Pre-Visit contact (telephone) *~Day -5 to Day -2*	Baseline Week 0 Visit *Day 0*	Between study visit contact (telephone) *~Day 10-20*	Pre-Visit contact (telephone) *~Day 23 to Day 26*[1]	Week 4 Visit *Day 28 +/- 10*
Informed Consent	+					
History	+		+			+
Exam	+		+			+
Joint Count	+		+			+
Disease activity score based on 28 joints (DAS28)/ Clinical disease activity score (CDAI)			+			+
Office blood pressure			+			+
24-hour blood pressure			+			+
Modified health assessment questionnaire (mHAQ)			+			+
Randomization			+			
Adverse event inquiry		+	+	+	+	+
Pill count						+
Rheumatoid factor[2]	+					
Anti-CCP antibody[2]	+					
Urine pregnancy test[3]	+		+			
Complete blood count[4]			+			+
Complete metabolic panel[4]	+		+			+
hs-C-reactive protein			+			+
Erythrocyte sedimentation rate			+			+
Plasma storage			+			+
Serum storage			+			+
Urine storage			+			+
DNA storage			+			
Study Completion						+
Cellular isoLG-adducts (measured after study completion)			+			+
Plasma isoLG-adducts (measured after study completion)			+			+
Olink inflammation panel (measured after study completion)			+			+
Plasma 2-HOBA concentration (measured after study completion)			+			+

[1] The pre-visit contact prior to the Week 4 visit should occur ~2-5 days prior to the scheduled Week 4 visit. [2]Rheumatoid factor and anti-CCP antibody tested only if information is not available in EHR. [3] Urine pregnancy test only in patients of childbearing capacity. [4] Complete blood count and complete metabolic panel tested if not available in the last 30 days at the time of screening and/or baseline visit.

7.0 RISKS OF INVESTIGATIONAL AGENTS/ DEVICES (SIDE EFFECTS)

2-HOBA is currently available in the food chain as both a compound found naturally in buckwheat as well as used as a self-affirmed generally recognized as safe "GRAS" ingredient in food. Based upon the currently available data, 2-HOBA acetate is well-tolerated in healthy individuals at doses within the expected therapeutic range, and there is a low risk of toxicity or adverse reactions.

There are signs of prenatal or reproductive toxicity in preclinical studies at human equivalent doses above the proposed dose for this study. Thus, women of childbearing capacity are asked to maintain effective birth control throughout the study and pregnant or breastfeeding women will be excluded.

In preclinical in vitro studies 2-HOBA was not cytotoxic or mutagenic; however, the characterization of the genotoxic potential of 2-HOBA is incomplete. In preclinical in vitro studies 2-HOBA did not induce CYP1A2, CYP2B6, or CYP3A4 enzyme mRNA expression; and had low risk of QT prolongation based on human ether a-go-go (hERG) inhibition[35]. In healthy control subjects 2-HOBA did not affect liver function studies; however, the effect of hepatic impairment on the pharmacokinetics of 2-HOBA has not been evaluated in humans. Thus, subjects with known liver cirrhosis or those with AST or ALT greater than 1.5x the upper limit of normal or with total bilirubin ≥1.5 mg/dl will be excluded. 2-HOBA had low binding to plasma proteins, suggesting low risk for plasma protein-mediated drug-drug interactions. It has no preferential binding to red blood cells, permitting accurate measurement of plasma concentration[35]. The main 2-HOBA metabolites were salicylic acid, which is also a metabolite of aspirin, and the glycoside conjugate of 2-HOBA, which is an which is a pathway for urinary or bile excretion of compounds[35].

Because of these findings we have conservatively excluded those with aspirin allergy because salicylic acid is a metabolite of 2-HOBA, and excluded those using monoamine oxidase inhibitors (MAO-I) due to some inhibition of MAO-A in the anticipated therapeutic range of 2-HOBA (See Safety of 2-HOBA – in vitro), and estimated creatinine clearance <30 ml/min due to likelihood of renal clearance of 2-HOBA[35].

Risk of the study include pain and bruising at the site of the blood draw, and on occasion a person can faint from a blood draw. 24-hour blood pressure monitoring may be annoying, possibly interfere with sleep, and could leave some skin irritation or bruising. There is a theoretic risk of loss of privacy/confidentiality. However, as described above, each participant will receive a code which will be used on biospecimen and the research database. Thus, it is unlikely that breach of privacy would occur. There is the possibility that 2-HOBA may have side effects that have not so far been identified.

Risks of the study will be minimized by performing studies in the Vanderbilt Clinical Research Center (CRC). All protocols must undergo approval by the Vanderbilt IRB. A complete history, physical examination and routine laboratory tests will be performed to ensure that subjects fulfill the entry criteria as defined. Vanderbilt University/Hospital does not provide coverage for injury occurring as a result of research. The CRC and its staff will provide emergency treatment for illness which results directly from the study but provides no other compensation. There are no alternative procedures which would allow us to obtain the information outlined in this proposal.

