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MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION: a randomized, double-blind, placebo-controlled, parallel-group clinical trial

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ABSTRACT

Introduction: New medication classes are needed to improve treatment effectiveness in the depressed phase of bipolar disorder (BD). Extant evidence suggests that BD is characterized by neural changes such as dendritic remodeling and glial and neuronal cell loss. These changes have been hypothesized to result from chronic inflammation. The principal aims of the proposed research is to evaluate the antidepressant efficacy in bipolar depression of minocycline, a drug with neuroprotective and immune-modulating properties, and of aspirin, at doses expected to selectively inhibit cyclooxygenase 1 (COX-1). **Methods and Analysis:** One hundred and twenty outpatients between 18 and 55 years of age, who meet DSM-IV-TR criteria for BD (type I or II) and for a current major depressive episode will be recruited to take part in a randomized, double-blind, placebo-controlled, parallel-group clinical trial following a 2 x 2 design. As adjuncts to existing treatment, subjects will be randomized to receive one of four treatment combinations: placebo-minocycline plus placebo-aspirin, active-minocycline plus placebo-aspirin, placebo-minocycline plus active-aspirin, or active-minocycline plus active-aspirin. The dose of minocycline and aspirin is 100mg bid and 81mg bid, respectively. Antidepressant response will be evaluated by assessing changes in the Montgomery-Asberg Depression Rating Scale (MADRS) scores between baseline and the end of the 6 week trial. As secondary outcome measures, the anti-inflammatory effects of minocycline and aspirin will be tested by measuring pre-and-post treatment levels of CRP and inflammatory cytokines. **Ethics and Dissemination:** Minocycline has been widely used as an antibiotic in doses up to 400 mg/day. Low dose aspirin has been safely used on a worldwide scale for its role as an anti-thrombotic and thrombolytic. The study progress will be overseen by a Data, Safety and Monitoring Board which will meet once every 6 months. Results of the study will be published in peer-reviewed publications. **Registration:** Clinical Trials.gov: NCT01429272.

INTRODUCTION

The treatment of bipolar depression remains a major challenge for psychiatry. The US FDA has not approved any of the ~25 standard antidepressants for the treatment of bipolar depression, partly because these agents have not been robustly effective in BD patients[1]. Thus, currently approved treatments for bipolar depression include lithium, quetiapine, and the combination of olanzapine and fluoxetine[2]. Other treatments used include lamotrigine, conventional antidepressant agents, other atypical antipsychotics, pramipexole or riluzole (reviewed in [3]). Unfortunately, the effectiveness of these options also is limited. For example, in a placebo-controlled study in which subjects receiving lithium were randomized to receive either standard antidepressant pharmacotherapy (paroxetine or imipramine) or placebo, those receiving lithium plus an antidepressant did not show a significant improvement over those receiving lithium plus placebo[4]. Similarly, in the STEP-BD trial, 42 of 179 subjects (23.5%) receiving a mood stabilizer plus adjunctive antidepressant drug treatment had a durable recovery, which did not differ significantly from 51 of 187 subjects (27.3%) receiving mood stabilizer plus placebo. Mallinger et al. reported a similar durable recovery rate in BD depressives treated with mood stabilizer plus paroxetine (27%), but found a higher rate for adjunctive monoamine oxidase inhibitors (MAOIs; 53%)[5], consistent with the findings of previous studies comparing MAOIs vs imipramine[6 7] . Unfortunately MAOIs are commonly unacceptable to patients.

New classes of antidepressant drugs are needed for bipolar depression. Existing agents exert their primary actions on monoaminergic systems. The efficacy of these agents contributed to the monoamine-deficiency hypothesis of depression, which continues to receive empirical support. Nevertheless, the field is in the early stages of a paradigm shift driven by evidence of dendritic remodeling and neuronal atrophy in animal models of depression, and of reductions in gray matter (GM) volume, and glial cell loss at *postmortem* in BD[8]. The neurotrophic effects of lithium, coupled with longitudinal studies demonstrating volumetric changes over time, raise the possibility that mood disorders are underpinned by a neurotoxic process[8 9]. The final common pathway through

which neurotoxic agents exert their effect is hypothesized to involve excess glutamatergic signaling[10].

The glutamatergic model of mood disorders is based on the premise that excessive stimulation of NMDA-glutamatergic receptors, results in neuronal atrophy and apoptosis of glial and/or neuronal cells, and *ipso facto*, depression. Evidence for this hypothesis derives from multiple sources. In preclinical models, riluzole, which inhibits neuronal release of glutamate, ceftriaxone, which increases glutamate reuptake, and NMDA receptor antagonists such as ketamine, ameliorate behavioral analogs of depression[11]. In addition, rats bred to be genetically sensitive to stress show differential expression of NMDA receptors[12], and behavioral analogs of depression are abrogated in NMDA receptor subunit knockout mice[13]. In humans, increased serum levels of glutamate that resolve with antidepressant treatment were reported in MDD, and extended to the CSF post mortem[11]. Polymorphisms of the metabotropic glutamate receptor genes, GRM2 and GRM3, and a haplotype of the glutamic acid decarboxylase (GAD2) gene were associated with MDD[14]. Finally, ketamine induced a rapid, sustained antidepressant effect in BD[15 16] and riluzole showed promising results in treatment-resistant depression[15 16].

One potential cause of the disruption in glutamatergic signaling in BD is dysregulation of the immune system. Increased levels of proinflammatory cytokines such as interleukin 6 (IL-6), IL-1β, interferon alpha (IFNα), tumor necrosis factor alpha (TNF-α) prostaglandinE2 (PGE2), and chemokine ligand 2 (CCL2) are consistently observed in the blood and CSF of patients with mood disorders, both at baseline and after exposure to stressors[17 18]. Elevated serum levels of (pro-inflammatory) positive acute-phase proteins (e.g., haptoglobin, α1-antitrypsin, ceruloplasmin, C-reactive protein), but reduced levels of negative acute-phase proteins (e.g., albumin and retinal-binding protein) also are reported in mood disorders[19-21]. Further, treatment of hepatitis C with IFNα is known to induce the major depressive syndrome and/or manic symptoms in a significant minority of patients, and the efficacy of conventional antidepressant drugs is associated with a reduction in inflammation[18]. Moreover, anti–tumor necrosis factor (TNF) therapy (for

psoriasis) can improve mood[22]. Since proinflammatory cytokines can alter brain function, these data are compatible with evidence that an activated inflammatory response system exists in mood disorders which plays a role in their pathophysiology[23-26].

The over-activity of the hypothalamic-pituitary-adrenal axis in mood disorders may play a role in inflammation, since hypersecretion of corticotrophin-releasing hormone (CRH) activates the transcription factor, nuclear factor kappa B (NF-κB). NF-κB regulates the expression of proinflammatory cytokines in immune cells in the CNS and periphery, and the expression of genes involved in apoptosis[27]. In addition, NF-κB may result in the expression of the class 1 major histocompatibility complex (MHC I), labeling cells for removal by cytotoxic T-cells[27]. Usually, cortisol suppresses this inflammatory response, but chronic stress appears to desensitize the glucocorticoid receptor (GR) and by extension, the anti-inflammatory effects of cortisol[27]. Cytokines play a role in desensitizing the system to cortisol. For example, IL1 and TNF-α retard dexamethasone-induced translocation of the GR receptor from the cytoplasm to the nucleus[28].

The immunologic and glutamatergic models of BD are complementary because a proinflammatory state is one potential cause of excitotoxicity[27]. Peripheral inflammatory signals activate microglia in the brain, inducing an inflammatory cascade of cytokines and free radicals. Cytokines and reactive oxygen and nitrogen species exert a direct toxic, apoptotic effect on oligodendrocytes. Potentially through the loss of oligodendrocytes, oxidative stress can lead to demyelination. Such a process conceivably may account for the reduction in oligodendroglia found *postmortem* in the prefrontal cortex[29] in mood disorders. The inflammatory milieu also compromises astrocyte function, leading to down-regulation of glutamate transporters and impaired glutamate reuptake into astrocytes, further amplifying inflammatory signaling[27].

In addition, cytokines such as interleukin 1 (IL-1), IL-6, and TNF-α activate indoleamine 2, 3-dioxygenase (IDO). IDO catalyzes the breakdown of tryptophan, the amino-acid precursor of serotonin, and an important regulator of T-cell function, into kynurenine (Kyn)[30]. Activation of the Kyn pathway shunts tryptophan away from 5-HT synthesis,

putatively reducing serotonergic transmission. Kyn is in turn metabolized into quinolinic acid (Quin), a potent NMDA receptor agonist, and neuromodulator involved in lipid peroxidation, which can induce neuronal damage via oxidative stress and overstimulation of NMDA receptors[30]. Consistent with inflammation-related shunt towards Kyn metabolism, the plasma tryptophan-Kyn ratio was found to correlate inversely with striatal total choline (a putative cell membrane turnover biomarker) in adolescents with melancholic depression[31].

The mRNA transcripts for proinflammatory genes appear particularly sensitive for discriminating BD patients. Microarray gene expression profiles in purified CD14+ monocytes from whole blood of BD subjects, offspring of BD parents, and healthy controls (HC) displayed a distinct mRNA signature representing genes from inflammatory and inflammation-related pathways[32]. The signature showed >80% sensitivity and specificity in BD subjects who were not receiving lithium or antipsychotic drugs (n=11), and in affected offspring of a BD parent (n=13, of whom 10 had only manifested depression). A positive signature also was present in 17 of 38 unaffected offspring (45%) versus 13 of 70 healthy children (19%). Cross-sectional comparisons suggested lithium and antipsychotic drugs—but not conventional antidepressant drugs--down-regulated expression of most inflammatory genes. Thus, when medicated and unmedicated subjects were considered together only 23 of 42 BD patients (55%) had a positive signature versus 7 of 38 HCs (18%). Notably, the IL6 mRNA level remained elevated in medicated BD subjects and did not differ significantly from unmedicated subjects (table 1), suggesting that this assay identifies a proinflammatory diathesis even in treated cases.

Table 1: Magnitude of difference in mRNA expression between mood disordered and healthy control (HC) samples from Padmos et al.[32], showing selected transcripts in unmedicated subjects vs HCs, relative to that of medicated BD subjects.

Gene Symbol	**Unmedicated BD vs HC**		**Medicated BD vs HC**		**Affected offspring# vs HC**	
	fold change	p-value	fold change	p-value	fold change	p-value
PDE4B	13.73*	<.001	3.42	<.001	5.79	<.001
IL6	37.92	.005	9.56	.006	935.7	<.001
CCL20	55.49	.006	6.02	.10	400.1	<.001

Legend: * - difference significant between unmedicated vs medicated BD samples; # - affected with respect to having manifested either a depressive or a manic episode
Sample sizes: unmedicated BD n=11, medicated BD n=31, affected offspring n=13, HCs n=25 for comparisons against BD adults, n=70 for comparisons of offspring. Abbrev: BD – bipolar disorder; HC – healthy control; PDE4B - phosphodiesterase type 4B; IL6 - interleukin 6; CCL20-chemokine ligand 20

Minocycline is a second-generation tetracycline that may prevent both glutamate-induced excitotoxicity and cytokine-induced inflammation in the CNS and periphery.

Minocycline has high lipophilicity enabling efficient transfer across the blood brain barrier (BBB)[33] - its concentration in CSF reaches 11–56% of plasma concentrations[34]. Minocycline inhibits the microglial-mediated release of proinflammatory cytokines IL-1β, TNF-α, IL-6, and p38[35], while promoting release of the anti-inflammatory cytokine, IL-10[34]. Moreover, minocycline inhibits matrix metalloproteinases which process

cytokines such as TNF-α and IL-1β into their biologically active forms[35]. Minocycline is also an effective scavenger of proapoptotic reactive oxygen species and protects against excitotoxicity by preventing glutamate-induced activation of nitric oxide synthase[36]. Nitric oxide facilitates glutamate release from presynaptic neurons and inhibits glial glutamate transporters, amplifying glutamatergic signaling, and contributing to excitotoxic cell death[10]. Minocycline also upregulates a key molecular factor in the apoptosis pathway, B-cell CLL/lymphoma 2 (BCL-2)[37], an effect shared by lithium, valproate[38] and certain antidepressant drugs[39]. BCL-2 represses apoptosis induced by cytotoxic insults[40].

Minocycline has neuroprotective and anti-inflammatory properties.

Minocycline prevents glutamate-induced apoptosis of neurons *in vitro*[41], prevents ischemia-induced activation of microglia in gerbils[42], increases hippocampal neuron survival[43], reduces lesion-volume and improves neurological function in mice with traumatic brain injury[44] and in fragile X syndrome[45], reduces pro-inflammatory cytokine expression and improves neurological function and locomotor activity in rats with spinal cord injury[46], attenuates MDMA-induced neurotoxicity of serotonin and dopamine systems in the cerebral cortex and hippocampus of mice[47], reduces inflammation in a rat-model of rheumatoid arthritis (RA)[48], and delays disease progression and demyelination in rodent models of encephalitis[49], amyotrophic lateral sclerosis (ALS)[50] and Huntington's Disease (HD)[51]. Based on these data, minocycline was employed, and has shown promise as, a therapeutic agent in human diseases including HD[52], rheumatoid arthritis (RA)[53], and stroke[54].

Minocycline has been used to treat psychiatric disorders.

Miyaoka et al.[55] discussed 2 patients with catatonic schizophrenia who benefited from minocycline. This group then conducted a 4-week trial with minocycline (150 mg/day) in 22 patients with schizophrenia to evaluate its efficacy as an adjunct to antipsychotic drugs[56]. Patients showed a significant improvement in positive and negative symptoms.

Levkovitz et al.[57] recently studied 54 patients with early-stage schizophrenia treated for 6 months with antipsychotic medication and either minocycline (200 mg/day) or placebo in a double-blind trial. Minocycline was associated with a reduction in negative symptoms and improved attention/ memory.

The efficacy of minocycline has not been formally tested in mood disorders. In rodents, minocycline reduced immobility during the forced-swim test[58], and co-administration of minocycline synergized the antidepressant-like actions of desipramine (but not fluoxetine)[59]. Minocycline also abrogated the depression-like behavior of rodents exposed to lipopolysaccharide (LPS)[60]. Levine et al.[61] presented the case of a 66-year old woman with severe BD, who observed that the tetracycline she took for an infection alleviated her depression. When her depression returned post-treatment, minocycline was reinitiated (150 mg/day). After one week her HAM-D score fell from 25 to 8.

Aspirin (Acetyl-salicylic acid, ASA) also holds potential efficacy in bipolar disorder.

The second aim of this study is to assess the antidepressant efficacy of ASA in bipolar depression. Using a 2 x 2 design we will obtain data providing estimates of the effect size of ASA relative to placebo, ASA relative to minocycline, and ASA in combination with minocycline relative to placebo. These data also will explore the specificity of any effect found for minocycline. The clinical use of low dose ASA primarily has been driven by its role as an anti-thrombotic and thrombolytic. Given the exaggerated death rate from cardiovascular events in BD, this action potentially is advantageous in the management of BD. Nevertheless, the recent literature also supports a role for low dose ASA in the management of the mood disorder itself, specifically in the amelioration of depressive symptoms.

The mechanism of ASA relates to its capacity to inactivate irreversibly the cyclooxygenase (COX) activity of prostaglandin (PG) H-synthase-1 and PGH-synthase 2 (referred to as COX-1 and COX-2, respectively). Although ASA has a short half-life (15 to 20 min) ASA's permanent inhibition of COX-1 allows once daily dosing for anucleate

platelets. In contrast, because nucleated cells rapidly regenerate this enzyme a shorter dosing interval is required to persistently impact COX activity in cells that mediate inflammatory processes. Moreover, ASA is 50- to 100-fold more potent in inhibiting platelet COX-1 than monocyte COX-2 activity[62], so there is nearly a 100-fold variation in the daily dose of aspirin, as higher doses are used to target COX-2 in the management of treating peripheral inflammation (e.g., arthritis) or pain. As reviewed below, preliminary evidence obtained in BD suggests beneficial effects are achieved using ASA in low doses, where aspirin would inhibit COX-1, but not COX-2.

Aspirin has neuroprotective and anti-inflammatory properties.

In the brain, recent data indicate that genetic manipulation of COX-1 and COX-2 differentially modulate leukocyte recruitment during neuroinflammation, and suggest that reduction of COX-1 activity is neuroprotective, whereas reduction in COX-2 activity is detrimental, during a primary neuroinflammatory response (reviewed in [63]). Choi et al.[63] propose that these distinct roles reflect the predominant localization of COX-1 in microglia, which play a major role in mediating neuroinflammation, in contrast to the predominant localization of COX-2 in pyramidal neurons. For example, Choi et al.[64] examined the effects of COX-1 or COX-2 deficiency on intracerebroventricular lipopolysaccharide (LPS)-induced neuroinflammation by comparing COX-1 (-/-) and COX-2 (-/-) knockout mice to wild-type (WT) (+/+) control animals. After LPS, leukocyte infiltration and inflammatory response were attenuated in the COX-1 (-/-) mice but increased in the COX-2 (-/-) mice, compared with WT controls. In another study, Choi et al.[65] examined the effect of COX□1 genetic deletion on the inflammatory response and neurodegeneration induced by β□amyloid, and found that in COX□1 (□/□) mice, the Aβ1□42□induced inflammatory response and associated neuronal damage were attenuated compared to WT mice. Compatible with these results, in pharmacoepidemiological studies investigating whether chronic NSAID use reduced the risk of developing Alzheimer's disease (AD), indomethacin, a preferential COX□1 inhibitor, showed beneficial effects, while COX□2 selective inhibitors, failed to show any beneficial effect in AD patients with mild to severe cognitive impairment. These data

suggest the hypothesis that inhibition of COX□1 activity may be a valid therapeutic strategy to reduce the cerebral inflammatory response and neurodegeneration in neuropsychiatric diseases in which neuroinflammatory components play a role in pathophysiology.

Other researchers hypothesized that NSAIDs would be beneficial in BD more specifically because of their ability to down-regulate activity in the brain arachidonic acid (AA) cascade by via interfering with phospholipase A2 (PLA2) and/or COX function. In rodents Rapoport and colleagues[66-68] demonstrated that conventional mood stabilizers decrease the AA turnover in phospholipids and the expression of PLA2 and/or COX enzymes. The PLA2 and COX enzymes catalyze, respectively, release of AA from membrane phospholipid and AA conversion to eicosanoids such as prostaglandin E2 and thromboxane B2. The AA cascade is involved in neuroreceptor-initiated signaling and can be pathologically upregulated by neuroinflammation and excitotoxicity.

Nevertheless, aspirin has additional mechanisms that may underlie benefits in neuropsychiatric illness. While low-dose aspirin down-regulates AA cascade activity via inhibition of COX-1 activity, in higher doses it also down-regulates COX-2 gene transcription, increases levels of lipoxygenase-derived eicosanoids such as the anti-inflammatory lipoxin A4, and acetylates COX-2 protein to a modified enzyme that can convert unesterified AA to anti-inflammatory mediators such as 15-epi-lipoxin A4 (reviewed in [69]). The acylated enzyme also can convert docosahexaenoic acid (DHA) to 17-(R)-OH-DHA, which, like its metabolites di(R)-OH-DHA (neuroprotectin (R) D1) and tri(R)-OH-DHA (resolvin (R) D1), is highly anti-inflammatory (reviewed in [69]). Lithium given chronically to rats with lipopolysaccharide-induced neuroinflammation also increases the brain concentration of 17-OH-DHA. Thus, there may be a synergy between aspirin and lithium in forming anti-inflammatory brain DHA metabolites.

Aspirin appears effective in preliminary studies of mood disorders.

Pharmaco-epidemiological data in BD supportive of these hypotheses were published by Stolk et al.[69]. Using the Netherlands based PHARMO Record Linkage System (which connects pharmacy dispensing records to hospital discharge records of > two million individuals since 1985), these researchers tested whether non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids would ameliorate bipolar symptoms. The target sample consisted of 5,145 patients receiving lithium (mean age = 48.6 $\pm$ 15 yrs; mean duration of lithium use=847 days), based upon the assumption that lithium treatment is relatively specific to individuals with BD. The main outcome measure was a calculated incidence density (ID) of medication events (change in the type or numbers of psychotropic medications prescribed, or increase [>30%] in the psychotropic drug dose). Subjects receiving low-dose ($\leq$80 mg/day) aspirin were 17% less likely to have a medication event, a finding that remained significant after adjusting for age, sex, chronic disease score and health care utilization. This effect was selective for low-dose ASA. In contrast, high-dose aspirin or non-selective NSAIDs (i.e., regimens expected to inhibit both COX-1 and -2), selective COX-2 inhibitors and glucocorticoids did not produce a statistically significant protection. Instead, the co-administration of non-selective NSAIDS and glucocorticoids was associated with statistically significant increases in medication events, suggesting destabilization of bipolar illness. The finding that low-dose aspirin decreased the number of medication events was particularly noteworthy since aspirin does not significantly augment serum lithium levels in contrast to selective COX-2 inhibitors which can raise lithium levels[70]. These preliminary observations thus appeared consistent with the hypothesis that COX-1 inhibitors can reduce neuroinflammatory processes and thus benefit BD patients.

Notably, the observation that beneficial effects in BD were conferred by low-dose ASA, but not by nonselective COX inhibitors, COX-2 inhibitors or glucocorticoids, appeared inconsistent with the hypothesis that drugs that down-regulate AA cascade activity in general hold therapeutic potential in BD. Thus the putative neuroprotective effects associated with COX-1 inhibition may contribute specifically to the benefits of low-dose aspirin in BD observed by Stolk et al. For example, as reviewed above, aspirin and

lithium may exert synergistic effects in forming anti-inflammatory brain DHA metabolites (reviewed in [69]).

Other data suggest that aspirin exerts antidepressant effects within the context of MDD or cardiovascular illness. Mendlewicz et al.[71] examined the effect of aspirin augmentation of conventional antidepressant pharmacotherapy in 24 patients with MDD who had proven non-responsive after 4 weeks of SSRI treatment. Participants were treated openly during the subsequent 4 weeks with aspirin 160 mg/day in addition to their SSRI regimen. The combined administration of SSRI plus aspirin was associated with a response rate of 52.4%. Remission was achieved in 43% of the total sample and 82% of the responder sample. In the responder group, a significant improvement was observed within week 1 and this benefit persisted through day 28. In another study Ketterer et al.[72] reported that in 174 males undergoing coronary angiography (of whom 99 were taking low-dose aspirin), aspirin use was associated with less depression and anxiety symptoms.

In contrast, a preliminary study of the selective COX-2 inhibitor, celecoxib, was negative in bipolar depression[73], potentially compatible with the negative results of COX-2 inhibitors reported by Stolk et al.[69]. In a double-blind, randomized, add-on clinical trial of celecoxib in patients (n = 28) studied during a depressed or mixed episode of BD, no significant difference was observed between the celecoxib and placebo add-on groups at study endpoint[73]. These results contrasted with those obtained using celecoxib in unipolar depression, however. In MDD, celecoxib augmentation of either reboxetine[74] or fluoxetine[75] was associated with a significant therapeutic effect on depressive symptoms in randomized, double-blind, add-on clinical trials.

METHODS AND ANALYSIS

Participants

One hundred and twenty male or female outpatients between 18 and 55 years of age, who meet DSM-IV-TR criteria for BD (type I or II) and for a current major depressive episode

will be recruited. The depressive syndrome must have been present for at least 4 weeks and the minimum threshold for depression severity will be set at a 17-item HAM-D score ≥18. Subjects will provide written informed consent as approved by the Western Institutional Review Board.

Concurrent Medications

At study entry type I BD subjects must have been taking a stable dose of a mood-stabilizing medication (lithium, valproate, carbamazepine, lamotrigine, antipsychotic agents), for at least 4 weeks, dosed clinically to target the therapeutic range. Type II BD subjects will be included irrespective of whether they present on a mood stabilizer. To investigate the utility of this augmentation strategy in the population for whom minocycline is most likely to prove therapeutically relevant, volunteers receiving stable doses of mood stabilizing, antipsychotic, antidepressant, and/or anxiolytic drugs for at least 4 weeks will be included. However, volunteers who currently are receiving more than 4 psychotropic medications in a daily regimen will be excluded, since this condition may signify a more brittle or complex clinical state. Subjects may remain in psychotherapy or have no psychosocial intervention. Volunteers will be excluded if they currently are receiving medications likely to have adverse interactions with minocycline or aspirin, including warfarin, digoxin, penicillins, and isotretinoin products.

For participants who enter the study, the preferred strategy will be for subjects to maintain the same regimen of concurrent medications throughout the six week study so that only the study drug regimen will be altered per protocol. Nevertheless, changes to concurrent medications will not affect study status, so long as the medication change does not target a depressive or manic symptom. If changes to concurrent medication regimens are clinically required to address worsening depressive symptoms or the development of manic symptoms, then the subject will be dropped from the study.

Study Design

Patients will participate in a randomized, double-blind, placebo-controlled, trial with a 2 x 2 design. As adjuncts to existing treatment, subjects will receive placebo-minocycline plus placebo-aspirin, active-minocycline plus placebo-aspirin, placebo-minocycline plus active-aspirin, or active-minocycline plus active-aspirin. The randomization sequences will be determined by a research staff-member who is not obtaining clinical information from the research subject and will be assigned by subject number at consenting. The trial will be conducted over 6 weeks and will comprise 7 assessment sessions (figure 1). The subject will be seen at the prescribed time intervals within a window of two business days on either side of visit target date to complete the specified visits.

At each session, a clinical assessment will be conducted using the rating scales listed below, and treatment side-effects will be assessed and rated for severity. To preserve the rater blind, the research staff member who conducts the clinical ratings will not be the research staff member who assesses the presence of side effects, and will remain blind to the information pertaining to side effects. Subjects who experience severe adverse effects or who develop treatment-associated hypomania or mania will be dropped from the study, instructed to discontinue the study medication, and referred for appropriate clinical management of these adverse events.

The primary outcome measure will be the change in the Montgomery-Asberg Depression Rating Scale (MADRS) scores.

Medication

This pilot proof-of-concept study will adhere to the dosing limits and route of administration for the FDA indications for minocycline's and aspirin's use in other conditions (thus an IND is not required). A fixed dose design will be followed, and all medications will be administered via the p.o. route. The pilot data extant for both study drugs supports an onset of improvement within two weeks, so the six week study duration is expected to provide sufficient time to detect an antidepressant effect, to

provide information about the persistence of the antidepressant effect over about one month from the anticipated onset of effect, and to minimize dropouts.

For minocycline the starting dose will be 100 mg b.i.d. (total daily dose=200 mg). This dose of 100 mg b.i.d. has been shown by a substantial literature to produce consistent anti-inflammatory effects in rheumatoid arthritis and other inflammatory disorders. This also is the dose used in a recent schizophrenia treatment trial[57]. The associated placebo capsules match the appearance of the 100 mg minocycline capsule.

The starting dose of aspirin will be 81 mg p.o. b.i.d. This dose is sufficient to inhibit COX-1, and appeared beneficial in stabilizing the course of BD in the pharmaco-epidemiological study of Stolk et al.[69]. When aspirin is used as an anti-platelet drug once daily dosing is sufficient since anucleate platelets do not produce enough COX-1 to overcome the irreversible inhibition of COX-1 within a 24-hour period. In contrast, in nucleated cells COX-1 is replenished, so more frequent dosing is required to persistently inhibit COX-1. Thus we will administer the dose in a b.i.d. regimen, according to the guidelines described above. A total daily dose of 160 mg was administered in the preliminary study which reported that aspirin significantly augmented the antidepressant effects of fluoxetine in MDD[71]. The relevant placebo matches the appearance of the aspirin tablet.

Participants will be advised that one of the study drugs may reduce the efficacy of oral contraceptives, and to avoid taking the study drugs within 3 hours of iron products or of antacids containing calcium, magnesium or aluminum. They also will be advised that one study drug can increase their risk for bleeding during surgical procedure or if combined with other drugs or herbal preparations that reduce hemostasis.

Compensation

Participants will be compensated for participation in the amount of $300.00.

Treatment Compliance

To enhance compliance, study participants will be given an information sheet to take home detailing the procedure to be followed in the case of a missed dose, and requesting that this information be recorded for the investigators. The number of capsules and tablets remaining in each supply given to the patients will also be counted to evaluate treatment compliance. In cases where treatment compliance is poor, subjects will be excluded from the data analysis, using conventional criteria for defining adequate compliance in a clinical trial.

[Fig. 1, here]

Psychiatric Assessment and Clinical Ratings

Patients will be evaluated and followed in the outpatient clinics at LIBR or Oklahoma University School of Community Medicine in Tulsa, OK, or at the University of Kansas Medical Center Research Institute (KUMCRI) in Wichita, KS. The diagnosis of BD will be established using DSM-IV-TR criteria on the basis of an unstructured interview conducted by a psychiatrist and the MINI-Plus administered by trained psychiatric interviewers. The following rating scales will be administered: MADRS, Quick Inventory of Depressive Symptomatology (QUIDS; 16 item), Hamilton Anxiety Rating Scale (HAM-A), Young Mania Rating Scale (YMRS), Universal Fagerstrom (to assess nicotine use), Hollingshead socioeconomic scale, Sheehan Disability Scale (SDS) and the Family Interview for Genetic Studies (FIGS). Medical assessment will include a physical examination, electrocardiogram, complete blood count (CBC), electrolytes and liver-function assays (SMA 20), thyroid panel, and urinalysis, serum drug and pregnancy tests at study entry and study completion. At each follow-up session, the MADRS, HAM-A, YMRS, and Clinical Global Impressions (CGI) scale will be repeated. Physical and psychiatric symptoms will be evaluated and recorded in order to measure the side-effect profiles of minocycline and aspirin. Participants will be questioned about adverse reactions, including dizziness, photosensitivity, hyperpigmentation, gastrointestinal

distress or bleeding at each assessment and will be withdrawn from the study if medically necessary. Vital signs will be measured at entry and at each session.

Immune System Measures

The activity of peripheral cytokines correlates with inflammatory processes in the CNS. Peripheral cytokines cross the BBB, and can propagate signals across the BBB in the form of small, freely diffusible lipophilic molecules such as prostaglandins, which induce the production of cytokines from glia[76]. The measurement of peripheral markers of inflammation thus serves as a valid, if indirect assessment of CNS inflammation.

To explore predictors and correlates of treatment outcome, blood will be sampled for testing plasma and whole blood peripheral blood monocyte (PBM) based markers of inflammation at baseline and study end. These markers will include 10 cytokine proteins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFNγ, and TNF, high sensitivity (hs) CRP, and RNA expression of candidate genes from PBMCs. Candidate genes include IL-6, TNF, and IRF5 (a factor that mediates monocyte polarization). The 10 inflammation-related cytokines and the PBMC mRNA will be assayed from plasma at baseline and study end. We selected the markers IL-6, TNF and CRP because they are the most widely implicated in mood disorders. The other cytokines included in the cytokine bead array assays are measured simultaneously with IL-6 and have all been implicated in the general regulation of inflammation. A meta-analysis of >100 studies found that IL-6 and CRP each were significantly elevated in depressed patients with standardized mean difference scores (d) of 0.71 and 0.26, respectively[77]. The associations remained significant after adjustment for body-mass index (BMI) and smoking. Moreover, IL-6 has been shown to modulate HPA axis function by inducing CRH release, adrenocorticotrophic hormone synthesis, and corticosteroid production[78]. CRP production is induced by the proinflammatory cytokines, IL-1, IL-6, and IL-17, and is thus a non-specific marker of systemic inflammation.

Three blood samples will be transported to the immunology lab in the Department of Surgery at the University of Oklahoma College of Medicine for each participant at each of the sampling time-points (sessions 1 and 7). One sample will be centrifuged to obtain plasma which will be stored at −80°C until analyzed. Serum CRP, IL-6, TNF, and the other cytokines listed above will be assayed in duplicate with ELISA (CRP high-sensitivity kit, R & D Systems, Oxford, UK) or enhanced cytokine bead array flex kits (Becton Dickinson) using the manufacturer's reagents and standards. The other two samples will be used to isolate PBMCs and plasma and will be frozen until processed. Monocytes will be isolated from the PBMCs in order to assess mRNA levels similar to the method used by Padmos et al.[32]. This procedure utilizes monoclonal antibodies directed against human CD14 to isolate monocytes in peripheral blood monocyte cell suspensions. A magnetic cell sorting system will be used for the separation of the monocytes and flow cytometry will be used to gauge the purity of the population. Once purity is established, total RNA will be isolated from the monocytes using an RNeasy kits (Qiagen) according to the manufacturer's directions. RNA will then be reverse transcribed to cDNA using standard commercial kits. rtPCR reactions will be performed using the Dynamo Sybr Green HS Master Mix (New England Biolabs) and custom primers will be synthesized by a commercial laboratory. Real-time rtPCR reactions will be run using a Cepheid Smart Cycler II or similar instrument. Additional aliquots of serum and plasma will be stored so that other inflammatory markers can be tested in the future using Luminex bead arrays and/or additional available technologies.

Source of Compounds Tested

Minocycline and aspirin are available on a generic basis, and are manufactured within the USA by several companies. The identity of the active medicines and placebos will be blinded using placebos that match the appearance of the active drugs. The medications and placebos have been formulated by Wedgewood Pharmacy, Swedesboro, NJ. The study minocycline capsule and chewable aspirin tablet are identical in appearance to their corresponding placebos.

Outcome Measures and Data Analysis

Antidepressant response will be evaluated by assessing changes in MADRS scores. The *a priori* hypothesis that minocycline and/or aspirin plus existing medication will exert greater antidepressant effects than placebo plus existing medication will be tested in an intent-to-treat analysis using last observation carried forward for study dropouts. A secondary analysis will be performed to assess clinical improvement only in the study completers. These analyses will be statistically assessed using a group (for the four treatment cells)-by-session (1 vs. 7) repeated measures analysis of variance (ANOVA). If the ANOVA statistic is significant, between- and within-group t tests will be used in planned comparisons to identify the nature of the effect leading to the significant overall ANOVA statistic. We expect to find a significant group-by-session interaction, attributable to a greater reduction in MADRS scores in the minocycline and aspirin groups compared to the placebo group between session 1 and session 7.

In order to test whether the putative antidepressant effects of minocycline or aspirin have a rapid onset, as a *post hoc* analysis the ANOVA will be repeated using MADRS ratings from the assessment that follows the first week of exposure to active drug versus the corresponding change under placebo; i.e. session 2. *Post hoc* tests will be performed to assess the significance of changes in the secondary clinical outcome measures (QUIDS 16, HAM-A, YMRS, CGI-I). The rate of completion in the two cells also will be considered an outcome measure. The completion rate in the minocycline arm may be influenced more by dropouts due to side effects while the completion rate in the placebo group may be influenced more by dropouts due to non-response.

We will test the hypothesis that minocycline and aspirin reduce inflammation (e.g. CRP, IL-6, IL-6 mRNA) more than placebo using statistical analyses similar to those described above. If the assay results are normally distributed then a group-by-session repeated-measures ANOVA with CRP, IL-6 and nine other cytokine levels as dependent variables, and BMI, smoking status, and time of blood draw as covariates, will be used to assess anti-inflammatory effects of minocycline and aspirin. If the CRP or inflammatory

cytokine data are not normally distributed (Kolmogorov-Smirnov test) or if the equality of statistical variance assumption across assessments is violated (Levene's test), then Friedman's ANOVA will be used to test for CRP or inflammatory cytokine differences between groups. If the Friedman's ANOVA statistic is significant, Wilcoxon sign-ranked tests will be used for post-hoc analysis of group differences. Nonspecific factors that influence CRP and inflammatory cytokine levels include time of day, presence of infection, treatment with anti-inflammatory medications, smoking, obesity, and alcohol abuse. We will attempt to control for these potential confounds by measuring BMI and recording NSAID and nicotine use (Universal Fagerstrom scale), and by excluding individuals who have recently abused substances or who have intercurrent infections. The serum CRP concentration shows minimal diurnal variability in adults[79] but IL-6 and other cytokine levels vary across time of day[80]. To minimize cytokine measurement variability due to circadian fluctuations, we will schedule patient assessment sessions at the same time each day. Since this may not always be possible, we will record the time of day that each blood-draw is made, divide the day into quartiles: 7am-10am, 10am-12pm; 12pm-3pm, and 3pm-6pm, and use these data as a covariate in the statistical analyses.

To test whether baseline levels of CRP and inflammatory cytokines predict response to minocycline or aspirin, we will subclassify the participants using conventional criteria[81] as achieving full response (≥50% reduction in MADRS score from baseline), partial response (<50% but ≥25% reduction), or nonresponse (<25% reduction). Patients achieving remission (post-treatment MADRS score ≤10) will also be identified. A non-parametric alternative to the ANOVA statistic, the Mann-Whitney test, will be used to compare remitted and non-remitted groups in baseline levels of inflammatory cytokines and CRP if the data are not normally distributed.

Statistical Power

A recent meta-analysis of 96 antidepressant treatment studies found that the average effect size of a placebo treatment is 1.69 compared with 2.50 for an antidepressant treatment[82]. We calculated that in order to detect a difference in-group means of 0.81

(2.50-1.69) with an 80% probability (2-sided test, α=0.05), we will require a sample size of 26 subjects per group (http://hedwig.mgh.harvard.edu/sample_size/size.html). Thus given our sample size of 30 per group we should have sufficient power to test Specific Aim 1, allowing for a 13% drop-out rate.

As discussed above, a recent meta-analysis[77] of cross-sectional studies of serum-derived IL-6 and CRP in depression calculated effect sizes of 0.71 for IL-6 and 0.26 for CRP. Based on these effect sizes a sample size of 26 would yield >80% probability of detecting significant depression-related changes in IL-6, but only a 60% probability of detecting a depression-related change in CRP. There are 3 reasons why we believe that these CRP power estimations are not applicable to this study. Firstly, the effect sizes derived from the meta-analysis are based on cross-sectional studies. Given the effect of variables such as smoking, diet, exercise, and BMI on proinflammatory cytokines, a within-subjects design is likely to reduce non-depression-related sources of variance, and substantially increase statistical power. Secondly, we are not only examining the effect of mood on IL-6 and CRP levels, but are treating patients with minocycline and aspirin, drugs known to possess anti-inflammatory properties. We therefore suggest that our proposed study is likely adequately powered to detect any true changes in plasma IL-6, CRP, and the other inflammatory cytokines across treatment blocks.

Regarding IL-6 mRNA gene expression in peripheral blood monocytes, Padmos et al.[32] reported a 38-fold increase in IL-6 mRNA levels in unmedicated patients with BD compared with HC. Since minocycline reduces IL-6 levels (see above) we expect our study to have very high power to detect differences between groups, as well as changes in response to minocycline. The simultaneous detection of nine other inflammation-related cytokines, in addition to IL-6 (using newer more sensitive technology) will provide much finer resolution of the effects on inflammatory cascades than that measured in previous studies.

ETHICS AND DISSEMINATION

Gender/Minority/Pediatric Inclusion for Research

Women and Minorities will be included in the study without prejudice according to their representation in the study population. Participants will be recruited from the greater metropolitan areas of Tulsa, OK and Wichita, KS and efforts will be made to ensure that our subject population resembles the gender, ethnic and racial composition of these areas.

Exclusion Criteria

The following exclusion criteria apply: 1) inability to provide informed consent; 2) age of onset of BD>40 years; 3) serious risk of suicide; 4) current delusions or hallucinations sufficient to interfere with the capacity to provide informed consent; 5) current manic symptoms [depressed BD patients with concurrent manic symptoms have been found to be more likely to experience adverse reactions in antidepressant treatment trials[83]]; 6) medical illness including as hepatic impairment, renal dysfunction, bleeding diatheses (e.g., hemophilia), cerebrovascular disease or heart disease, hypertension that is inadequately controlled by medication, diabetes mellitus, or known peptic ulcer disease; 7) abuse of drugs or alcohol within the preceding 6 months, or substance dependence within the last 5 years; 8) daily alcoholic beverage consumption equivalent to ≥3 oz. of alcohol; 9) asthma or known allergies or hypersensitivities to tetracycline antibiotics, aspirin or other NSAIDs; 10) current use of drugs that could increase the risks associated with aspirin or minocycline administration, namely other antibiotic medications, other NSAIDs or anticoagulants (e.g., warfarin), acetazolamide, or methotrexate; 11) known HIV or other chronic infection including, but not limited to viral hepatitis. 12) Pregnant or nursing women, and women who are attempting to conceive during the 6 week study period, will also be excluded.

Specimens, Records, and Data Collection

A physician, registered nurse, or trained phlebotomist will utilize a sterile technique to draw 60 ml of blood by venipuncture. Participants will also be asked to submit a urine

sample. A physician, registered nurse, or trained technician will collect EKG data from the subject in a private exam room.

Recruitment and Consent Procedure

Volunteers will be recruited from the community as well as from the clinical services at the Laureate Psychiatric Clinic and Hospital and the Oklahoma University School of Community Medicine in Tulsa, OK, and from the clinical services affiliated with the KUMCRI. Volunteers may be referred from sources that include physicians, newspaper advertising, self-help organizations, self-referral, and WIRB approved flyers posted at local universities, schools, churches and grocery stores. Participants may be pre-screened through screening protocols based at LIBR or KUCRI. We plan to recruit a total of 120 participants.

All participant interactions including consenting will be conducted in private interview / exam rooms. These rooms are secured from public areas via combination locked doors that are only accessible to authorized personnel. Prospective participants will receive an explanation of the objectives, procedures, and hazards of this protocol that is appropriate to their level of understanding. The right of the subject to decline to participate or to withdraw from the study at any time will be made clear.

Non-English speaking participants will not be recruited.

After the consent form is verbally explained to the participant, and any questions have been answered, the researcher will leave the room to allow the participant to read the consent form thoroughly. Family members will be allowed to be present and to discuss the consenting process with the participant. After the consent is read, the researcher will return and answer any additional questions the participant may have. The researcher will remind the subject that participation is strictly voluntary and that they have the right to withdraw at any time. Participants will be asked to arrive 30 minutes early in order to have sufficient time for the consenting process.