8.0 REPORTING OF ADVERSE EVENTS OR UNANTICIPATED PROBLEMS INVOLVING RISK TO PARTICIPANTS OR OTHERS

Data and Safety Monitoring Plan

The National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) has determined that independent data and safety monitoring oversite will be performed by dual Safety Officers (SOs), which NIAMS will appoint. NIAMS utilizes a contract research organization, Navitas Clinical Research, Inc (NCR) which serves as an Executive Secretary. Information on adverse events, adverse drug reactions, data quality, and study recruitment will be provided to NCR, and NCR will provide the information to the dual SOs at least twice a year. These data will be sent to the IRB at least yearly.
The dual SOs will review all serious adverse events (AEs). Any serious AE will be reported to the IRB and FDA and NCR as soon as possible, but not more than 48 hours from the investigators' awareness of the event. NCR will provide the information to the dual SOs and NIAMS. Any untoward medical event will be classified as an AE, regardless of its causal relationship with the study.

Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others:

Definitions of adverse events: an adverse event (AE) is "any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment".

Adverse events include:

- Worsening (change in nature, severity, or frequency) of conditions present at the onset of the trial
- Patient/subject deterioration due to the primary illness
- Intercurrent illnesses
- Drug interactions
- Events related or possibly related to concomitant medications
- Abnormal laboratory values or changes of vital signs, as well as significant shifts from baseline within the range of normal, which the Investigator considers clinically significant.

A serious adverse event/experience (SAE) or reaction is any untoward medical occurrence that at any dose:

- results in death
- is life-threatening
- requires inpatient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability/ incapacity (as per reporter's opinion)
- is a congenital anomaly/birth defect
- is another medically important condition
- The term "life-threatening" in the definition of "serious" refers to an event in which the patient is at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death if it were more severe.

Important medical conditions that may not result in death, be life-threatening or require hospitalization may be considered SAEs when, based upon appropriate medical judgment, they may jeopardize the patient or may require intervention to prevent one of the outcomes listed in the definition above. Examples of such events are intensive treatment in an emergency room or at home for allergic

bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

Unanticipated Problem: An unanticipated problem is any incident, experience or outcome that meets all the following requirements:

1. Unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the IRB-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;
2. Related or possibly related to participation in the research. Possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
3. Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

Definition of severity of adverse events:

Mild: Causing no limitation of usual activities; the subject/patient may experience slight discomfort.

Moderate: Causing some limitation of usual activities; the subject/patient may experience annoying discomfort.

Severe: Causing inability to carry out usual activities; the subject/patient may experience intolerable comfort or pain.

Definition of adverse event causality:

The Investigator will determine causality of each adverse event by using the classification criteria: not related, possibly/probably related, definitely related.

Not related: The AE is considered by the Investigator to be due to a pre-existing condition, a known manifestation of the target disease, a recurrent condition, or is likely explained by environmental or diagnostic therapeutic factors or was pre-existing and did not deteriorate.

Possibly/Probably related: The AE occurred during or after administration of the study treatment or a preexisting event worsened within an appropriate period of time, and at least one of the following criteria is applicable:

- the event could not be explained by the clinical condition or history of the subject, environmental or toxic factors, or other diagnostic or therapeutic measures;
- AE subsided or disappeared after withdrawal or dose reduction of study treatment; or
- AE recurred after re-exposure to study treatment.

Definitely related: The AE is a known side effect. (Prior studies to date have not demonstrated specific side effects of 2-HOBA which are observed more than placebo.)

Definition of unexpected adverse events: An unexpected adverse event is "an adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g., Investigator's Brochure for an unapproved investigational medicinal product)".

Adverse event reporting:

Any non-serious adverse events (AEs) will be reported to the PI within 72 hours of notification of the event. The PI will notify NCR who will notify the dual SOs of any serious adverse events as discussed below. Non-serious AEs and incidences of noncompliance with the protocol will be reported to the IRB at the time of annual review. All AEs are reported in aggregate as part of the routine data and safety monitoring report provided to the Monitoring Body and the NIAMS (through the NIAMS Executive Secretary) twice per year.

The study team will use the Redcap AE form and the separate AE report form to collect the information and the PI will assess each event.

Serious Adverse Events (SAEs) and unanticipated problems will be reported according to the following procedure:

The occurrence of serious adverse events will be reported to the Investigator within 24 hours after notification of their occurrence. The Investigator will report SAEs to the dual SOs and the Vanderbilt University Medical Center Institutional Review Board within 48 hours of the Investigator's notification of the event.