Subject Risks

The risks of behavioral testing are minimal. The risks of blood drawing are also minimal. Possible mild side effects of the blood draw include mild pain or bruising at the site of the venipuncture.

Minocycline has been used a broad-spectrum antibiotic for many years in doses up to 400 mg/day[34]. It has been used on a chronic basis to treat acne and rheumatoid arthritis, often for many years, in hundreds of thousands of patients. The most commonly encountered side effects are upset stomach, diarrhea, dizziness, drowsiness, ataxia, vertigo, headache and vomiting. Prolonged use can be associated with pigmentation of the skin, gums or teeth. Between 1975 and 2006, the World Health Organization Collaborating Center for International Drug Monitoring listed 122 cases of adverse drug reactions to intravenous minocycline; most commonly, abnormal hepatic function and thrombocytopenia[34]. These included cases of serious liver injury, including irreversible drug-induced hepatitis and fulminant hepatic failure that was fatal in two cases, thought to be due to triggering or unmasking autoimmune hepatitis. One case of autoimmune-related glomerulonephritis has been reported. The role of oral minocycline in precipitating these conditions has not been clearly established. Minocycline also has been associated with idiopathic intracranial hypertension (pseudotumor cerebri). Long-term trials have shown that minocycline is well tolerated. In a 2-year trial of minocycline (200 mg/day) for RA, 3 of 30 patients withdrew due to finger-nail discoloration, dizziness, or erythematous rash[53]. Of 11 patients with HD treated with minocycline (100 mg/day) for 2 years, one complained of nausea in the first 3 weeks, and two of sedation[52], while in a 6-month trial of minocycline for ALS, the mean tolerated dose was 387 mg/day and the most common adverse effects were gastrointestinal[84]. Five of 36 patients with schizophrenia withdrew from a 6-month trial of minocycline (200 mg/day) due to indigestion (n=2), pigmentation (n=2), or a suicide attempt (n=1)[57].

Low dose aspirin has been safely used in many millions of patients on a worldwide scale for its role as an anti-thrombotic and thrombolytic. A meta-analysis of >100 randomized trials in high-risk patients indicated that low-dose ASA reduced cardiovascular death by 15% and prevented nonfatal vascular events by about 30%[85]. These data stand in striking contrast to the data obtained in COX-2 inhibitors, which can increase cardiovascular risk. In clinical trials of several COX-2 selective and nonselective NSAIDs of up to three years duration have shown an increased risk of serious cardiovascular (CV) thrombotic events, myocardial infarction, and stroke, which have in many cases been fatal[86]. Patients with known CV disease or risk factors for CV disease are at greater risk for such events during chronic treatment with COX-2 inhibitors. Evidence from human pharmacology and genetics, genetically manipulated rodents, and other animal models and randomized trials indicates that this is consequent to suppression of COX-2-dependent cardioprotective prostaglandins, particularly prostacyclin[87].

Aspirin does not cause a generalized bleeding abnormality unless given to patients with an underlying hemostatic defect (e.g., hemophilia, uremia, or that induced by anticoagulant therapy). Aspirin-induced impairment of primary hemostasis cannot be separated from its antithrombotic effect and is similar at all doses $\geq$ 75 mg/d[88]. The risk of intracranial bleeding is exceedingly rare (<0.1% in high risk populations), but is higher in individuals with cerebrovascular disease[85]. Hypertension that is inadequately controlled by medication often is considered a contraindication to aspirin because of the concern that possible benefits in the prevention of cardiovascular events may be counterbalanced by an increased risk of cerebral bleeding. However, hypertensive patients whose blood pressure is well-controlled appear protected from myocardial infarction by aspirin therapy without an increase in the number of cerebral hemorrhages or strokes[89]. Moreover, aspirin therapy does not affect blood pressure or the response of hypertension to antihypertensive agents[88 90].

NSAIDs as a class can cause serious gastrointestinal (GI) adverse events including inflammation, bleeding, ulceration, and perforation of the stomach, small intestine, or large intestine, which rarely have proven fatal. In controlled clinical trials the percentage

of patients reporting one or more gastrointestinal complaints has ranged from 4% to 16%[88]. The mechanism underlying this adverse effect appears attributable to the inhibition of COX-1. Thus, the incidence of GI side effects has been higher for NSAIDs with more potent effects at COX-1, such as aspirin and indomethicin. For example, in controlled trials the incidence of GI side effects for aspirin and indomethacin have been about twice as high as that for ibuprofen, a nonselective COX inhibitor, in equally effective doses for arthritis. Nevertheless, the incidence of GI side effects associated with aspirin is dose-dependent, and thus is markedly lower when using aspirin in the low dose range planned for the current study. Notably, the risk of GI bleeding is not reduced by using the enterically coated aspirin formulations, but is thought to be lower during concomitant use of omeprazole[88]. The effects of warfarin and NSAIDs on GI bleeding are synergistic, such that the users of both drugs together have a risk of serious GI bleeding higher than users of either drug alone. Fortunately, the risk of GI bleeding, which reflects the inhibition of prostaglandins in the stomach (from systemic rather than local exposure) is much smaller when using low-dose as opposed to high-dose aspirin.

Low-dose aspirin has not been reported to alter renal function, and does not reduce effectiveness of ACE inhibitors for HTN (in contrast to other NSAIDs)[90 91]. However, aspirin can inhibit the renal clearance of acetazolamide and methotrexate potentially leading to increased blood concentrations of and toxicity from these agents. Salicylate can displace other drugs which are protein-bound, especially phenytoin and valproic acid, increasing their free drug concentrations in plasma. This may increase side effects, toxicity and/or efficacy for displaced drugs. If the BD subjects are currently receiving valproic acid preparations (e.g., divalproex) then the plasma levels of these agents will be monitored for potential changes.

Aspirin may cause a severe allergic reaction that may include: hives, asthma (wheezing), facial swelling, shock. Aspirin overdose can be fatal at 30 g or higher.

PHI Protection

Paper copies of consents, screening forms, the Research Privacy Form, and any other forms, testing results or papers containing Protected Health Information (PHI) will be stored in a secured medical records room with access granted only to authorized personnel.

Electronic data that contain PHI will be managed in accordance with ISO 27000 series information security standards with policies developed from current NIST guidelines (SP 800-66) for HIPAA and HITECH compliance. Specific controls implemented to protect PHI are derived from NIST 800-122, and include (but not limited to):

1) Access Enforcement (AC-3) – Individual user accounts, role based access control, access control lists;
2) Separation of Duties (AC-5) – de-identification of data as appropriate, acquire/analyze/manage firewall;
3) Least Privilege (AC-6) – to ensure PHI data is only available to persons with established need for access;
4) Remote Access (AC-17) – Secure VPN, encrypted end devices;
5) Access Control for Mobile Devices (AC-19) – Password login, remote destruction capabilities;
6) Auditable Events (AU-2) + Monitoring: Log detailed server and network information, alert for problems;
7) Analysis, and Reporting (AU-6) – Procedures to audit system records for inappropriate activity.
8) User Identification and Authentication (IA-2) – username/secure password and two factor authentication will be required when appropriate.
9) Media Access, Marking, Storage, and Transport (MP-2,3,4,5) – Records will be asset tagged and marked to their PHI status, PHI data will be secured and managed by professional system administrators, and will be transported via encryption (VPN, USB, File);
10) Media Sanitization (MP-6) – Data will be destroyed by SFHS in accordance with their policies and procedures;

11) Transmission Confidentiality (SC-9) – Encryption will be used when needed for all avenues of data transmission (wireless, network, etc.).

To protect subject confidentiality, blood samples will be anonymized as follows:

1. Last name: All participants will be assigned the last name "LIBR."
2. First name: The first name will be a secure alpha cryptographic hash based on LIBR user ID. This technique is the gold standard in computer security for one-way correlation of data.

Benefits versus Risks

The participant may benefit from participation if either study drug produces an antidepressant effect. Participants will also receive a free clinical evaluation; more frequent treatment visits than are typical in practice, diligent follow-up in terms of symptoms and side effects, and physical and psychiatric monitoring during the study. The risks of delaying alternative treatments are minimal in relation to the potential long-term benefits to the subjects and the importance of knowledge that may reasonably result. The importance of the knowledge that will likely be gained from this study clearly exceeds the associated potential risks.

Alternative Treatment

It is possible that some patients may feel better with talk therapy. Participating in any type of talk therapy with their psychiatrist or psychologist does not require dropping out of this study. Subjects will be encouraged to contact the study investigators, particularly the physician in the study, with any questions they may have regarding alternatives to treatment through this research study. The study investigators will assist in referring the subject to another physician for treatment after their participation in the study has ended. Physical and psychological testing, blood draws, urine samples, and EKG data provide no known risks to persons other than those listed in the exclusion criteria whereas the combinatory power of these measures may provide information relevant to understanding the pathophysiology of bipolar disorder.

Data and Safety Monitoring Plan

This study involves more than minimal risk. The study progress will be overseen by a Data, Safety and Monitoring Board (DSMB). The DSMB is composed of three members who will meet in person or per telephone at least once every 6 months to review relevant study data including adverse events and dropout rates.

Any unanticipated adverse events will be reported immediately to the IRB of record and to the LIBR Human Protection Administrator. Any adverse events will be included in the annual IRB report.

Dissemination of Results

The study results will be presented at national and/or international biomedical scientific meetings and published in peer-reviewed journals.

REGISTRATION

In accordance with the recommendations of the International Committee of Medical Journal Editors[92], the proposed trial is registered in a public registry (www.clinicaltrials.gov Identifier: NCT01429272).

Figure Legend

Figure 1: Schematic of Study Design

Legend: Each session number (total of 7) is encircled, with the timing between sessions indicated in weeks with a 2 business day window on either side of visit target date to complete the visit. Session 1 is the baseline (green star) and session 7 is the study end

(purple star). Peripheral blood will be sampled at baseline and study end to assay markers of inflammation. The study duration is 6 weeks.

Author Contributions

The protocol was written by Drs. Savitz and W. Drevets and was critically reviewed by Drs. Preskorn, Teague, D. Drevets, and Yates.

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Competing Interests

Wayne Drevets, M.D. has consulted for Johnson & Johnson, Pfizer, Rules-Based Medicine, and Eisai. None of the other authors have conflicts of interest to declare.

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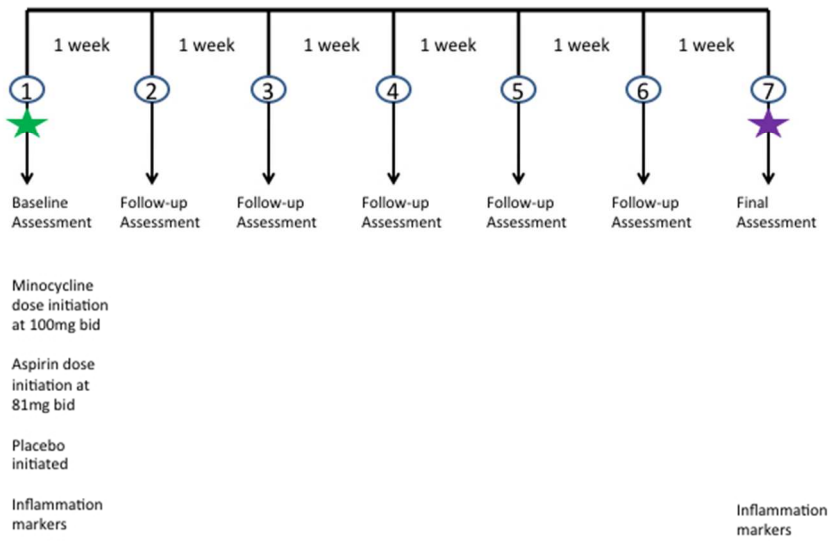

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INVESTIGATOR: Wayne Drevets M.D.
6655 South Yale Avenue
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BOARD ACTION DATE: 08/22/2011
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STUDY APPROVAL EXPIRES: 08/05/2012
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SPONSOR: Laureate Institute for Brain Research (LIBR)
PROTOCOL NUM: 2011-002-00
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TITLE:
MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION

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CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	3-13
	2b	Specific objectives or hypotheses	20
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	14-15
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	13-14
	4b	Settings and locations where the data were collected	17
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	15-16
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	20-21
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	21
	7b	When applicable, explanation of any interim analyses and stopping guidelines	30
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	15
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	15
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	15
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	15

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	20-21
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	20-21
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	
	13b	For each group, losses and exclusions after randomisation, together with reasons	
Recruitment	14a	Dates defining the periods of recruitment and follow-up	
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	
Other information			
Registration	23	Registration number and name of trial registry	1, 30
Protocol	24	Where the full trial protocol can be accessed, if available	19
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

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MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION: a protocol for a proof-of-concept randomized, double-blind, placebo-controlled, 2x2, clinical trial

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MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION: a protocol for a proof-of-concept randomized, double-blind, placebo-controlled, 2x2, clinical trial

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Key Words

Bipolar Depression, Clinical Trial, Minocycline, Aspirin, Inflammation

ABSTRACT

Introduction: New medication classes are needed to improve treatment effectiveness in the depressed phase of bipolar disorder (BD). Extant evidence suggests that BD is characterized by neural changes such as dendritic remodeling and glial and neuronal cell loss. These changes have been hypothesized to result from chronic inflammation. The principal aims of the proposed research is to evaluate the antidepressant efficacy in bipolar depression of minocycline, a drug with neuroprotective and immune-modulating properties, and of aspirin, at doses expected to selectively inhibit cyclooxygenase 1 (COX-1). **Methods and Analysis:** One hundred and twenty outpatients between 18 and 55 years of age, who meet DSM-IV-TR criteria for BD (type I or II) and for a current major depressive episode will be recruited to take part in a randomized, double-blind, placebo-controlled, parallel-group, proof-of-concept, clinical trial following a 2 x 2 design. As adjuncts to existing treatment, subjects will be randomized to receive one of four treatment combinations: placebo-minocycline plus placebo-aspirin, active-minocycline plus placebo-aspirin, placebo-minocycline plus active-aspirin, or active-minocycline plus active-aspirin. The dose of minocycline and aspirin is 100mg bid and 81mg bid, respectively. Antidepressant response will be evaluated by assessing changes in the Montgomery-Asberg Depression Rating Scale (MADRS) scores between baseline and the end of the 6 week trial. As secondary outcome measures, the anti-inflammatory effects of minocycline and aspirin will be tested by measuring pre-and-post treatment levels of CRP and inflammatory cytokines. **Ethics and Dissemination:** Minocycline has been widely used as an antibiotic in doses up to 400 mg/day. Low dose aspirin has been safely used on a worldwide scale for its role as an anti-thrombotic and thrombolytic. The study progress will be overseen by a Data, Safety and Monitoring Board which will meet once every 6 months. Results of the study will be published in peer-reviewed publications. **Registration:** Clinical Trials.gov: NCT01429272.

INTRODUCTION

The treatment of bipolar depression remains a major challenge for psychiatry. The US FDA has not approved any of the ~25 standard antidepressants for the treatment of bipolar depression, partly because these agents have not been robustly effective in BD patients[1]. Thus, currently approved treatments for bipolar depression include lithium, quetiapine, and the combination of olanzapine and fluoxetine[2]. Other treatments used include lamotrigine, conventional antidepressant agents, other atypical antipsychotics, pramipexole or riluzole (reviewed in [3]). Unfortunately, the effectiveness of these options also is limited. For example, in a placebo-controlled study in which subjects receiving lithium were randomized to receive either standard antidepressant pharmacotherapy (paroxetine or imipramine) or placebo, those receiving lithium plus an antidepressant did not show a significant improvement over those receiving lithium plus placebo[4]. Similarly, in the STEP-BD trial, 42 of 179 subjects (23.5%) receiving a mood stabilizer plus adjunctive antidepressant drug treatment had a durable recovery, which did not differ significantly from 51 of 187 subjects (27.3%) receiving mood stabilizer plus placebo. Mallinger et al. reported a similar durable recovery rate in BD depressives treated with mood stabilizer plus paroxetine (27%), but found a higher rate for adjunctive monoamine oxidase inhibitors (MAOIs; 53%)[5], consistent with the findings of previous studies comparing MAOIs vs imipramine[6 7] . Unfortunately MAOIs are commonly unacceptable to patients.

New classes of antidepressant drugs are needed for bipolar depression. Existing agents exert their primary actions on monoaminergic systems. The efficacy of these agents contributed to the monoamine-deficiency hypothesis of depression, which continues to receive empirical support. Nevertheless, the field is in the early stages of a paradigm shift driven by evidence of dendritic remodeling and neuronal atrophy in animal models of depression, and of reductions in gray matter (GM) volume, and glial cell loss at *postmortem* in BD[8]. The neurotrophic effects of lithium, coupled with longitudinal studies demonstrating volumetric changes over time, raise the possibility that mood disorders are underpinned by a neurotoxic process[8 9]. The final common pathway through

which neurotoxic agents exert their effect is hypothesized to involve excess glutamatergic signaling[10].

The glutamatergic model of mood disorders is based on the premise that excessive stimulation of NMDA-glutamatergic receptors, results in neuronal atrophy and apoptosis of glial and/or neuronal cells, and *ipso facto*, depression. Evidence for this hypothesis derives from multiple sources. In preclinical models, riluzole, which inhibits neuronal release of glutamate, ceftriaxone, which increases glutamate reuptake, and NMDA receptor antagonists such as ketamine, ameliorate behavioral analogs of depression[11]. In addition, rats bred to be genetically sensitive to stress show differential expression of NMDA receptors[12], and behavioral analogs of depression are abrogated in NMDA receptor subunit knockout mice[13]. In humans, increased serum levels of glutamate that resolve with antidepressant treatment were reported in MDD, and extended to the CSF post mortem[11]. Polymorphisms of the metabotropic glutamate receptor genes, GRM2 and GRM3, and a haplotype of the glutamic acid decarboxylase (GAD2) gene were associated with MDD[14]. Finally, ketamine induced a rapid, sustained antidepressant effect in BD[15 16] and riluzole showed promising results in treatment-resistant depression[15 16].

One potential cause of the disruption in glutamatergic signaling in BD is dysregulation of the immune system. Increased levels of proinflammatory cytokines such as interleukin 6 (IL-6), IL-1β, interferon alpha (IFNα), tumor necrosis factor alpha (TNF-α) prostaglandinE2 (PGE2), and chemokine ligand 2 (CCL2) are consistently observed in the blood and CSF of patients with mood disorders, both at baseline and after exposure to stressors[17 18]. Elevated serum levels of (pro-inflammatory) positive acute-phase proteins (e.g., haptoglobin, α1-antitrypsin, ceruloplasmin, C-reactive protein), but reduced levels of negative acute-phase proteins (e.g., albumin and retinal-binding protein) also are reported in mood disorders[19-21]. Further, treatment of hepatitis C with IFNα is known to induce the major depressive syndrome and/or manic symptoms in approximately 40% of patients, and the efficacy of conventional antidepressant drugs is associated with a reduction in inflammation[18]. Moreover, anti–tumor necrosis factor (TNF) therapy (for

psoriasis) can improve mood[22]. Since proinflammatory cytokines can alter brain function, these data are compatible with evidence that an activated inflammatory response system exists in mood disorders which plays a role in their pathophysiology[23-26].

The over-activity of the hypothalamic-pituitary-adrenal axis in mood disorders may play a role in inflammation, since hypersecretion of corticotrophin-releasing hormone (CRH) activates the transcription factor, nuclear factor kappa B (NF-κB). NF-κB regulates the expression of proinflammatory cytokines in immune cells in the CNS and periphery, and the expression of genes involved in apoptosis[27]. In addition, NF-κB may result in the expression of the class 1 major histocompatibility complex (MHC I), labeling cells for removal by cytotoxic T-cells[27]. Usually, cortisol suppresses this inflammatory response, but chronic stress appears to desensitize the glucocorticoid receptor (GR) and by extension, the anti-inflammatory effects of cortisol[27]. Cytokines play a role in desensitizing the system to cortisol. For example, IL1 and TNF-α retard dexamethasone-induced translocation of the GR receptor from the cytoplasm to the nucleus[28].

The immunologic and glutamatergic models of BD are complementary because a proinflammatory state is one potential cause of excitotoxicity[27]. Peripheral inflammatory signals activate microglia in the brain, inducing an inflammatory cascade of cytokines and free radicals. Cytokines and reactive oxygen and nitrogen species exert a direct toxic, apoptotic effect on oligodendrocytes. Potentially through the loss of oligodendrocytes, oxidative stress can lead to demyelination. Such a process conceivably may account for the reduction in oligodendroglia found *postmortem* in the prefrontal cortex[29] in mood disorders. The inflammatory milieu also compromises astrocyte function, leading to down-regulation of glutamate transporters and impaired glutamate reuptake into astrocytes, further amplifying inflammatory signaling[27].

In addition, cytokines such as interleukin 1 (IL-1), IL-6, and TNF-α activate indoleamine 2, 3-dioxygenase (IDO). IDO catalyzes the breakdown of tryptophan, the amino-acid precursor of serotonin, and an important regulator of T-cell function, into kynurenine (Kyn)[30]. Activation of the Kyn pathway shunts tryptophan away from 5-HT synthesis,

putatively reducing serotonergic transmission. Kyn is in turn metabolized into quinolinic acid (Quin), a potent NMDA receptor agonist, and neuromodulator involved in lipid peroxidation, which can induce neuronal damage via oxidative stress and overstimulation of NMDA receptors[30]. Consistent with inflammation-related shunt towards Kyn metabolism, the plasma tryptophan-Kyn ratio was found to correlate inversely with striatal total choline (a putative cell membrane turnover biomarker) in adolescents with melancholic depression[31].

The mRNA transcripts for proinflammatory genes appear particularly sensitive for discriminating BD patients. Microarray gene expression profiles in purified CD14+ monocytes from whole blood of BD subjects, offspring of BD parents, and healthy controls (HC) displayed a distinct mRNA signature representing genes from inflammatory and inflammation-related pathways[32]. The signature showed >80% sensitivity and specificity in BD subjects who were not receiving lithium or antipsychotic drugs (n=11), and in affected offspring of a BD parent (n=13, of whom 10 had only manifested depression). A positive signature also was present in 17 of 38 unaffected offspring (45%) versus 13 of 70 healthy children (19%). Cross-sectional comparisons suggested lithium and antipsychotic drugs—but not conventional antidepressant drugs--down-regulated expression of most inflammatory genes. Thus, when medicated and unmedicated subjects were considered together only 23 of 42 BD patients (55%) had a positive signature versus 7 of 38 HCs (18%). Notably, the IL6 mRNA level remained elevated in medicated BD subjects and did not differ significantly from unmedicated subjects (table 1), suggesting that this assay identifies a proinflammatory diathesis even in treated cases.

Table 1: Magnitude of difference in mRNA expression between mood disordered and healthy control (HC) samples from Padmos et al.[32], showing selected transcripts in unmedicated subjects vs HCs, relative to that of medicated BD subjects.

Gene Symbol	**Unmedicated BD vs HC**		**Medicated BD vs HC**		**Affected offspring# vs HC**	
	fold change	p-value	fold change	p-value	fold change	p-value
PDE4B	13.73*	<.001	3.42	<.001	5.79	<.001
IL6	37.92	.005	9.56	.006	935.7	<.001
CCL20	55.49	.006	6.02	.10	400.1	<.001

Legend: * - difference significant between unmedicated vs medicated BD samples; # - affected with respect to having manifested either a depressive or a manic episode
Sample sizes: unmedicated BD n=11, medicated BD n=31, affected offspring n=13, HCs n=25 for comparisons against BD adults, n=70 for comparisons of offspring. Abbrev: BD – bipolar disorder; HC – healthy control; PDE4B - phosphodiesterase type 4B; IL6 - interleukin 6; CCL20-chemokine ligand 20

Minocycline is a second-generation tetracycline that may prevent both glutamate-induced excitotoxicity and cytokine-induced inflammation in the CNS and periphery.

Minocycline has high lipophilicity enabling efficient transfer across the blood brain barrier (BBB)[33] - its concentration in CSF reaches 11–56% of plasma concentrations[34]. Minocycline inhibits the microglial-mediated release of proinflammatory cytokines IL-1β, TNF-α, IL-6, and p38[35], while promoting release of the anti-inflammatory cytokine, IL-10[34]. Moreover, minocycline inhibits matrix metalloproteinases which process

cytokines such as TNF-α and IL-1β into their biologically active forms[35]. Minocycline is also an effective scavenger of proapoptotic reactive oxygen species and protects against excitotoxicity by preventing glutamate-induced activation of nitric oxide synthase (NOS)[36]. Nitric oxide facilitates glutamate release from presynaptic neurons and inhibits glial glutamate transporters, amplifying glutamatergic signaling, and contributing to excitotoxic cell death[10]. Minocycline also upregulates a key molecular factor in the apoptosis pathway, B-cell CLL/lymphoma 2 (BCL-2)[37], an effect shared by lithium, valproate[38] and certain antidepressant drugs[39]. BCL-2 represses apoptosis induced by cytotoxic insults[40]. Conceivably, minocycline may additionally reduce inflammation indirectly by blocking the translocation of bacteria across the intestinal barrier. In mice exposed to a social stressor, bacteria translocated across the intestinal barrier stimulating the release of circulating cytokines such as IL6, and increasing microbicidal activity via inducible NOS[41]. Additionally, stress induced a change in the community structure of the microflora in the cecum with a decrease the relative abundance of bacteria in the genus Bacteroides and an increase the relative abundance of bacteria in the genus Clostridium. Notably, these effects were blocked by pretreatment with a broad spectrum antibiotic[41].

Minocycline has neuroprotective and anti-inflammatory properties.

Minocycline prevents glutamate-induced apoptosis of neurons *in vitro*[42], prevents ischemia-induced activation of microglia in gerbils[43], increases hippocampal neuron survival[44], reduces lesion-volume and improves neurological function in mice with traumatic brain injury[45] and in fragile X syndrome[46], reduces pro-inflammatory cytokine expression and improves neurological function and locomotor activity in rats with spinal cord injury[47], attenuates MDMA-induced neurotoxicity of serotonin and dopamine systems in the cerebral cortex and hippocampus of mice[48], reduces inflammation in a rat-model of rheumatoid arthritis (RA)[49], and delays disease progression and demyelination in rodent models of encephalitis[50], amyotrophic lateral sclerosis (ALS)[51] and Huntington's Disease (HD)[52]. Based on these data, minocycline was employed, and has shown promise as, a therapeutic agent in human diseases including HD[53], rheumatoid arthritis (RA)[54], and stroke[55].

Minocycline has been used to treat psychiatric disorders.

Miyaoka et al.[56] discussed 2 patients with catatonic schizophrenia who benefited from minocycline. This group then conducted a 4-week trial with minocycline (150 mg/day) in 22 patients with schizophrenia to evaluate its efficacy as an adjunct to antipsychotic drugs[57]. Patients showed a significant improvement in positive and negative symptoms. Levkovitz et al.[58] recently studied 54 patients with early-stage schizophrenia treated for 6 months with antipsychotic medication and either minocycline (200 mg/day) or placebo in a double-blind trial. Minocycline was associated with a reduction in negative symptoms and improved attention/ memory.

The efficacy of minocycline has not been formally tested in mood disorders. In rodents, minocycline reduced immobility during the forced-swim test[59], and co-administration of minocycline synergized the antidepressant-like actions of desipramine (but not fluoxetine)[60]. Minocycline also abrogated the depression-like behavior of rodents exposed to lipopolysaccharide (LPS)[61]. Levine et al.[62] presented the case of a 66-year old woman with severe BD, who observed that the tetracycline she took for an infection alleviated her depression. When her depression returned post-treatment, minocycline was reinitiated (150 mg/day). After one week her HAM-D score fell from 25 to 8.

Aspirin (Acetyl-salicylic acid, ASA) also holds potential efficacy in bipolar disorder.

The second aim of this study is to assess the antidepressant efficacy of ASA in bipolar depression. Using a 2 x 2 design we will obtain data providing estimates of the effect size of ASA relative to placebo, ASA relative to minocycline, and ASA in combination with minocycline relative to placebo. These data also will explore the specificity of any effect found for minocycline. The clinical use of low dose ASA primarily has been driven by its role as an anti-thrombotic and thrombolytic. Given the exaggerated death rate from cardiovascular events in BD, this action potentially is advantageous in the management of BD. Nevertheless, the recent literature also supports a role for low dose ASA in the

management of the mood disorder itself, specifically in the amelioration of depressive symptoms.

The mechanism of ASA relates to its capacity to inactivate irreversibly the cyclooxygenase (COX) activity of prostaglandin (PG) H-synthase-1 and PGH-synthase 2 (referred to as COX-1 and COX-2, respectively). Although ASA has a short half-life (15 to 20 min) ASA's permanent inhibition of COX-1 allows once daily dosing for anucleate platelets. In contrast, because nucleated cells rapidly regenerate this enzyme a shorter dosing interval is required to persistently impact COX activity in cells that mediate inflammatory processes. Moreover, ASA is 50- to 100-fold more potent in inhibiting platelet COX-1 than monocyte COX-2 activity[63], so there is nearly a 100-fold variation in the daily dose of aspirin, as higher doses are used to target COX-2 in the management of treating peripheral inflammation (e.g., arthritis) or pain. As reviewed below, preliminary evidence obtained in BD suggests beneficial effects are achieved using ASA in low doses, where aspirin would inhibit COX-1, but not COX-2.

Aspirin has neuroprotective and anti-inflammatory properties.

In the brain, recent data indicate that genetic manipulation of COX-1 and COX-2 differentially modulate leukocyte recruitment during neuroinflammation, and suggest that reduction of COX-1 activity is neuroprotective, whereas reduction in COX-2 activity is detrimental, during a primary neuroinflammatory response (reviewed in [64]). Choi et al.[64] propose that these distinct roles reflect the predominant localization of COX-1 in microglia, which play a major role in mediating neuroinflammation, in contrast to the predominant localization of COX-2 in pyramidal neurons. For example, Choi et al.[65] examined the effects of COX-1 or COX-2 deficiency on intracerebroventricular lipopolysaccharide (LPS)-induced neuroinflammation by comparing COX-1 (-/-) and COX-2 (-/-) knockout mice to wild-type (WT) (+/+) control animals. After LPS, leukocyte infiltration and inflammatory response were attenuated in the COX-1 (-/-) mice but increased in the COX-2 (-/-) mice, compared with WT controls. In another study, Choi et al.[66] examined the effect of COX□1 genetic deletion on the inflammatory

response and neurodegeneration induced by β□amyloid, and found that in COX□1 (□/□) mice, the Aβ1□42□induced inflammatory response and associated neuronal damage were attenuated compared to WT mice. Compatible with these results, in pharmacoepidemiological studies investigating whether chronic NSAID use reduced the risk of developing Alzheimer's disease (AD), indomethacin, a preferential COX□1 inhibitor, showed beneficial effects, while COX□2 selective inhibitors, failed to show any beneficial effect in AD patients with mild to severe cognitive impairment. These data suggest the hypothesis that inhibition of COX□1 activity may be a valid therapeutic strategy to reduce the cerebral inflammatory response and neurodegeneration in neuropsychiatric diseases in which neuroinflammatory components play a role in pathophysiology.

Other researchers hypothesized that NSAIDs would be beneficial in BD more specifically because of their ability to down-regulate activity in the brain arachidonic acid (AA) cascade by via interfering with phospholipase A2 (PLA2) and/or COX function. In rodents Rapoport and colleagues[67-69] demonstrated that conventional mood stabilizers decrease the AA turnover in phospholipids and the expression of PLA2 and/or COX enzymes. The PLA2 and COX enzymes catalyze, respectively, release of AA from membrane phospholipid and AA conversion to eicosanoids such as prostaglandin E2 and thromboxane B2. The AA cascade is involved in neuroreceptor-initiated signaling and can be pathologically upregulated by neuroinflammation and excitotoxicity.

Nevertheless, aspirin has additional mechanisms that may underlie benefits in neuropsychiatric illness. While low-dose aspirin down-regulates AA cascade activity via inhibition of COX-1 activity, in higher doses it also down-regulates COX-2 gene transcription, increases levels of lipoxygenase-derived eicosanoids such as the anti-inflammatory lipoxin A4, and acetylates COX-2 protein to a modified enzyme that can convert unesterified AA to anti-inflammatory mediators such as 15-epi-lipoxin A4 (reviewed in [70]). The acylated enzyme also can convert docosahexaenoic acid (DHA) to 17-(R)-OH-DHA, which, like its metabolites di(R)-OH-DHA (neuroprotectin (R) D1) and tri(R)-OH-DHA (resolvin (R) D1), is highly anti-inflammatory (reviewed in [70]).

Lithium given chronically to rats with lipopolysaccharide-induced neuroinflammation also increases the brain concentration of 17-OH-DHA. Thus, there may be a synergy between aspirin and lithium in forming anti-inflammatory brain DHA metabolites.

Aspirin appears effective in preliminary studies of mood disorders.

Pharmaco-epidemiological data in BD supportive of these hypotheses were published by Stolk et al.[70]. Using the Netherlands based PHARMO Record Linkage System (which connects pharmacy dispensing records to hospital discharge records of > two million individuals since 1985), these researchers tested whether non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids would ameliorate bipolar symptoms. The target sample consisted of 5,145 patients receiving lithium (mean age = 48.6 $\pm$ 15 yrs; mean duration of lithium use=847 days), based upon the assumption that lithium treatment is relatively specific to individuals with BD. The main outcome measure was a calculated incidence density (ID) of medication events (change in the type or numbers of psychotropic medications prescribed, or increase [>30%] in the psychotropic drug dose). Subjects receiving low-dose ($\leq$80 mg/day) aspirin were 17% less likely to have a medication event, a finding that remained significant after adjusting for age, sex, chronic disease score and health care utilization. This effect was selective for low-dose ASA. In contrast, high-dose aspirin or non-selective NSAIDs (i.e., regimens expected to inhibit both COX-1 and -2), selective COX-2 inhibitors and glucocorticoids did not produce a statistically significant protection. Instead, the co-administration of non-selective NSAIDS and glucocorticoids was associated with statistically significant increases in medication events, suggesting destabilization of bipolar illness. The finding that low-dose aspirin decreased the number of medication events was particularly noteworthy since aspirin does not significantly augment serum lithium levels in contrast to selective COX-2 inhibitors which can raise lithium levels[71]. These preliminary observations thus appeared consistent with the hypothesis that COX-1 inhibitors can reduce neuroinflammatory processes and thus benefit BD patients.

Notably, the observation that beneficial effects in BD were conferred by low-dose ASA, but not by nonselective COX inhibitors, COX-2 inhibitors or glucocorticoids, appeared inconsistent with the hypothesis that drugs that down-regulate AA cascade activity in general hold therapeutic potential in BD. Thus the putative neuroprotective effects associated with COX-1 inhibition may contribute specifically to the benefits of low-dose aspirin in BD observed by Stolk et al. For example, as reviewed above, aspirin and lithium may exert synergistic effects in forming anti-inflammatory brain DHA metabolites (reviewed in [70]).

Other data suggest that aspirin exerts antidepressant effects within the context of MDD or cardiovascular illness. Mendlewicz et al.[72] examined the effect of aspirin augmentation of conventional antidepressant pharmacotherapy in 24 patients with MDD who had proven non-responsive after 4 weeks of SSRI treatment. Participants were treated openly during the subsequent 4 weeks with aspirin 160 mg/day in addition to their SSRI regimen. The combined administration of SSRI plus aspirin was associated with a response rate of 52.4%. Remission was achieved in 43% of the total sample and 82% of the responder sample. In the responder group, a significant improvement was observed within week 1 and this benefit persisted through day 28. In another study Ketterer et al.[73] reported that in 174 males undergoing coronary angiography (of whom 99 were taking low-dose aspirin), aspirin use was associated with less depression and anxiety symptoms.

In contrast, a preliminary study of the selective COX-2 inhibitor, celecoxib, was negative in bipolar depression[74], potentially compatible with the negative results of COX-2 inhibitors reported by Stolk et al.[70]. In a double-blind, randomized, add-on clinical trial of celecoxib in patients (n = 28) studied during a depressed or mixed episode of BD, no significant difference was observed between the celecoxib and placebo add-on groups at study endpoint[74]. These results contrasted with those obtained using celecoxib in unipolar depression, however. In MDD, celecoxib augmentation of either reboxetine[75] or fluoxetine[76] was associated with a significant therapeutic effect on depressive symptoms in randomized, double-blind, add-on clinical trials.

METHODS AND ANALYSIS

Participants

One hundred and twenty male or female outpatients between 18 and 55 years of age, who meet DSM-IV-TR criteria for BD (type I or II) and for a current major depressive episode will be recruited. The depressive syndrome must have been present for at least 4 weeks and the minimum threshold for depression severity will be set at a 17-item HAM-D score ≥18. Subjects will provide written informed consent as approved by the Western Institutional Review Board.

Concurrent Medications

At study entry type I BD subjects must have been taking a stable dose of a mood-stabilizing medication (lithium, valproate, carbamazepine, lamotrigine, antipsychotic agents), for at least 4 weeks, dosed clinically to target the therapeutic range. Type II BD subjects will be included irrespective of whether they present on a mood stabilizer. To investigate the utility of this augmentation strategy in the population for whom minocycline is most likely to prove therapeutically relevant, volunteers receiving stable doses of mood stabilizing, antipsychotic, antidepressant, and/or anxiolytic drugs for at least 4 weeks will be included. However, volunteers who currently are receiving more than 4 psychotropic medications in a daily regimen will be excluded, since this condition may signify a more brittle or complex clinical state. Subjects may remain in psychotherapy or have no psychosocial intervention. Volunteers will be excluded if they currently are receiving medications likely to have adverse interactions with minocycline or aspirin, including NSAIDS, warfarin, digoxin, penicillins, and isotretinoin products.

For participants who enter the study, the preferred strategy will be for subjects to maintain the same regimen of concurrent medications throughout the six week study so that only the study drug regimen will be altered per protocol. Nevertheless, changes to concurrent medications will not affect study status, so long as the medication change does

not target a depressive or manic symptom. If changes to concurrent medication regimens are clinically required to address worsening depressive symptoms or the development of manic symptoms, then the subject will be dropped from the study.

Study Design

Patients will participate in a randomized, double-blind, placebo-controlled, trial with a 2 x 2 design. As adjuncts to existing treatment, subjects will receive placebo-minocycline plus placebo-aspirin, active-minocycline plus placebo-aspirin, placebo-minocycline plus active-aspirin, or active-minocycline plus active-aspirin. The randomization sequences will be determined by a research staff-member who is not obtaining clinical information from the research subject and will be assigned by subject number at consenting. A restricted randomization (permuted block randomization) method will be used in which subjects are randomly allocated to each block (n=30) to ensure that equal numbers of participants receive each drug/placebo combination. In order to ensure that experimental group assignment is not skewed across the two trial sites, the study progress will be monitored by individuals who are not involved in the data collection, and in the case of "drift", adjustments will be made as necessary.

The trial will be conducted over 6 weeks and will comprise 7 assessment sessions (figure 1). The subject will be seen at the prescribed time intervals within a window of two business days on either side of visit target date to complete the specified visits.

At each session, a clinical assessment will be conducted using the rating scales listed below, and treatment side-effects will be assessed and rated for severity. To preserve the rater blind, the research staff member who conducts the clinical ratings will not be the research staff member who assesses the presence of side effects, and will remain blind to the information pertaining to side effects. Subjects who experience severe adverse effects or who develop treatment-associated hypomania or mania will be dropped from the study, instructed to discontinue the study medication, and referred for appropriate clinical management of these adverse events.

The primary outcome measure will be the change in the Montgomery-Asberg Depression Rating Scale (MADRS) scores at the seventh assessment session (week 6).

Medication

This pilot proof-of-concept study will adhere to the dosing limits and route of administration for the FDA indications for minocycline's and aspirin's use in other conditions (thus an IND is not required). A fixed dose design will be followed, and all medications will be administered via the p.o. route. The pilot data extant for both study drugs supports an onset of improvement within two weeks, so the six week study duration is expected to provide sufficient time to detect an antidepressant effect, to provide information about the persistence of the antidepressant effect over about one month from the anticipated onset of effect, and to minimize dropouts.

For minocycline the starting dose will be 100 mg b.i.d. (total daily dose=200 mg). This dose of 100 mg b.i.d. has been shown by a substantial literature to produce consistent anti-inflammatory effects in rheumatoid arthritis and other inflammatory disorders. This also is the dose used in a recent schizophrenia treatment trial[58]. The associated placebo capsules match the appearance of the 100 mg minocycline capsule.

The starting dose of aspirin will be 81 mg p.o. b.i.d. This dose is sufficient to inhibit COX-1, and appeared beneficial in stabilizing the course of BD in the pharmaco-epidemiological study of Stolk et al.[70]. When aspirin is used as an anti-platelet drug once daily dosing is sufficient since anucleate platelets do not produce enough COX-1 to overcome the irreversible inhibition of COX-1 within a 24-hour period. In contrast, in nucleated cells COX-1 is replenished, so more frequent dosing is required to persistently inhibit COX-1. Thus we will administer the dose in a b.i.d. regimen, according to the guidelines described above. A total daily dose of 160 mg was administered in the preliminary study which reported that aspirin significantly augmented the antidepressant

effects of fluoxetine in MDD[72]. The relevant placebo matches the appearance of the aspirin tablet.

Participants will be advised that one of the study drugs may reduce the efficacy of oral contraceptives, and to avoid taking the study drugs within 3 hours of iron products or of antacids containing calcium, magnesium or aluminum. They also will be advised that one study drug can increase their risk for bleeding during surgical procedure or if combined with other drugs or herbal preparations that reduce hemostasis.

Compensation

Participants will be compensated for participation in the amount of $300.00.

Treatment Compliance

To enhance compliance, study participants will be given an information sheet to take home detailing the procedure to be followed in the case of a missed dose, and requesting that this information be recorded for the investigators. The number of capsules and tablets remaining in each supply given to the patients will also be counted to evaluate treatment compliance. In cases where treatment compliance is poor, subjects will be excluded from the data analysis, using conventional criteria for defining adequate compliance in a clinical trial.