In an unanticipated event of prolonged side effect, requiring prolongation of hospital stay, patients will be retained in the hospital until side effects have resolved. For minor side effects, where inpatient care is deemed unnecessary, follow-up will be maintained via phone or as outpatient if necessary. Patient and their families will be given the PI's contact number for reporting any other effects of medication following discharge.

Any newly discovered information which may affect the subject or their caregiver's decision to continue to participate in the study will be passed on to them as soon as possible. This may also result in a change to the consent form and review by the IRB.

9.0 STUDY WITHDRAWAL/ DISCONTINUATION

Participants may withdraw from the study at any time by informing the study staff verbally or in writing. Contact information for the PI and study staff will be made available to the participant upon enrollment in the consent document. Subjects will be encouraged to conduct an exit study visit prior to withdrawing from the study. Any data or biological samples prior to their withdrawal request will not be withdrawn and destroyed.

A participant may be withdrawn from the study by the PI if the participant does not/ cannot comply with the study protocol, such as failure to attend study visits or needing to change RA treatment (changing DMARD or oral glucocorticoid).

Participants who elect to prematurely discontinue the study drug or must discontinue study drug per the PI recommendation due to adverse event, will discontinue study drug but will be asked to still complete the trial study visits.

10.0 STATISTICAL CONSIDERATIONS

Statistics:

Baseline characteristics will be compared between subjects in the active and placebo arms using Student's t-tests or Wilcoxon rank sum tests for continuous variables, and chi-square tests or Fisher's exact tests for proportions. Adverse events, pill counts, and drop-out rate will be presented as summary statistics for comparison between active and placebo arms. A modified intention-to-treat analysis using last observation carried forward method will be used on all randomized patients who take at least one dose of study drug. Thus subject data from Baseline Week 0 or an early exit visit will be carried forward to the week 4 study visit if not completed. Subjects who never receive drug will be excluded from analysis. Subjects who discontinue drug either by choice or request by PI due to adverse event and complete the week 4 study visit will be included in the modified intention-to-treat analysis. Separately an exploratory on-drug analysis will be conducted for subjects who continue drug through the week 4 study visit.

The primary analysis will compare cellular isoLGs from week 0 to week 4 in active versus placebo arms adjusting for baseline cellular isoLGs. Other exploratory prespecified outcomes will similarly compare week 0 and 4 (for active drug analysis) (**Table 4**) and use repeated measures analysis for cellular isoLGs and available secondary outcomes using repeated measure ANOVA or other mixed-effect models to assess treatment effect over time. Treatment by time interaction effect will be estimated and graphically summarized. We will perform a per protocol analysis of those completing the full study. We will report the adjusted 95% confidence intervals (CIs) of the mean difference between study groups and time points.

The study is not powered for subgroup analyses based on specific clinical features.

Table 4. Minimal detectible difference of outcomes

	Mean SD	Detectable difference
Primary outcome		
Cellular isoLGs, % cells	7.3 ± 3.0	3.65
Other prespecified outcomes		
Plasma isoLG-adducts, pmol/mg	*unavailable*	
DAS28, units	4.58 ± 1.1	1.66
CRP, mg/dl	12.69 ± 24.57	37.11
ESR, mm/hr	22.8 ± 21.3	32.17
TNFα, pg/ml	15.77 ± 17.86	26.98
IL6, pg/ml	15.66 ± 31.94	48.25
24hr SBP, mmHg	124 ± 14	17

Sample Size and Power

We plan to study up to 32 RA patients, assuming up to 25% dropout, to provide complete data on ≥24 RA patients. This is feasible based on a clinical trial of similar size and eligibility criteria we conducted previously[39]. The sample size is based on a change in cellular isoLGs. In our pilot study mean ± SD of isoLG$^+$ dendritic cells was 7.3% ± 3.0%. 24 RA patients (12 each in active and placebo arms) would provide 80% power to detect a 50% reduction in cellular isoLGs from week 0 to week 4 at a 2-sided 5% significance level. Estimated detectible differences for secondary outcomes are in **Table 4**.

11.0 PRIVACY/ CONFIDENTIALITY ISSUES

Each participant will have a unique code to de-identify the participant's data. Participant medical information will be stored in a REDCap database. Protected information such as names and medical record numbers of the study participants will be designated as non-exportable, protected fields. Access to this information will be granted only to members of the study team.

Biospecimens will be labeled with each participant's unique code.

If the results of this study are published, only the participant's unique code will be used for identification purposes. Participants will not be identified by name.

12.0 FOLLOW-UP AND RECORD RETENTION

Participant data and biospecimen data will be stored in the RedCap database for an indefinite period of time. Follow-up with the participant will be completed after exiting the study.

13.0 REFERENCES

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