[Fig. 1, here]

Psychiatric Assessment and Clinical Ratings

Patients will be evaluated and followed in the outpatient clinics at LIBR or Oklahoma University School of Community Medicine in Tulsa, OK, or at the University of Kansas Medical Center Research Institute (KUMCRI) in Wichita, KS. The diagnosis of BD will be established using DSM-IV-TR criteria on the basis of an unstructured interview

conducted by a psychiatrist and the MINI-Plus administered by trained psychiatric interviewers. The following rating scales will be administered: MADRS, Quick Inventory of Depressive Symptomatology (QUIDS; 16 item), Hamilton Anxiety Rating Scale (HAM-A), Young Mania Rating Scale (YMRS), Universal Fagerstrom (to assess nicotine use), Hollingshead socioeconomic scale, Sheehan Disability Scale (SDS) and the Family Interview for Genetic Studies (FIGS). Medical assessment will include a physical examination, electrocardiogram, complete blood count (CBC), electrolytes and liver-function assays (SMA 20), thyroid panel, and urinalysis, serum drug and pregnancy tests at study entry and study completion. At each follow-up session, the MADRS, HAM-A, YMRS, and Clinical Global Impressions (CGI) scale will be repeated. Physical and psychiatric symptoms will be evaluated and recorded in order to measure the side-effect profiles of minocycline and aspirin. Participants will be questioned about adverse reactions, including dizziness, photosensitivity, hyperpigmentation, gastrointestinal distress or bleeding at each assessment and will be withdrawn from the study if medically necessary. Vital signs will be measured at entry and at each session.

Immune System Measures

The activity of peripheral cytokines correlates with inflammatory processes in the CNS. Peripheral cytokines cross the BBB, and can propagate signals across the BBB in the form of small, freely diffusible lipophilic molecules such as prostaglandins, which induce the production of cytokines from glia[77]. The measurement of peripheral markers of inflammation thus serves as a valid, if indirect assessment of CNS inflammation.

To explore predictors and correlates of treatment outcome, blood will be sampled for testing plasma and whole blood peripheral blood monocyte (PBM) based markers of inflammation at baseline and study end. These markers will include 10 cytokine proteins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFNγ, and TNF, high sensitivity (hs) CRP, and RNA expression of candidate genes from PBMCs. Candidate genes include IL-6, TNF, and IRF5 (a factor that mediates monocyte polarization). The 10 inflammation-related cytokines and the PBMC mRNA will be assayed from plasma at

baseline and study end. We selected the markers IL-6, TNF and CRP because they are the most widely implicated in mood disorders. The other cytokines included in the cytokine bead array assays are measured simultaneously with IL-6 and have all been implicated in the general regulation of inflammation. A meta-analysis of >100 studies found that IL-6 and CRP each were significantly elevated in depressed patients with standardized mean difference scores (d) of 0.71 and 0.26, respectively[78]. The associations remained significant after adjustment for body-mass index (BMI) and smoking. Moreover, IL-6 has been shown to modulate HPA axis function by inducing CRH release, adrenocorticotrophic hormone synthesis, and corticosteroid production[79]. CRP production is induced by the proinflammatory cytokines, IL-1, IL-6, and IL-17, and is thus a non-specific marker of systemic inflammation.

Three blood samples will be transported to the immunology lab in the Department of Surgery at the University of Oklahoma College of Medicine for each participant at each of the sampling time-points (sessions 1 and 7). One sample will be centrifuged to obtain plasma which will be stored at −80°C until analyzed. Serum CRP, IL-6, TNF, and the other cytokines listed above will be assayed in duplicate with ELISA (CRP high-sensitivity kit, R & D Systems, Oxford, UK) or enhanced cytokine bead array flex kits (Becton Dickinson) using the manufacturer's reagents and standards. The other two samples will be used to isolate PBMCs and plasma and will be frozen until processed. Monocytes will be isolated from the PBMCs in order to assess mRNA levels similar to the method used by Padmos et al.[32]. This procedure utilizes monoclonal antibodies directed against human CD14 to isolate monocytes in peripheral blood monocyte cell suspensions. A magnetic cell sorting system will be used for the separation of the monocytes and flow cytometry will be used to gauge the purity of the population. Once purity is established, total RNA will be isolated from the monocytes using an RNeasy kits (Qiagen) according to the manufacturer's directions. RNA will then be reverse transcribed to cDNA using standard commercial kits. rtPCR reactions will be performed using the Dynamo Sybr Green HS Master Mix (New England Biolabs) and custom primers will be synthesized by a commercial laboratory. Real-time rtPCR reactions will be run using a Cepheid Smart Cycler II or similar instrument. Additional aliquots of

serum and plasma will be stored so that other inflammatory markers can be tested in the future using Luminex bead arrays and/or additional available technologies.

Source of Compounds Tested

Minocycline and aspirin are available on a generic basis, and are manufactured within the USA by several companies. The identity of the active medicines and placebos will be blinded using placebos that match the appearance of the active drugs. The medications and placebos have been formulated by Wedgewood Pharmacy, Swedesboro, NJ. The study minocycline capsule and chewable aspirin tablet are identical in appearance to their corresponding placebos.

Outcome Measures and Data Analysis

Antidepressant response will be evaluated by assessing changes in MADRS scores at assessment session # 7 (i.e. 6 weeks). Our *a priori* hypothesis is that minocycline and/or aspirin plus existing medication will exert greater antidepressant effects than placebo plus existing medication by study completion. Assuming that there are equal numbers of subjects in each treatment group, this hypothesis will be statistically assessed using a group (for the four treatment cells)-by-session (1 vs. 7) repeated measures analysis of variance (ANOVA). If the ANOVA statistic is significant, between- and within-group t tests will be used in planned comparisons to identify the nature of the effect leading to the significant overall ANOVA statistic. We expect to find a significant group-by-session interaction, attributable to a greater reduction in MADRS scores in the minocycline and aspirin groups compared to the placebo group between session 1 and session 7. If there is an imbalance in the number of subjects across groups, (e.g., due to differential dropout rates during the first treatment week), the data analysis will be conducted with a mixed-effects model.

A Mixed Effect Model Repeated Measure (MMRM)[80] will be used to impute missing data points as this method has been shown to be superior to last observation carried

forward (LOCF) which can inflate the Type I error rates[81]. The LOCF and observed cases (OC) approaches to data imputation will be used *post-hoc* to provide further confirmation of the results obtained under the MMRM analysis.

In order to test whether the putative antidepressant effects of minocycline or aspirin have a rapid onset, as a *post hoc* analysis the ANOVA will be repeated using MADRS ratings from the assessment that follows the first week of exposure to active drug versus the corresponding change under placebo; i.e. session 2. *Post hoc* tests will be performed to assess the significance of changes in the secondary clinical outcome measures (QUIDS 16, HAM-A, YMRS, CGI-I).

The rate of completion in the four cells also will be considered an outcome measure. The completion rate in the minocycline and/or aspirin arms may be influenced more by dropouts due to side effects while the completion rate in the placebo group may be influenced more by dropouts due to non-response. Two different measures of completion rate will be obtained: completion of week 1 of the study (baseline to week 1) and completion of the study (baseline to week 6). Differences between the groups in completion rates will be assessed with an ANOVA.

We will test the hypothesis that minocycline and aspirin reduce inflammation (e.g. CRP, IL-6, IL-6 mRNA) more than placebo using statistical analyses similar to those described above. If the assay results are normally distributed then a group-by-session repeated-measures ANOVA with CRP, IL-6 and nine other cytokine levels as dependent variables, and BMI, smoking status, and time of blood draw as covariates, will be used to assess anti-inflammatory effects of minocycline and aspirin. Mixed-effect models will be used if necessary. If the CRP or inflammatory cytokine data are not normally distributed (Kolmogorov-Smirnov test) or if the equality of statistical variance assumption across assessments is violated (Levene's test), then Friedman's ANOVA will be used to test for CRP or inflammatory cytokine differences between groups. If the Friedman's ANOVA statistic is significant, Wilcoxon sign-ranked tests will be used for post-hoc analysis of group differences. Nonspecific factors that influence CRP and inflammatory cytokine

levels include time of day, presence of infection, treatment with anti-inflammatory medications, smoking, obesity, and alcohol abuse. We will attempt to control for these potential confounds by measuring BMI and recording NSAID and nicotine use (Universal Fagerstrom scale), and by excluding individuals who have recently abused substances or who have intercurrent infections. The serum CRP concentration shows minimal diurnal variability in adults[82] but IL-6 and other cytokine levels vary across time of day[83]. To minimize cytokine measurement variability due to circadian fluctuations, we will schedule patient assessment sessions at the same time each day. Since this may not always be possible, we will record the time of day that each blood-draw is made, divide the day into quartiles: 7am-10am, 10am-12pm; 12pm-3pm, and 3pm-6pm, and use these data as a covariate in the statistical analyses.

To test whether baseline levels of CRP and inflammatory cytokines predict response to minocycline or aspirin, we will subclassify the participants using conventional criteria[84] as achieving full response (≥50% reduction in MADRS score from baseline), partial response (<50% but ≥25% reduction), or nonresponse (<25% reduction). Patients achieving remission (post-treatment MADRS score ≤10) will also be identified. A non-parametric alternative to the ANOVA statistic, the Mann-Whitney test, will be used to compare remitted and non-remitted groups in baseline levels of inflammatory cytokines and CRP if the data are not normally distributed. Ideally, the impact of baseline levels of inflammation on treatment response would be tested more rigorously using a formal stratified design. However, in order to conduct a stratified trial with for example, 8 experimental groups (4 x high versus low inflammation), the sample size of the study would have to be doubled, which would significantly increase costs and decrease feasibility. Nevertheless, this stratification approach would be important to consider for future studies if promising results are obtained in this clinical trial.

Statistical Power

A recent meta-analysis of 96 antidepressant treatment studies found that the average effect size of a placebo treatment is 1.69 compared with 2.50 for an antidepressant

treatment[85]. We calculated that in order to detect an effect size of 0.81 (i.e. the difference between 2.50 and 1.69) with an 80% probability (2-sided test, α=0.05), we will require a sample size of 26 subjects per group (http://hedwig.mgh.harvard.edu/sample_size/size.html). Thus given our sample size of 30 per group we should have sufficient power to test Specific Aim 1, allowing for a 13% drop-out rate during week 1 of the study (dropouts after completion of study week 1 will be included in the analysis under the MMRM approach described above).

As discussed above, a recent meta-analysis[78] of cross-sectional studies of serum-derived IL-6 and CRP in depression calculated effect sizes of 0.71 for IL-6 and 0.26 for CRP. Based on these effect sizes a sample size of 26 would yield >80% probability of detecting significant depression-related changes in IL-6, but only a 60% probability of detecting a depression-related change in CRP. There are 3 reasons why we believe that these CRP power estimations are not applicable to this study. Firstly, the effect sizes derived from the meta-analysis are based on cross-sectional studies. Given the effect of variables such as smoking, diet, exercise, and BMI on proinflammatory cytokines, a within-subjects design is likely to reduce non-depression-related sources of variance, and substantially increase statistical power. Secondly, we are not only examining the effect of mood on IL-6 and CRP levels, but are treating patients with minocycline and aspirin, drugs known to possess anti-inflammatory properties. We therefore suggest that our proposed study is likely adequately powered to detect any true changes in plasma IL-6, CRP, and the other inflammatory cytokines across treatment blocks.

Regarding IL-6 mRNA gene expression in peripheral blood monocytes, Padmos et al.[32] reported a 38-fold increase in IL-6 mRNA levels in unmedicated patients with BD compared with HC. Since minocycline reduces IL-6 levels (see above) we expect our study to have very high power to detect differences between groups, as well as changes in response to minocycline. The simultaneous detection of nine other inflammation-related cytokines, in addition to IL-6 (using newer more sensitive technology) will provide much finer resolution of the effects on inflammatory cascades than that measured in previous studies.

ETHICS AND DISSEMINATION

Gender/Minority/Pediatric Inclusion for Research

Women and Minorities will be included in the study without prejudice according to their representation in the study population. Participants will be recruited from the greater metropolitan areas of Tulsa, OK and Wichita, KS and efforts will be made to ensure that our subject population resembles the gender, ethnic and racial composition of these areas.

Exclusion Criteria

The following exclusion criteria apply: 1) inability to provide informed consent; 2) age of onset of BD>40 years; 3) serious risk of suicide; 4) current delusions or hallucinations sufficient to interfere with the capacity to provide informed consent; 5) current manic symptoms [depressed BD patients with concurrent manic symptoms have been found to be more likely to experience adverse reactions in antidepressant treatment trials[86]]; 6) medical illness including as hepatic impairment, renal dysfunction, bleeding diatheses (e.g., hemophilia), cerebrovascular disease or heart disease, hypertension that is inadequately controlled by medication, diabetes mellitus, or known peptic ulcer disease; 7) abuse of drugs or alcohol within the preceding 6 months, or substance dependence within the last 5 years; 8) daily alcoholic beverage consumption equivalent to $\geq$3 oz. of alcohol; 9) asthma or known allergies or hypersensitivities to tetracycline antibiotics, aspirin or other NSAIDs; 10) current use of drugs that could increase the risks associated with aspirin or minocycline administration, namely other antibiotic medications, other NSAIDs or anticoagulants (e.g., warfarin), acetazolamide, or methotrexate; 11) known HIV or other chronic infection including, but not limited to viral hepatitis. 12) Pregnant or nursing women, and women who are attempting to conceive during the 6 week study period, will also be excluded.

Specimens, Records, and Data Collection

A physician, registered nurse, or trained phlebotomist will utilize a sterile technique to draw 60 ml of blood by venipuncture. Participants will also be asked to submit a urine sample. A physician, registered nurse, or trained technician will collect EKG data from the subject in a private exam room.

Recruitment and Consent Procedure

Volunteers will be recruited from the community as well as from the clinical services at the Laureate Psychiatric Clinic and Hospital and the Oklahoma University School of Community Medicine in Tulsa, OK, and from the clinical services affiliated with the KUMCRI. Volunteers may be referred from sources that include physicians, newspaper advertising, self-help organizations, self-referral, and WIRB approved flyers posted at local universities, schools, churches and grocery stores. Participants may be pre-screened through screening protocols based at LIBR or KUCRI. We plan to recruit a total of 120 participants.

All participant interactions including consenting will be conducted in private interview / exam rooms. These rooms are secured from public areas via combination locked doors that are only accessible to authorized personnel. Prospective participants will receive an explanation of the objectives, procedures, and hazards of this protocol that is appropriate to their level of understanding. The right of the subject to decline to participate or to withdraw from the study at any time will be made clear.

Non-English speaking participants will not be recruited.

After the consent form is verbally explained to the participant, and any questions have been answered, the researcher will leave the room to allow the participant to read the consent form thoroughly. Family members will be allowed to be present and to discuss the consenting process with the participant. After the consent is read, the researcher will return and answer any additional questions the participant may have. The researcher will

remind the subject that participation is strictly voluntary and that they have the right to withdraw at any time. Participants will be asked to arrive 30 minutes early in order to have sufficient time for the consenting process.

Subject Risks

The risks of behavioral testing are minimal. The risks of blood drawing are also minimal. Possible mild side effects of the blood draw include mild pain or bruising at the site of the venipuncture.

Minocycline has been used a broad-spectrum antibiotic for many years in doses up to 400 mg/day[34]. It has been used on a chronic basis to treat acne and rheumatoid arthritis, often for many years, in hundreds of thousands of patients. The most commonly encountered side effects are upset stomach, diarrhea, dizziness, drowsiness, ataxia, vertigo, headache and vomiting. Prolonged use can be associated with pigmentation of the skin, gums or teeth. Between 1975 and 2006, the World Health Organization Collaborating Center for International Drug Monitoring listed 122 cases of adverse drug reactions to intravenous minocycline; most commonly, abnormal hepatic function and thrombocytopenia[34]. These included cases of serious liver injury, including irreversible drug-induced hepatitis and fulminant hepatic failure that was fatal in two cases, thought to be due to triggering or unmasking autoimmune hepatitis. One case of autoimmune-related glomerulonephritis has been reported. The role of oral minocycline in precipitating these conditions has not been clearly established. Minocycline also has been associated with idiopathic intracranial hypertension (pseudotumor cerebri). Long-term trials have shown that minocycline is well tolerated. In a 2-year trial of minocycline (200 mg/day) for RA, 3 of 30 patients withdrew due to finger-nail discoloration, dizziness, or erythematous rash[54]. Of 11 patients with HD treated with minocycline (100 mg/day) for 2 years, one complained of nausea in the first 3 weeks, and two of sedation[53], while in a 6-month trial of minocycline for ALS, the mean tolerated dose was 387 mg/day and the most common adverse effects were gastrointestinal[87]. Five of 36 patients with schizophrenia withdrew

from a 6-month trial of minocycline (200 mg/day) due to indigestion (n=2), pigmentation (n=2), or a suicide attempt (n=1)[58].

Low dose aspirin has been safely used in many millions of patients on a worldwide scale for its role as an anti-thrombotic and thrombolytic. A meta-analysis of >100 randomized trials in high-risk patients indicated that low-dose ASA reduced cardiovascular death by 15% and prevented nonfatal vascular events by about 30%[88]. These data stand in striking contrast to the data obtained in COX-2 inhibitors, which can increase cardiovascular risk. In clinical trials of several COX-2 selective and nonselective NSAIDs of up to three years duration have shown an increased risk of serious cardiovascular (CV) thrombotic events, myocardial infarction, and stroke, which have in many cases been fatal[89]. Patients with known CV disease or risk factors for CV disease are at greater risk for such events during chronic treatment with COX-2 inhibitors. Evidence from human pharmacology and genetics, genetically manipulated rodents, and other animal models and randomized trials indicates that this is consequent to suppression of COX-2-dependent cardioprotective prostaglandins, particularly prostacyclin[90].

Aspirin does not cause a generalized bleeding abnormality unless given to patients with an underlying hemostatic defect (e.g., hemophilia, uremia, or that induced by anticoagulant therapy). Aspirin-induced impairment of primary hemostasis cannot be separated from its antithrombotic effect and is similar at all doses $\geq$ 75 mg/d[91]. The risk of intracranial bleeding is exceedingly rare (<0.1% in high risk populations), but is higher in individuals with cerebrovascular disease[88]. Hypertension that is inadequately controlled by medication often is considered a contraindication to aspirin because of the concern that possible benefits in the prevention of cardiovascular events may be counterbalanced by an increased risk of cerebral bleeding. However, hypertensive patients whose blood pressure is well-controlled appear protected from myocardial infarction by aspirin therapy without an increase in the number of cerebral hemorrhages or strokes[92]. Moreover, aspirin therapy does not affect blood pressure or the response of hypertension to antihypertensive agents[91 93].

NSAIDs as a class can cause serious gastrointestinal (GI) adverse events including inflammation, bleeding, ulceration, and perforation of the stomach, small intestine, or large intestine, which rarely have proven fatal. In controlled clinical trials the percentage of patients reporting one or more gastrointestinal complaints has ranged from 4% to 16%[91]. The mechanism underlying this adverse effect appears attributable to the inhibition of COX-1. Thus, the incidence of GI side effects has been higher for NSAIDs with more potent effects at COX-1, such as aspirin and indomethicin. For example, in controlled trials the incidence of GI side effects for aspirin and indomethacin have been about twice as high as that for ibuprofen, a nonselective COX inhibitor, in equally effective doses for arthritis. Nevertheless, the incidence of GI side effects associated with aspirin is dose-dependent, and thus is markedly lower when using aspirin in the low dose range planned for the current study. Notably, the risk of GI bleeding is not reduced by using the enterically coated aspirin formulations, but is thought to be lower during concomitant use of omeprazole[91]. The effects of warfarin and NSAIDs on GI bleeding are synergistic, such that the users of both drugs together have a risk of serious GI bleeding higher than users of either drug alone. Fortunately, the risk of GI bleeding, which reflects the inhibition of prostaglandins in the stomach (from systemic rather than local exposure) is much smaller when using low-dose as opposed to high-dose aspirin.

Low-dose aspirin has not been reported to alter renal function, and does not reduce effectiveness of ACE inhibitors for HTN (in contrast to other NSAIDs)[93 94]. However, aspirin can inhibit the renal clearance of acetazolamide and methotrexate potentially leading to increased blood concentrations of and toxicity from these agents. Salicylate can displace other drugs which are protein-bound, especially phenytoin and valproic acid, increasing their free drug concentrations in plasma. This may increase side effects, toxicity and/or efficacy for displaced drugs. If the BD subjects are currently receiving valproic acid preparations (e.g., divalproex) then the plasma levels of these agents will be monitored for potential changes.

Aspirin may cause a severe allergic reaction that may include: hives, asthma (wheezing), facial swelling, shock. Aspirin overdose can be fatal at 30 g or higher.

In sum, we believe that our two-by-two design is appropriate for trials involving experimental drugs that already have been well-studied with respect to toxicity, as is the case with aspirin and minocycline. A parallel arm design, as opposed to a 2 x 2 factorial design, would be more clearly informative in the case of an experimental drug for which the toxicity and drug interaction potential have not been thoroughly studied in human subjects

PHI Protection

Paper copies of consents, screening forms, the Research Privacy Form, and any other forms, testing results or papers containing Protected Health Information (PHI) will be stored in a secured medical records room with access granted only to authorized personnel.

Electronic data that contain PHI will be managed in accordance with ISO 27000 series information security standards with policies developed from current NIST guidelines (SP 800-66) for HIPAA and HITECH compliance. Specific controls implemented to protect PHI are derived from NIST 800-122, and include (but not limited to):

1) Access Enforcement (AC-3) – Individual user accounts, role based access control, access control lists;
2) Separation of Duties (AC-5) – de-identification of data as appropriate, acquire/analyze/manage firewall;
3) Least Privilege (AC-6) – to ensure PHI data is only available to persons with established need for access;
4) Remote Access (AC-17) – Secure VPN, encrypted end devices;
5) Access Control for Mobile Devices (AC-19) – Password login, remote destruction capabilities;
6) Auditable Events (AU-2) + Monitoring: Log detailed server and network information, alert for problems;

7) Analysis, and Reporting (AU-6) – Procedures to audit system records for inappropriate activity.
8) User Identification and Authentication (IA-2) – username/secure password and two factor authentication will be required when appropriate.
9) Media Access, Marking, Storage, and Transport (MP-2,3,4,5) – Records will be asset tagged and marked to their PHI status, PHI data will be secured and managed by professional system administrators, and will be transported via encryption (VPN, USB, File);
10) Media Sanitization (MP-6) – Data will be destroyed by SFHS in accordance with their policies and procedures;
11) Transmission Confidentiality (SC-9) – Encryption will be used when needed for all avenues of data transmission (wireless, network, etc.).

To protect subject confidentiality, blood samples will be anonymized as follows:

1. Last name: All participants will be assigned the last name "LIBR."
2. First name: The first name will be a secure alpha cryptographic hash based on LIBR user ID. This technique is the gold standard in computer security for one-way correlation of data.

Benefits versus Risks

The participant may benefit from participation if either study drug produces an antidepressant effect. Participants will also receive a free clinical evaluation; more frequent treatment visits than are typical in practice, diligent follow-up in terms of symptoms and side effects, and physical and psychiatric monitoring during the study. The risks of delaying alternative treatments are minimal in relation to the potential long-term benefits to the subjects and the importance of knowledge that may reasonably result. The importance of the knowledge that will likely be gained from this study clearly exceeds the associated potential risks.

Alternative Treatment

It is possible that some patients may feel better with talk therapy. Participating in any type of talk therapy with their psychiatrist or psychologist does not require dropping out of this study. Subjects will be encouraged to contact the study investigators, particularly the physician in the study, with any questions they may have regarding alternatives to treatment through this research study. The study investigators will assist in referring the subject to another physician for treatment after their participation in the study has ended. Physical and psychological testing, blood draws, urine samples, and EKG data provide no known risks to persons other than those listed in the exclusion criteria whereas the combinatory power of these measures may provide information relevant to understanding the pathophysiology of bipolar disorder.

Data and Safety Monitoring Plan

This study involves more than minimal risk. The study progress will be overseen by a Data, Safety and Monitoring Board (DSMB). The DSMB is composed of three members who will meet in person or per telephone at least once every 6 months to review relevant study data including adverse events and dropout rates.

Any unanticipated adverse events will be reported immediately to the IRB of record and to the LIBR Human Protection Administrator. Any adverse events will be included in the annual IRB report.

Dissemination of Results

The study results will be presented at national and/or international biomedical scientific meetings and published in peer-reviewed journals.

REGISTRATION

In accordance with the recommendations of the International Committee of Medical Journal Editors[95], the proposed trial is registered in a public registry (www.clinicaltrials.gov Identifier: NCT01429272).

Figure Legend

Figure 1: Schematic of Study Design

Legend: Each session number (total of 7) is encircled, with the timing between sessions indicated in weeks with a 2 business day window on either side of visit target date to complete the visit. Session 1 is the baseline (green star) and session 7 is the study end (purple star). Peripheral blood will be sampled at baseline and study end to assay markers of inflammation. The study duration is 6 weeks.

Author Contributions

All authors made a significant contribution to the conception and design of the study protocol. The protocol was written by Drs. Savitz and W. Drevets and was critically reviewed by Drs. Preskorn, Teague, D. Drevets, and Yates. All authors gave approval for the publication.

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Competing Interests

None of the authors has a conflict of interest to declare.

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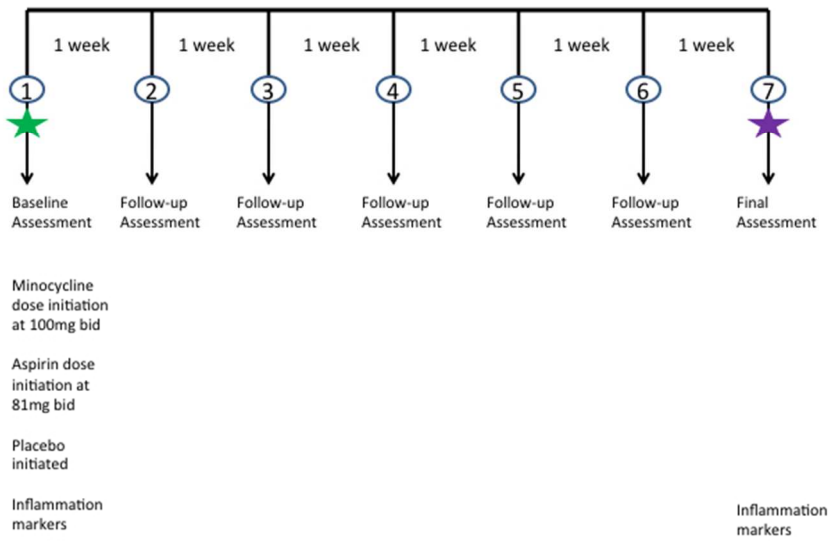

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PROTOCOL NUM: 2011-002-00
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TITLE:
MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION

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CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	3-13
	2b	Specific objectives or hypotheses	20
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	14-15
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	13-14
	4b	Settings and locations where the data were collected	17
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	15-16
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	20-21
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	21
	7b	When applicable, explanation of any interim analyses and stopping guidelines	30
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	15
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	15
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	15
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	15

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	20-21
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	20-21
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	
	13b	For each group, losses and exclusions after randomisation, together with reasons	
Recruitment	14a	Dates defining the periods of recruitment and follow-up	
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	
Other information			
Registration	23	Registration number and name of trial registry	1, 30
Protocol	24	Where the full trial protocol can be accessed, if available	19
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

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MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION: a protocol for a proof-of-concept randomized, double-blind, placebo-controlled, 2x2, clinical trial

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MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION: a protocol for a proof-of-concept randomized, double-blind, placebo-controlled, 2x2, clinical trial

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Key Words

Bipolar Depression, Clinical Trial, Minocycline, Aspirin, Inflammation

ABSTRACT

Introduction: New medication classes are needed to improve treatment effectiveness in the depressed phase of bipolar disorder (BD). Extant evidence suggests that BD is characterized by neural changes such as dendritic remodeling and glial and neuronal cell loss. These changes have been hypothesized to result from chronic inflammation. The principal aims of the proposed research is to evaluate the antidepressant efficacy in bipolar depression of minocycline, a drug with neuroprotective and immune-modulating properties, and of aspirin, at doses expected to selectively inhibit cyclooxygenase 1 (COX-1). **Methods and Analysis:** One hundred and twenty outpatients between 18 and 55 years of age, who meet DSM-IV-TR criteria for BD (type I or II) and for a current major depressive episode will be recruited to take part in a randomized, double-blind, placebo-controlled, parallel-group, proof-of-concept, clinical trial following a 2 x 2 design. As adjuncts to existing treatment, subjects will be randomized to receive one of four treatment combinations: placebo-minocycline plus placebo-aspirin, active-minocycline plus placebo-aspirin, placebo-minocycline plus active-aspirin, or active-minocycline plus active-aspirin. The dose of minocycline and aspirin is 100mg bid and 81mg bid, respectively. Antidepressant response will be evaluated by assessing changes in the Montgomery-Asberg Depression Rating Scale (MADRS) scores between baseline and the end of the 6 week trial. As secondary outcome measures, the anti-inflammatory effects of minocycline and aspirin will be tested by measuring pre-and-post treatment levels of CRP and inflammatory cytokines. **Ethics and Dissemination:** Minocycline has been widely used as an antibiotic in doses up to 400 mg/day. Low dose aspirin has been safely used on a worldwide scale for its role as an anti-thrombotic and thrombolytic. The study progress will be overseen by a Data, Safety and Monitoring Board which will meet once every 6 months. Results of the study will be published in peer-reviewed publications. **Registration:** Clinical Trials.gov: NCT01429272.

INTRODUCTION

The treatment of bipolar depression remains a major challenge for psychiatry. The US FDA has not approved any of the ~25 standard antidepressants for the treatment of bipolar depression, partly because these agents have not been robustly effective in BD patients[1]. Thus, currently approved treatments for bipolar depression include lithium, quetiapine, and the combination of olanzapine and fluoxetine[2]. Other treatments used include lamotrigine, conventional antidepressant agents, other atypical antipsychotics, pramipexole or riluzole (reviewed in [3]). Unfortunately, the effectiveness of these options also is limited. For example, in a placebo-controlled study in which subjects receiving lithium were randomized to receive either standard antidepressant pharmacotherapy (paroxetine or imipramine) or placebo, those receiving lithium plus an antidepressant did not show a significant improvement over those receiving lithium plus placebo[4]. Similarly, in the STEP-BD trial, 42 of 179 subjects (23.5%) receiving a mood stabilizer plus adjunctive antidepressant drug treatment had a durable recovery, which did not differ significantly from 51 of 187 subjects (27.3%) receiving mood stabilizer plus placebo. Mallinger et al. reported a similar durable recovery rate in BD depressives treated with mood stabilizer plus paroxetine (27%), but found a higher rate for adjunctive monoamine oxidase inhibitors (MAOIs; 53%)[5], consistent with the findings of previous studies comparing MAOIs vs imipramine[6 7] . Unfortunately MAOIs are commonly unacceptable to patients.

New classes of antidepressant drugs are needed for bipolar depression. Existing agents exert their primary actions on monoaminergic systems. The efficacy of these agents contributed to the monoamine-deficiency hypothesis of depression, which continues to receive empirical support. Nevertheless, the field is in the early stages of a paradigm shift driven by evidence of dendritic remodeling and neuronal atrophy in animal models of depression, and of reductions in gray matter (GM) volume, and glial cell loss at *postmortem* in BD[8]. The neurotrophic effects of lithium, coupled with longitudinal studies demonstrating volumetric changes over time, raise the possibility that mood disorders are underpinned by a neurotoxic process[8 9]. The final common pathway through

which neurotoxic agents exert their effect is hypothesized to involve excess glutamatergic signaling[10].

The glutamatergic model of mood disorders is based on the premise that excessive stimulation of NMDA-glutamatergic receptors, results in neuronal atrophy and apoptosis of glial and/or neuronal cells, and *ipso facto*, depression. Evidence for this hypothesis derives from multiple sources. In preclinical models, riluzole, which inhibits neuronal release of glutamate, ceftriaxone, which increases glutamate reuptake, and NMDA receptor antagonists such as ketamine, ameliorate behavioral analogs of depression[11]. In addition, rats bred to be genetically sensitive to stress show differential expression of NMDA receptors[12], and behavioral analogs of depression are abrogated in NMDA receptor subunit knockout mice[13]. In humans, increased serum levels of glutamate that resolve with antidepressant treatment were reported in MDD, and extended to the CSF post mortem[11]. Polymorphisms of the metabotropic glutamate receptor genes, GRM2 and GRM3, and a haplotype of the glutamic acid decarboxylase (GAD2) gene were associated with MDD[14]. Finally, ketamine induced a rapid, sustained antidepressant effect in BD[15 16] and riluzole showed promising results in treatment-resistant depression[15 16].

One potential cause of the disruption in glutamatergic signaling in BD is dysregulation of the immune system. Increased levels of proinflammatory cytokines such as interleukin 6 (IL-6), IL-1β, interferon alpha (IFNα), tumor necrosis factor alpha (TNF-α) prostaglandinE2 (PGE2), and chemokine ligand 2 (CCL2) are consistently observed in the blood and CSF of patients with mood disorders, both at baseline and after exposure to stressors[17 18]. Elevated serum levels of (pro-inflammatory) positive acute-phase proteins (e.g., haptoglobin, α1-antitrypsin, ceruloplasmin, C-reactive protein), but reduced levels of negative acute-phase proteins (e.g., albumin and retinal-binding protein) also are reported in mood disorders[19-21]. Further, treatment of hepatitis C with IFNα is known to induce the major depressive syndrome and/or manic symptoms in approximately 40% of patients, and the efficacy of conventional antidepressant drugs is associated with a reduction in inflammation[18]. Moreover, anti–tumor necrosis factor (TNF) therapy (for

psoriasis) can improve mood[22]. Since proinflammatory cytokines can alter brain function, these data are compatible with evidence that an activated inflammatory response system exists in mood disorders which plays a role in their pathophysiology[23-26].

The over-activity of the hypothalamic-pituitary-adrenal axis in mood disorders may play a role in inflammation, since hypersecretion of corticotrophin-releasing hormone (CRH) activates the transcription factor, nuclear factor kappa B (NF-κB). NF-κB regulates the expression of proinflammatory cytokines in immune cells in the CNS and periphery, and the expression of genes involved in apoptosis[27]. In addition, NF-κB may result in the expression of the class 1 major histocompatibility complex (MHC I), labeling cells for removal by cytotoxic T-cells[27]. Usually, cortisol suppresses this inflammatory response, but chronic stress appears to desensitize the glucocorticoid receptor (GR) and by extension, the anti-inflammatory effects of cortisol[27]. Cytokines play a role in desensitizing the system to cortisol. For example, IL1 and TNF-α retard dexamethasone-induced translocation of the GR receptor from the cytoplasm to the nucleus[28].

The immunologic and glutamatergic models of BD are complementary because a proinflammatory state is one potential cause of excitotoxicity[27]. Peripheral inflammatory signals activate microglia in the brain, inducing an inflammatory cascade of cytokines and free radicals. Cytokines and reactive oxygen and nitrogen species exert a direct toxic, apoptotic effect on oligodendrocytes. Potentially through the loss of oligodendrocytes, oxidative stress can lead to demyelination. Such a process conceivably may account for the reduction in oligodendroglia found *postmortem* in the prefrontal cortex[29] in mood disorders. The inflammatory milieu also compromises astrocyte function, leading to down-regulation of glutamate transporters and impaired glutamate reuptake into astrocytes, further amplifying inflammatory signaling[27].

In addition, cytokines such as interleukin 1 (IL-1), IL-6, and TNF-α activate indoleamine 2, 3-dioxygenase (IDO). IDO catalyzes the breakdown of tryptophan, the amino-acid precursor of serotonin, and an important regulator of T-cell function, into kynurenine (Kyn)[30]. Activation of the Kyn pathway shunts tryptophan away from 5-HT synthesis,

putatively reducing serotonergic transmission. Kyn is in turn metabolized into quinolinic acid (Quin), a potent NMDA receptor agonist, and neuromodulator involved in lipid peroxidation, which can induce neuronal damage via oxidative stress and overstimulation of NMDA receptors[30]. Consistent with inflammation-related shunt towards Kyn metabolism, the plasma tryptophan-Kyn ratio was found to correlate inversely with striatal total choline (a putative cell membrane turnover biomarker) in adolescents with melancholic depression[31].

The mRNA transcripts for proinflammatory genes appear particularly sensitive for discriminating BD patients. Microarray gene expression profiles in purified CD14+ monocytes from whole blood of BD subjects, offspring of BD parents, and healthy controls (HC) displayed a distinct mRNA signature representing genes from inflammatory and inflammation-related pathways[32]. The signature showed >80% sensitivity and specificity in BD subjects who were not receiving lithium or antipsychotic drugs (n=11), and in affected offspring of a BD parent (n=13, of whom 10 had only manifested depression). A positive signature also was present in 17 of 38 unaffected offspring (45%) versus 13 of 70 healthy children (19%). Cross-sectional comparisons suggested lithium and antipsychotic drugs—but not conventional antidepressant drugs--down-regulated expression of most inflammatory genes. Thus, when medicated and unmedicated subjects were considered together only 23 of 42 BD patients (55%) had a positive signature versus 7 of 38 HCs (18%). Notably, the IL6 mRNA level remained elevated in medicated BD subjects and did not differ significantly from unmedicated subjects (table 1), suggesting that this assay identifies a proinflammatory diathesis even in treated cases.

Table 1: Magnitude of difference in mRNA expression between mood disordered and healthy control (HC) samples from Padmos et al.[32], showing selected transcripts in unmedicated subjects vs HCs, relative to that of medicated BD subjects.

Gene Symbol	**Unmedicated BD vs HC**		**Medicated BD vs HC**		**Affected offspring# vs HC**	
	fold change	p-value	fold change	p-value	fold change	p-value
PDE4B	13.73*	<.001	3.42	<.001	5.79	<.001
IL6	37.92	.005	9.56	.006	935.7	<.001
CCL20	55.49	.006	6.02	.10	400.1	<.001

Legend: * - difference significant between unmedicated vs medicated BD samples; # - affected with respect to having manifested either a depressive or a manic episode
Sample sizes: unmedicated BD n=11, medicated BD n=31, affected offspring n=13, HCs n=25 for comparisons against BD adults, n=70 for comparisons of offspring. Abbrev: BD – bipolar disorder; HC – healthy control; PDE4B - phosphodiesterase type 4B; IL6 - interleukin 6; CCL20-chemokine ligand 20

Minocycline is a second-generation tetracycline that may prevent both glutamate-induced excitotoxicity and cytokine-induced inflammation in the CNS and periphery.

Minocycline has high lipophilicity enabling efficient transfer across the blood brain barrier (BBB)[33] - its concentration in CSF reaches 11–56% of plasma concentrations[34]. Minocycline inhibits the microglial-mediated release of proinflammatory cytokines IL-1β, TNF-α, IL-6, and p38[35], while promoting release of the anti-inflammatory cytokine,

IL-10[34]. Moreover, minocycline inhibits matrix metalloproteinases which process cytokines such as TNF-α and IL-1β into their biologically active forms[35]. Minocycline is also an effective scavenger of proapoptotic reactive oxygen species and protects against excitotoxicity by preventing glutamate-induced activation of nitric oxide synthase (NOS)[36]. Nitric oxide facilitates glutamate release from presynaptic neurons and inhibits glial glutamate transporters, amplifying glutamatergic signaling, and contributing to excitotoxic cell death[10]. Minocycline also upregulates a key molecular factor in the apoptosis pathway, B-cell CLL/lymphoma 2 (BCL-2)[37], an effect shared by lithium, valproate[38] and certain antidepressant drugs[39]. BCL-2 represses apoptosis induced by cytotoxic insults[40]. Conceivably, minocycline may additionally reduce inflammation indirectly by blocking the translocation of bacteria across the intestinal barrier. In mice exposed to a social stressor, bacteria translocated across the intestinal barrier stimulating the release of circulating cytokines such as IL6, and increasing microbicidal activity via inducible NOS[41]. Additionally, stress induced a change in the community structure of the microflora in the cecum with a decrease the relative abundance of bacteria in the genus Bacteroides and an increase the relative abundance of bacteria in the genus Clostridium. Notably, these effects were blocked by pretreatment with a broad spectrum antibiotic[41].

Minocycline has neuroprotective and anti-inflammatory properties.

Minocycline prevents glutamate-induced apoptosis of neurons *in vitro*[42], prevents ischemia-induced activation of microglia in gerbils[43], increases hippocampal neuron survival[44], reduces lesion-volume and improves neurological function in mice with traumatic brain injury[45] and in fragile X syndrome[46], reduces pro-inflammatory cytokine expression and improves neurological function and locomotor activity in rats with spinal cord injury[47], attenuates MDMA-induced neurotoxicity of serotonin and dopamine systems in the cerebral cortex and hippocampus of mice[48], reduces inflammation in a rat-model of rheumatoid arthritis (RA)[49], and delays disease progression and demyelination in rodent models of encephalitis[50], amyotrophic lateral sclerosis (ALS)[51] and Huntington's Disease (HD)[52]. Based on these data, minocycline was employed, and has

shown promise as, a therapeutic agent in human diseases including HD[53], rheumatoid arthritis (RA)[54], and stroke[55].

Minocycline has been used to treat psychiatric disorders.

Miyaoka et al.[56] discussed 2 patients with catatonic schizophrenia who benefited from minocycline. This group then conducted a 4-week trial with minocycline (150 mg/day) in 22 patients with schizophrenia to evaluate its efficacy as an adjunct to antipsychotic drugs[57]. Patients showed a significant improvement in positive and negative symptoms. Levkovitz et al.[58] recently studied 54 patients with early-stage schizophrenia treated for 6 months with antipsychotic medication and either minocycline (200 mg/day) or placebo in a double-blind trial. Minocycline was associated with a reduction in negative symptoms and improved attention/ memory.

The efficacy of minocycline has not been formally tested in mood disorders. In rodents, minocycline reduced immobility during the forced-swim test[59], and co-administration of minocycline synergized the antidepressant-like actions of desipramine (but not fluoxetine)[60]. Minocycline also abrogated the depression-like behavior of rodents exposed to lipopolysaccharide (LPS)[61]. Levine et al.[62] presented the case of a 66-year old woman with severe BD, who observed that the tetracycline she took for an infection alleviated her depression. When her depression returned post-treatment, minocycline was reinitiated (150 mg/day). After one week her HAM-D score fell from 25 to 8.

Aspirin (Acetyl-salicylic acid, ASA) also holds potential efficacy in bipolar disorder.

The second aim of this study is to assess the antidepressant efficacy of ASA in bipolar depression. Using a 2 x 2 design we will obtain data providing estimates of the effect size of ASA relative to placebo, ASA relative to minocycline, and ASA in combination with minocycline relative to placebo. These data also will explore the specificity of any effect found for minocycline. The clinical use of low dose ASA primarily has been driven by its role as an anti-thrombotic and thrombolytic. Given the exaggerated death rate from cardiovascular events in BD, this action potentially is advantageous in the management

of BD. Nevertheless, the recent literature also supports a role for low dose ASA in the management of the mood disorder itself, specifically in the amelioration of depressive symptoms.

The mechanism of ASA relates to its capacity to inactivate irreversibly the cyclooxygenase (COX) activity of prostaglandin (PG) H-synthase-1 and PGH-synthase 2 (referred to as COX-1 and COX-2, respectively). Although ASA has a short half-life (15 to 20 min) ASA's permanent inhibition of COX-1 allows once daily dosing for anucleate platelets. In contrast, because nucleated cells rapidly regenerate this enzyme a shorter dosing interval is required to persistently impact COX activity in cells that mediate inflammatory processes. Moreover, ASA is 50- to 100-fold more potent in inhibiting platelet COX-1 than monocyte COX-2 activity[63], so there is nearly a 100-fold variation in the daily dose of aspirin, as higher doses are used to target COX-2 in the management of treating peripheral inflammation (e.g., arthritis) or pain. As reviewed below, preliminary evidence obtained in BD suggests beneficial effects are achieved using ASA in low doses, where aspirin would inhibit COX-1, but not COX-2.

Aspirin has neuroprotective and anti-inflammatory properties.

In the brain, recent data indicate that genetic manipulation of COX-1 and COX-2 differentially modulate leukocyte recruitment during neuroinflammation, and suggest that reduction of COX-1 activity is neuroprotective, whereas reduction in COX-2 activity is detrimental, during a primary neuroinflammatory response (reviewed in [64]). Choi et al.[64] propose that these distinct roles reflect the predominant localization of COX-1 in microglia, which play a major role in mediating neuroinflammation, in contrast to the predominant localization of COX-2 in pyramidal neurons. For example, Choi et al.[65] examined the effects of COX-1 or COX-2 deficiency on intracerebroventricular lipopolysaccharide (LPS)-induced neuroinflammation by comparing COX-1 (-/-) and COX-2 (-/-) knockout mice to wild-type (WT) (+/+) control animals. After LPS, leukocyte infiltration and inflammatory response were attenuated in the COX-1 (-/-) mice but increased in the COX-2 (-/-) mice, compared with WT controls. In another study,

Choi et al.[66] examined the effect of COX□1 genetic deletion on the inflammatory response and neurodegeneration induced by β□amyloid, and found that in COX□1 (□/□) mice, the Aβ1□42□induced inflammatory response and associated neuronal damage were attenuated compared to WT mice. Compatible with these results, in pharmacoepidemiological studies investigating whether chronic NSAID use reduced the risk of developing Alzheimer's disease (AD), indomethacin, a preferential COX□1 inhibitor, showed beneficial effects, while COX□2 selective inhibitors, failed to show any beneficial effect in AD patients with mild to severe cognitive impairment. These data suggest the hypothesis that inhibition of COX□1 activity may be a valid therapeutic strategy to reduce the cerebral inflammatory response and neurodegeneration in neuropsychiatric diseases in which neuroinflammatory components play a role in pathophysiology.

Other researchers hypothesized that NSAIDs would be beneficial in BD more specifically because of their ability to down-regulate activity in the brain arachidonic acid (AA) cascade by via interfering with phospholipase A2 (PLA2) and/or COX function. In rodents Rapoport and colleagues[67-69] demonstrated that conventional mood stabilizers decrease the AA turnover in phospholipids and the expression of PLA2 and/or COX enzymes. The PLA2 and COX enzymes catalyze, respectively, release of AA from membrane phospholipid and AA conversion to eicosanoids such as prostaglandin E2 and thromboxane B2. The AA cascade is involved in neuroreceptor-initiated signaling and can be pathologically upregulated by neuroinflammation and excitotoxicity.

Nevertheless, aspirin has additional mechanisms that may underlie benefits in neuropsychiatric illness. While low-dose aspirin down-regulates AA cascade activity via inhibition of COX-1 activity, in higher doses it also down-regulates COX-2 gene transcription, increases levels of lipoxygenase-derived eicosanoids such as the anti-inflammatory lipoxin A4, and acetylates COX-2 protein to a modified enzyme that can convert unesterified AA to anti-inflammatory mediators such as 15-epi-lipoxin A4 (reviewed in [70]). The acylated enzyme also can convert docosahexaenoic acid (DHA) to 17-(R)-OH-DHA, which, like its metabolites di(R)-OH-DHA (neuroprotectin (R) D1)

and tri(R)-OH-DHA (resolvin (R) D1), is highly anti-inflammatory (reviewed in [70]). Lithium given chronically to rats with lipopolysaccharide-induced neuroinflammation also increases the brain concentration of 17-OH-DHA. Thus, there may be a synergy between aspirin and lithium in forming anti-inflammatory brain DHA metabolites.

Aspirin appears effective in preliminary studies of mood disorders.

Pharmaco-epidemiological data in BD supportive of these hypotheses were published by Stolk et al.[70]. Using the Netherlands based PHARMO Record Linkage System (which connects pharmacy dispensing records to hospital discharge records of > two million individuals since 1985), these researchers tested whether non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids would ameliorate bipolar symptoms. The target sample consisted of 5,145 patients receiving lithium (mean age = 48.6 $\pm$ 15 yrs; mean duration of lithium use=847 days), based upon the assumption that lithium treatment is relatively specific to individuals with BD. The main outcome measure was a calculated incidence density (ID) of medication events (change in the type or numbers of psychotropic medications prescribed, or increase [>30%] in the psychotropic drug dose). Subjects receiving low-dose ($\leq$80 mg/day) aspirin were 17% less likely to have a medication event, a finding that remained significant after adjusting for age, sex, chronic disease score and health care utilization. This effect was selective for low-dose ASA. In contrast, high-dose aspirin or non-selective NSAIDs (i.e., regimens expected to inhibit both COX-1 and -2), selective COX-2 inhibitors and glucocorticoids did not produce a statistically significant protection. Instead, the co-administration of non-selective NSAIDS and glucocorticoids was associated with statistically significant increases in medication events, suggesting destabilization of bipolar illness. The finding that low-dose aspirin decreased the number of medication events was particularly noteworthy since aspirin does not significantly augment serum lithium levels in contrast to selective COX-2 inhibitors which can raise lithium levels[71]. These preliminary observations thus appeared consistent with the hypothesis that COX-1 inhibitors can reduce neuroinflammatory processes and thus benefit BD patients.

Notably, the observation that beneficial effects in BD were conferred by low-dose ASA, but not by nonselective COX inhibitors, COX-2 inhibitors or glucocorticoids, appeared inconsistent with the hypothesis that drugs that down-regulate AA cascade activity in general hold therapeutic potential in BD. Thus the putative neuroprotective effects associated with COX-1 inhibition may contribute specifically to the benefits of low-dose aspirin in BD observed by Stolk et al. For example, as reviewed above, aspirin and lithium may exert synergistic effects in forming anti-inflammatory brain DHA metabolites (reviewed in [70]).

Other data suggest that aspirin exerts antidepressant effects within the context of MDD or cardiovascular illness. Mendlewicz et al.[72] examined the effect of aspirin augmentation of conventional antidepressant pharmacotherapy in 24 patients with MDD who had proven non-responsive after 4 weeks of SSRI treatment. Participants were treated openly during the subsequent 4 weeks with aspirin 160 mg/day in addition to their SSRI regimen. The combined administration of SSRI plus aspirin was associated with a response rate of 52.4%. Remission was achieved in 43% of the total sample and 82% of the responder sample. In the responder group, a significant improvement was observed within week 1 and this benefit persisted through day 28. In another study Ketterer et al.[73] reported that in 174 males undergoing coronary angiography (of whom 99 were taking low-dose aspirin), aspirin use was associated with less depression and anxiety symptoms.

In contrast, a preliminary study of the selective COX-2 inhibitor, celecoxib, was negative in bipolar depression[74], potentially compatible with the negative results of COX-2 inhibitors reported by Stolk et al.[70]. In a double-blind, randomized, add-on clinical trial of celecoxib in patients (n = 28) studied during a depressed or mixed episode of BD, no significant difference was observed between the celecoxib and placebo add-on groups at study endpoint[74]. These results contrasted with those obtained using celecoxib in unipolar depression, however. In MDD, celecoxib augmentation of either reboxetine[75] or fluoxetine[76] was associated with a significant therapeutic effect on depressive symptoms in randomized, double-blind, add-on clinical trials.

METHODS AND ANALYSIS

Participants

One hundred and twenty male or female outpatients between 18 and 55 years of age, who meet DSM-IV-TR criteria for BD (type I or II) and for a current major depressive episode will be recruited. The depressive syndrome must have been present for at least 4 weeks and the minimum threshold for depression severity will be set at a 17-item HAM-D score ≥18. Subjects will provide written informed consent as approved by the Western Institutional Review Board.

Concurrent Medications

At study entry type I BD subjects must have been taking a stable dose of a mood-stabilizing medication (lithium, valproate, carbamazepine, lamotrigine, antipsychotic agents), for at least 4 weeks, dosed clinically to target the therapeutic range. Type II BD subjects will be included irrespective of whether they present on a mood stabilizer. To investigate the utility of this augmentation strategy in the population for whom minocycline is most likely to prove therapeutically relevant, volunteers receiving stable doses of mood stabilizing, antipsychotic, antidepressant, and/or anxiolytic drugs for at least 4 weeks will be included. However, volunteers who currently are receiving more than 4 psychotropic medications in a daily regimen will be excluded, since this condition may signify a more brittle or complex clinical state. Subjects may remain in psychotherapy or have no psychosocial intervention. Volunteers will be excluded if they currently are receiving medications likely to have adverse interactions with minocycline or aspirin, including NSAIDS, warfarin, digoxin, penicillins, and isotretinoin products.

For participants who enter the study, the preferred strategy will be for subjects to maintain the same regimen of concurrent medications throughout the six week study so that only the study drug regimen will be altered per protocol. Nevertheless, changes to concurrent medications will not affect study status, so long as the medication change does

not target a depressive or manic symptom. If changes to concurrent medication regimens are clinically required to address worsening depressive symptoms or the development of manic symptoms, then the subject will be dropped from the study.

Study Design

Patients will participate in a randomized, double-blind, placebo-controlled, trial with a 2 x 2 design. As adjuncts to existing treatment, subjects will receive placebo-minocycline plus placebo-aspirin, active-minocycline plus placebo-aspirin, placebo-minocycline plus active-aspirin, or active-minocycline plus active-aspirin. The randomization sequences will be determined by a research staff-member who is not obtaining clinical information from the research subject and will be assigned by subject number at consenting. A restricted randomization (permuted block randomization) method will be used in which subjects are randomly allocated to each block (n=30) to ensure that equal numbers of participants receive each drug/placebo combination. In order to ensure that experimental group assignment is not skewed across the two trial sites, the study progress will be monitored by individuals who are not involved in the data collection, and in the case of "drift", adjustments will be made as necessary.

The trial will be conducted over 6 weeks and will comprise 7 assessment sessions (figure 1). The subject will be seen at the prescribed time intervals within a window of two business days on either side of visit target date to complete the specified visits.

At each session, a clinical assessment will be conducted using the rating scales listed below, and treatment side-effects will be assessed and rated for severity. To preserve the rater blind, the research staff member who conducts the clinical ratings will not be the research staff member who assesses the presence of side effects, and will remain blind to the information pertaining to side effects. Subjects who experience severe adverse effects or who develop treatment-associated hypomania or mania will be dropped from the study, instructed to discontinue the study medication, and referred for appropriate clinical management of these adverse events.

The primary outcome measure will be the change in the Montgomery-Asberg Depression Rating Scale (MADRS) scores at the seventh assessment session (week 6).

Medication

This pilot proof-of-concept study will adhere to the dosing limits and route of administration for the FDA indications for minocycline's and aspirin's use in other conditions (thus an IND is not required). A fixed dose design will be followed, and all medications will be administered via the p.o. route. The pilot data extant for both study drugs supports an onset of improvement within two weeks, so the six week study duration is expected to provide sufficient time to detect an antidepressant effect, to provide information about the persistence of the antidepressant effect over about one month from the anticipated onset of effect, and to minimize dropouts.

For minocycline the starting dose will be 100 mg b.i.d. (total daily dose=200 mg). This dose of 100 mg b.i.d. has been shown by a substantial literature to produce consistent anti-inflammatory effects in rheumatoid arthritis and other inflammatory disorders. This also is the dose used in a recent schizophrenia treatment trial[58]. The associated placebo capsules match the appearance of the 100 mg minocycline capsule.

The starting dose of aspirin will be 81 mg p.o. b.i.d. This dose is sufficient to inhibit COX-1, and appeared beneficial in stabilizing the course of BD in the pharmaco-epidemiological study of Stolk et al.[70]. When aspirin is used as an anti-platelet drug once daily dosing is sufficient since anucleate platelets do not produce enough COX-1 to overcome the irreversible inhibition of COX-1 within a 24-hour period. In contrast, in nucleated cells COX-1 is replenished, so more frequent dosing is required to persistently inhibit COX-1. Thus we will administer the dose in a b.i.d. regimen, according to the guidelines described above. A total daily dose of 160 mg was administered in the preliminary study which reported that aspirin significantly augmented the antidepressant

effects of fluoxetine in MDD[72]. The relevant placebo matches the appearance of the aspirin tablet.

Participants will be advised that one of the study drugs may reduce the efficacy of oral contraceptives, and to avoid taking the study drugs within 3 hours of iron products or of antacids containing calcium, magnesium or aluminum. They also will be advised that one study drug can increase their risk for bleeding during surgical procedure or if combined with other drugs or herbal preparations that reduce hemostasis.

Compensation

Participants will be compensated for participation in the amount of $300.00.

Treatment Compliance

To enhance compliance, study participants will be given an information sheet to take home detailing the procedure to be followed in the case of a missed dose, and requesting that this information be recorded for the investigators. The number of capsules and tablets remaining in each supply given to the patients will also be counted to evaluate treatment compliance. In cases where treatment compliance is poor, subjects will be excluded from the data analysis, using conventional criteria for defining adequate compliance in a clinical trial.

[Fig. 1, here]

Psychiatric Assessment and Clinical Ratings

Patients will be evaluated and followed in the outpatient clinics at LIBR or Oklahoma University School of Community Medicine in Tulsa, OK, or at the University of Kansas Medical Center Research Institute (KUMCRI) in Wichita, KS. The diagnosis of BD will be established using DSM-IV-TR criteria on the basis of an unstructured interview

conducted by a psychiatrist and the MINI-Plus administered by trained psychiatric interviewers. The following rating scales will be administered: MADRS, Quick Inventory of Depressive Symptomatology (QUIDS; 16 item), Hamilton Anxiety Rating Scale (HAM-A), Young Mania Rating Scale (YMRS), Universal Fagerstrom (to assess nicotine use), Hollingshead socioeconomic scale, Sheehan Disability Scale (SDS) and the Family Interview for Genetic Studies (FIGS). Medical assessment will include a physical examination, electrocardiogram, complete blood count (CBC), electrolytes and liver-function assays (SMA 20), thyroid panel, and urinalysis, serum drug and pregnancy tests at study entry and study completion. At each follow-up session, the MADRS, HAM-A, YMRS, and Clinical Global Impressions (CGI) scale will be repeated. Physical and psychiatric symptoms will be evaluated and recorded in order to measure the side-effect profiles of minocycline and aspirin. Participants will be questioned about adverse reactions, including dizziness, photosensitivity, hyperpigmentation, gastrointestinal distress or bleeding at each assessment and will be withdrawn from the study if medically necessary. Vital signs will be measured at entry and at each session.

Immune System Measures

The activity of peripheral cytokines correlates with inflammatory processes in the CNS. Peripheral cytokines cross the BBB, and can propagate signals across the BBB in the form of small, freely diffusible lipophilic molecules such as prostaglandins, which induce the production of cytokines from glia[77]. The measurement of peripheral markers of inflammation thus serves as a valid, if indirect assessment of CNS inflammation.

To explore predictors and correlates of treatment outcome, blood will be sampled for testing plasma and whole blood peripheral blood monocyte (PBM) based markers of inflammation at baseline and study end. These markers will include 10 cytokine proteins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFNγ, and TNF, high sensitivity (hs) CRP, and RNA expression of candidate genes from PBMCs. Candidate genes include IL-6, TNF, and IRF5 (a factor that mediates monocyte polarization). The 10 inflammation-related cytokines and the PBMC mRNA will be assayed from plasma at

baseline and study end. We selected the markers IL-6, TNF and CRP because they are the most widely implicated in mood disorders. The other cytokines included in the cytokine bead array assays are measured simultaneously with IL-6 and have all been implicated in the general regulation of inflammation. A meta-analysis of >100 studies found that IL-6 and CRP each were significantly elevated in depressed patients with standardized mean difference scores (d) of 0.71 and 0.26, respectively[78]. The associations remained significant after adjustment for body-mass index (BMI) and smoking. Moreover, IL-6 has been shown to modulate HPA axis function by inducing CRH release, adrenocorticotrophic hormone synthesis, and corticosteroid production[79]. CRP production is induced by the proinflammatory cytokines, IL-1, IL-6, and IL-17, and is thus a non-specific marker of systemic inflammation.

Three blood samples will be transported to the immunology lab in the Department of Surgery at the University of Oklahoma College of Medicine for each participant at each of the sampling time-points (sessions 1 and 7). One sample will be centrifuged to obtain plasma which will be stored at −80°C until analyzed. Serum CRP, IL-6, TNF, and the other cytokines listed above will be assayed in duplicate with ELISA (CRP high-sensitivity kit, R & D Systems, Oxford, UK) or enhanced cytokine bead array flex kits (Becton Dickinson) using the manufacturer's reagents and standards. The other two samples will be used to isolate PBMCs and plasma and will be frozen until processed. Monocytes will be isolated from the PBMCs in order to assess mRNA levels similar to the method used by Padmos et al.[32]. This procedure utilizes monoclonal antibodies directed against human CD14 to isolate monocytes in peripheral blood monocyte cell suspensions. A magnetic cell sorting system will be used for the separation of the monocytes and flow cytometry will be used to gauge the purity of the population. Once purity is established, total RNA will be isolated from the monocytes using an RNeasy kits (Qiagen) according to the manufacturer's directions. RNA will then be reverse transcribed to cDNA using standard commercial kits. rtPCR reactions will be performed using the Dynamo Sybr Green HS Master Mix (New England Biolabs) and custom primers will be synthesized by a commercial laboratory. Real-time rtPCR reactions will be run using a Cepheid Smart Cycler II or similar instrument. Additional aliquots of

serum and plasma will be stored so that other inflammatory markers can be tested in the future using Luminex bead arrays and/or additional available technologies.

Source of Compounds Tested

Minocycline and aspirin are available on a generic basis, and are manufactured within the USA by several companies. The identity of the active medicines and placebos will be blinded using placebos that match the appearance of the active drugs. The medications and placebos have been formulated by Wedgewood Pharmacy, Swedesboro, NJ. The study minocycline capsule and chewable aspirin tablet are identical in appearance to their corresponding placebos.

Outcome Measures and Data Analysis

Antidepressant response will be evaluated by assessing changes in MADRS scores at assessment session # 7 (i.e. 6 weeks). Our *a priori* hypothesis is that minocycline and/or aspirin plus existing medication will exert greater antidepressant effects than placebo plus existing medication by study completion. Assuming that there are equal numbers of subjects in each treatment group, this hypothesis will be statistically assessed using a group (for the four treatment cells)-by-session (1 vs. 7) repeated measures analysis of variance (ANOVA). If the ANOVA statistic is significant, between- and within-group t tests will be used in planned comparisons to identify the nature of the effect leading to the significant overall ANOVA statistic. We expect to find a significant group-by-session interaction, attributable to a greater reduction in MADRS scores in the minocycline and aspirin groups compared to the placebo group between session 1 and session 7. If there is an imbalance in the number of subjects across groups, (e.g., due to differential dropout rates during the first treatment week), the data analysis will be conducted with a mixed-effects model.

A Mixed Effect Model Repeated Measure (MMRM)[80] will be used to ~~impute~~ derive missing data points as this method has been shown to be superior to last observation

carried forward (LOCF) which can inflate the Type I error rates[81]. The LOCF and observed cases (OC) approaches to data imputation will be used *post-hoc* to provide further confirmation of the results obtained under the MMRM analysis.

In order to test whether the putative antidepressant effects of minocycline or aspirin have a rapid onset, as a *post hoc* analysis the ANOVA will be repeated using MADRS ratings from the assessment that follows the first week of exposure to active drug versus the corresponding change under placebo; i.e. session 2. *Post hoc* tests will be performed to assess the significance of changes in the secondary clinical outcome measures (QUIDS 16, HAM-A, YMRS, CGI-I).

The rate of completion in the four cells also will be considered an outcome measure. The completion rate in the minocycline and/or aspirin arms may be influenced more by dropouts due to side effects while the completion rate in the placebo group may be influenced more by dropouts due to non-response. Two different measures of completion rate will be obtained: completion of week 1 of the study (baseline to week 1) and completion of the study (baseline to week 6). Differences between the groups in completion rates will be assessed with a chi-squared test or a logistic regression. ~~a ANOVA~~.

We will test the hypothesis that minocycline and aspirin reduce inflammation (e.g. CRP, IL-6, IL-6 mRNA) more than placebo using statistical analyses similar to those described above. If the assay results are normally distributed then a group-by-session repeated-measures ANOVA with CRP, IL-6 and nine other cytokine levels as dependent variables, and BMI, smoking status, and time of blood draw as covariates, will be used to assess anti-inflammatory effects of minocycline and aspirin. Mixed-effect models will be used if necessary. If the CRP or inflammatory cytokine data are not normally distributed (Kolmogorov-Smirnov test) or if the equality of statistical variance assumption across assessments is violated (Levene's test), then Friedman's ANOVA will be used to test for CRP or inflammatory cytokine differences between groups. If the Friedman's ANOVA statistic is significant, Wilcoxon sign-ranked tests will be used for post-hoc analysis of

group differences. Nonspecific factors that influence CRP and inflammatory cytokine levels include time of day, presence of infection, treatment with anti-inflammatory medications, smoking, obesity, and alcohol abuse. We will attempt to control for these potential confounds by measuring BMI and recording NSAID and nicotine use (Universal Fagerstrom scale), and by excluding individuals who have recently abused substances or who have intercurrent infections. The serum CRP concentration shows minimal diurnal variability in adults[82] but IL-6 and other cytokine levels vary across time of day[83]. To minimize cytokine measurement variability due to circadian fluctuations, we will schedule patient assessment sessions at the same time each day. Since this may not always be possible, we will record the time of day that each blood-draw is made, divide the day into quartiles: 7am-10am, 10am-12pm; 12pm-3pm, and 3pm-6pm, and use these data as a covariate in the statistical analyses.

To test whether baseline levels of CRP and inflammatory cytokines predict response to minocycline or aspirin, we will subclassify the participants using conventional criteria[84] as achieving full response (≥50% reduction in MADRS score from baseline), partial response (<50% but ≥25% reduction), or nonresponse (<25% reduction). Patients achieving remission (post-treatment MADRS score ≤10) will also be identified. A non-parametric alternative to the ANOVA statistic, the Mann-Whitney test, will be used to compare remitted and non-remitted groups in baseline levels of inflammatory cytokines and CRP if the data are not normally distributed. Ideally, the impact of baseline levels of inflammation on treatment response would be tested more rigorously using a formal stratified design. However, in order to conduct a stratified trial with for example, 8 experimental groups (4 x high versus low inflammation), the sample size of the study would have to be doubled, which would significantly increase costs and decrease feasibility. Nevertheless, this stratification approach would be important to consider for future studies if promising results are obtained in this clinical trial.

Statistical Power

A recent meta-analysis of 96 antidepressant treatment studies found that the average effect size of a placebo treatment is 1.69 compared with 2.50 for an antidepressant treatment[85]. We calculated that in order to detect an effect size of 0.81 (i.e. the difference between 2.50 and 1.69) with an 80% probability (2-sided test, α=0.05), we will require a sample size of 26 subjects per group (http://hedwig.mgh.harvard.edu/sample_size/size.html). This effect size may correspond to approximately 3 points on the MADRS. Thus given our sample size of 30 per group we should have sufficient power to test Specific Aim 1, allowing for a 13% drop-out rate during week 1 of the study (dropouts after completion of study week 1 will be included in the analysis under the MMRM approach described above).

As discussed above, a recent meta-analysis[78] of cross-sectional studies of serum-derived IL-6 and CRP in depression calculated effect sizes of 0.71 for IL-6 and 0.26 for CRP. Based on these effect sizes a sample size of 26 would yield >80% probability of detecting significant depression-related changes in IL-6, but only a 60% probability of detecting a depression-related change in CRP. There are 3 reasons why we believe that these CRP power estimations are not applicable to this study. Firstly, the effect sizes derived from the meta-analysis are based on cross-sectional studies. Given the effect of variables such as smoking, diet, exercise, and BMI on proinflammatory cytokines, a within-subjects design is likely to reduce non-depression-related sources of variance, and substantially increase statistical power. Secondly, we are not only examining the effect of mood on IL-6 and CRP levels, but are treating patients with minocycline and aspirin, drugs known to possess anti-inflammatory properties. We therefore suggest that our proposed study is likely adequately powered to detect any true changes in plasma IL-6, CRP, and the other inflammatory cytokines across treatment blocks.

Regarding IL-6 mRNA gene expression in peripheral blood monocytes, Padmos et al.[32] reported a 38-fold increase in IL-6 mRNA levels in unmedicated patients with BD compared with HC. Since minocycline reduces IL-6 levels (see above) we expect our study to have very high power to detect differences between groups, as well as changes in response to minocycline. The simultaneous detection of nine other inflammation-related

cytokines, in addition to IL-6 (using newer more sensitive technology) will provide much finer resolution of the effects on inflammatory cascades than that measured in previous studies.

ETHICS AND DISSEMINATION

Gender/Minority/Pediatric Inclusion for Research

Women and Minorities will be included in the study without prejudice according to their representation in the study population. Participants will be recruited from the greater metropolitan areas of Tulsa, OK and Wichita, KS and efforts will be made to ensure that our subject population resembles the gender, ethnic and racial composition of these areas.

Exclusion Criteria

The following exclusion criteria apply: 1) inability to provide informed consent; 2) age of onset of BD>40 years; 3) serious risk of suicide; 4) current delusions or hallucinations sufficient to interfere with the capacity to provide informed consent; 5) current manic symptoms [depressed BD patients with concurrent manic symptoms have been found to be more likely to experience adverse reactions in antidepressant treatment trials[86]]; 6) medical illness including as hepatic impairment, renal dysfunction, bleeding diatheses (e.g., hemophilia), cerebrovascular disease or heart disease, hypertension that is inadequately controlled by medication, diabetes mellitus, or known peptic ulcer disease; 7) abuse of drugs or alcohol within the preceding 6 months, or substance dependence within the last 5 years; 8) daily alcoholic beverage consumption equivalent to ≥3 oz. of alcohol; 9) asthma or known allergies or hypersensitivities to tetracycline antibiotics, aspirin or other NSAIDs; 10) current use of drugs that could increase the risks associated with aspirin or minocycline administration, namely other antibiotic medications, other NSAIDs or anticoagulants (e.g., warfarin), acetazolamide, or methotrexate; 11) known HIV or other chronic infection including, but not limited to viral hepatitis. 12) Pregnant

or nursing women, and women who are attempting to conceive during the 6 week study period, will also be excluded.

Specimens, Records, and Data Collection

A physician, registered nurse, or trained phlebotomist will utilize a sterile technique to draw 60 ml of blood by venipuncture. Participants will also be asked to submit a urine sample. A physician, registered nurse, or trained technician will collect EKG data from the subject in a private exam room.

Recruitment and Consent Procedure

Volunteers will be recruited from the community as well as from the clinical services at the Laureate Psychiatric Clinic and Hospital and the Oklahoma University School of Community Medicine in Tulsa, OK, and from the clinical services affiliated with the KUMCRI. Volunteers may be referred from sources that include physicians, newspaper advertising, self-help organizations, self-referral, and WIRB approved flyers posted at local universities, schools, churches and grocery stores. Participants may be pre-screened through screening protocols based at LIBR or KUCRI. We plan to recruit a total of 120 participants.

All participant interactions including consenting will be conducted in private interview / exam rooms. These rooms are secured from public areas via combination locked doors that are only accessible to authorized personnel. Prospective participants will receive an explanation of the objectives, procedures, and hazards of this protocol that is appropriate to their level of understanding. The right of the subject to decline to participate or to withdraw from the study at any time will be made clear.

Non-English speaking participants will not be recruited.

After the consent form is verbally explained to the participant, and any questions have been answered, the researcher will leave the room to allow the participant to read the consent form thoroughly. Family members will be allowed to be present and to discuss the consenting process with the participant. After the consent is read, the researcher will return and answer any additional questions the participant may have. The researcher will remind the subject that participation is strictly voluntary and that they have the right to withdraw at any time. Participants will be asked to arrive 30 minutes early in order to have sufficient time for the consenting process.

Subject Risks

The risks of behavioral testing are minimal. The risks of blood drawing are also minimal. Possible mild side effects of the blood draw include mild pain or bruising at the site of the venipuncture.

Minocycline has been used a broad-spectrum antibiotic for many years in doses up to 400 mg/day[34]. It has been used on a chronic basis to treat acne and rheumatoid arthritis, often for many years, in hundreds of thousands of patients. The most commonly encountered side effects are upset stomach, diarrhea, dizziness, drowsiness, ataxia, vertigo, headache and vomiting. Prolonged use can be associated with pigmentation of the skin, gums or teeth. Between 1975 and 2006, the World Health Organization Collaborating Center for International Drug Monitoring listed 122 cases of adverse drug reactions to intravenous minocycline; most commonly, abnormal hepatic function and thrombocytopenia[34]. These included cases of serious liver injury, including irreversible drug-induced hepatitis and fulminant hepatic failure that was fatal in two cases, thought to be due to triggering or unmasking autoimmune hepatitis. One case of autoimmune-related glomerulonephritis has been reported. The role of oral minocycline in precipitating these conditions has not been clearly established. Minocycline also has been associated with idiopathic intracranial hypertension (pseudotumor cerebri). Long-term trials have shown that minocycline is well tolerated. In a 2-year trial of minocycline (200 mg/day) for RA, 3 of 30 patients withdrew due to finger-nail discoloration, dizziness, or erythematous rash[54].

Of 11 patients with HD treated with minocycline (100 mg/day) for 2 years, one complained of nausea in the first 3 weeks, and two of sedation[53], while in a 6-month trial of minocycline for ALS, the mean tolerated dose was 387 mg/day and the most common adverse effects were gastrointestinal[87]. Five of 36 patients with schizophrenia withdrew from a 6-month trial of minocycline (200 mg/day) due to indigestion (n=2), pigmentation (n=2), or a suicide attempt (n=1)[58].

Low dose aspirin has been safely used in many millions of patients on a worldwide scale for its role as an anti-thrombotic and thrombolytic. A meta-analysis of >100 randomized trials in high-risk patients indicated that low-dose ASA reduced cardiovascular death by 15% and prevented nonfatal vascular events by about 30%[88]. These data stand in striking contrast to the data obtained in COX-2 inhibitors, which can increase cardiovascular risk. In clinical trials of several COX-2 selective and nonselective NSAIDs of up to three years duration have shown an increased risk of serious cardiovascular (CV) thrombotic events, myocardial infarction, and stroke, which have in many cases been fatal[89]. Patients with known CV disease or risk factors for CV disease are at greater risk for such events during chronic treatment with COX-2 inhibitors. Evidence from human pharmacology and genetics, genetically manipulated rodents, and other animal models and randomized trials indicates that this is consequent to suppression of COX-2-dependent cardioprotective prostaglandins, particularly prostacyclin[90].

Aspirin does not cause a generalized bleeding abnormality unless given to patients with an underlying hemostatic defect (e.g., hemophilia, uremia, or that induced by anticoagulant therapy). Aspirin-induced impairment of primary hemostasis cannot be separated from its antithrombotic effect and is similar at all doses $\geq$ 75 mg/d[91]. The risk of intracranial bleeding is exceedingly rare (<0.1% in high risk populations), but is higher in individuals with cerebrovascular disease[88]. Hypertension that is inadequately controlled by medication often is considered a contraindication to aspirin because of the concern that possible benefits in the prevention of cardiovascular events may be counterbalanced by an increased risk of cerebral bleeding. However, hypertensive patients whose blood pressure is well-controlled appear protected from myocardial

infarction by aspirin therapy without an increase in the number of cerebral hemorrhages or strokes[92]. Moreover, aspirin therapy does not affect blood pressure or the response of hypertension to antihypertensive agents[91 93].

NSAIDs as a class can cause serious gastrointestinal (GI) adverse events including inflammation, bleeding, ulceration, and perforation of the stomach, small intestine, or large intestine, which rarely have proven fatal. In controlled clinical trials the percentage of patients reporting one or more gastrointestinal complaints has ranged from 4% to 16%[91]. The mechanism underlying this adverse effect appears attributable to the inhibition of COX-1. Thus, the incidence of GI side effects has been higher for NSAIDs with more potent effects at COX-1, such as aspirin and indomethicin. For example, in controlled trials the incidence of GI side effects for aspirin and indomethacin have been about twice as high as that for ibuprofen, a nonselective COX inhibitor, in equally effective doses for arthritis. Nevertheless, the incidence of GI side effects associated with aspirin is dose-dependent, and thus is markedly lower when using aspirin in the low dose range planned for the current study. Notably, the risk of GI bleeding is not reduced by using the enterically coated aspirin formulations, but is thought to be lower during concomitant use of omeprazole[91]. The effects of warfarin and NSAIDs on GI bleeding are synergistic, such that the users of both drugs together have a risk of serious GI bleeding higher than users of either drug alone. Fortunately, the risk of GI bleeding, which reflects the inhibition of prostaglandins in the stomach (from systemic rather than local exposure) is much smaller when using low-dose as opposed to high-dose aspirin.

Low-dose aspirin has not been reported to alter renal function, and does not reduce effectiveness of ACE inhibitors for HTN (in contrast to other NSAIDs)[93 94]. However, aspirin can inhibit the renal clearance of acetazolamide and methotrexate potentially leading to increased blood concentrations of and toxicity from these agents. Salicylate can displace other drugs which are protein-bound, especially phenytoin and valproic acid, increasing their free drug concentrations in plasma. This may increase side effects, toxicity and/or efficacy for displaced drugs. If the BD subjects are currently receiving

valproic acid preparations (e.g., divalproex) then the plasma levels of these agents will be monitored for potential changes.

Aspirin may cause a severe allergic reaction that may include: hives, asthma (wheezing), facial swelling, shock. Aspirin overdose can be fatal at 30 g or higher.

In sum, we believe that our two-by-two design is appropriate for trials involving experimental drugs that already have been well-studied with respect to toxicity, as is the case with aspirin and minocycline. A parallel arm design, as opposed to a 2 x 2 factorial design, would be more clearly informative in the case of an experimental drug for which the toxicity and drug interaction potential have not been thoroughly studied in human subjects

PHI Protection

Paper copies of consents, screening forms, the Research Privacy Form, and any other forms, testing results or papers containing Protected Health Information (PHI) will be stored in a secured medical records room with access granted only to authorized personnel.

Electronic data that contain PHI will be managed in accordance with ISO 27000 series information security standards with policies developed from current NIST guidelines (SP 800-66) for HIPAA and HITECH compliance. Specific controls implemented to protect PHI are derived from NIST 800-122, and include (but not limited to):

1) Access Enforcement (AC-3) – Individual user accounts, role based access control, access control lists;
2) Separation of Duties (AC-5) – de-identification of data as appropriate, acquire/analyze/manage firewall;
3) Least Privilege (AC-6) – to ensure PHI data is only available to persons with established need for access;
4) Remote Access (AC-17) – Secure VPN, encrypted end devices;

5) Access Control for Mobile Devices (AC-19) – Password login, remote destruction capabilities;
6) Auditable Events (AU-2) + Monitoring: Log detailed server and network information, alert for problems;
7) Analysis, and Reporting (AU-6) – Procedures to audit system records for inappropriate activity.
8) User Identification and Authentication (IA-2) – username/secure password and two factor authentication will be required when appropriate.
9) Media Access, Marking, Storage, and Transport (MP-2,3,4,5) – Records will be asset tagged and marked to their PHI status, PHI data will be secured and managed by professional system administrators, and will be transported via encryption (VPN, USB, File);
10) Media Sanitization (MP-6) – Data will be destroyed by SFHS in accordance with their policies and procedures;
11) Transmission Confidentiality (SC-9) – Encryption will be used when needed for all avenues of data transmission (wireless, network, etc.).

To protect subject confidentiality, blood samples will be anonymized as follows:

1. Last name: All participants will be assigned the last name "LIBR."
2. First name: The first name will be a secure alpha cryptographic hash based on LIBR user ID. This technique is the gold standard in computer security for one-way correlation of data.

Benefits versus Risks

The participant may benefit from participation if either study drug produces an antidepressant effect. Participants will also receive a free clinical evaluation; more frequent treatment visits than are typical in practice, diligent follow-up in terms of symptoms and side effects, and physical and psychiatric monitoring during the study. The risks of delaying alternative treatments are minimal in relation to the potential long-term benefits to the subjects and the importance of knowledge that may reasonably result. The

importance of the knowledge that will likely be gained from this study clearly exceeds the associated potential risks.

Alternative Treatment

It is possible that some patients may feel better with talk therapy. Participating in any type of talk therapy with their psychiatrist or psychologist does not require dropping out of this study. Subjects will be encouraged to contact the study investigators, particularly the physician in the study, with any questions they may have regarding alternatives to treatment through this research study. The study investigators will assist in referring the subject to another physician for treatment after their participation in the study has ended. Physical and psychological testing, blood draws, urine samples, and EKG data provide no known risks to persons other than those listed in the exclusion criteria whereas the combinatory power of these measures may provide information relevant to understanding the pathophysiology of bipolar disorder.

Data and Safety Monitoring Plan

This study involves more than minimal risk. The study progress will be overseen by a Data, Safety and Monitoring Board (DSMB). The DSMB is composed of three members who will meet in person or per telephone at least once every 6 months to review relevant study data including adverse events and dropout rates.

Any unanticipated adverse events will be reported immediately to the IRB of record and to the LIBR Human Protection Administrator. Any adverse events will be included in the annual IRB report.

Dissemination of Results

The study results will be presented at national and/or international biomedical scientific meetings and published in peer-reviewed journals.

REGISTRATION

In accordance with the recommendations of the International Committee of Medical Journal Editors[95], the proposed trial is registered in a public registry (www.clinicaltrials.gov Identifier: NCT01429272).

Figure Legend

Figure 1: Schematic of Study Design

Legend: Each session number (total of 7) is encircled, with the timing between sessions indicated in weeks with a 2 business day window on either side of visit target date to complete the visit. Session 1 is the baseline (green star) and session 7 is the study end (purple star). Peripheral blood will be sampled at baseline and study end to assay markers of inflammation. The study duration is 6 weeks.

Author Contributions

All authors made a significant contribution to the conception and design of the study protocol. The protocol was written by Drs. Savitz and W. Drevets and was critically reviewed by Drs. Preskorn, Teague, D. Drevets, and Yates. All authors gave approval for the publication.

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Competing Interests

None of the authors has a conflict of interest to declare.

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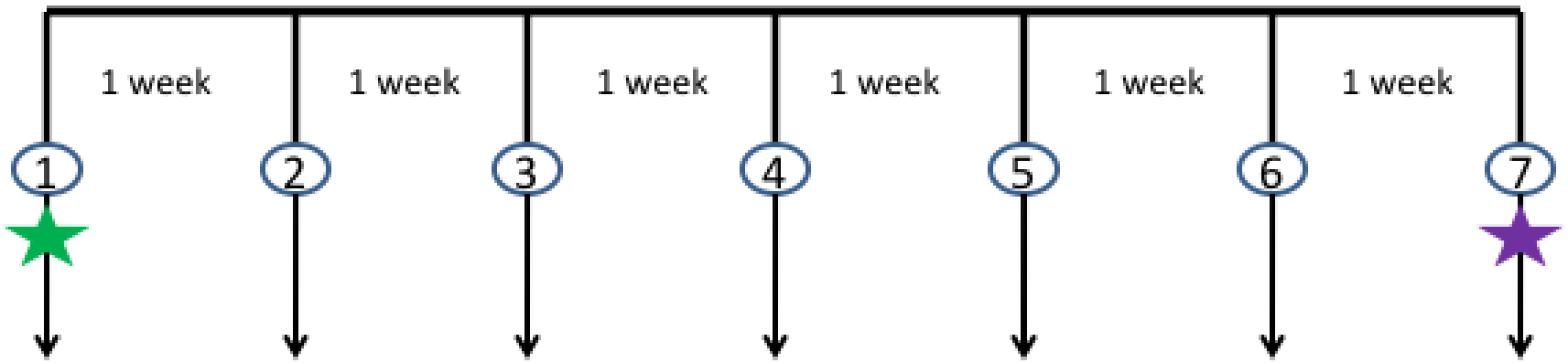

Minocycline dose initiation at 100mg bid

Aspirin dose initiation at 81mg bid

Placebo initiated

Inflammation markers

Inflammation markers

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	3-13
	2b	Specific objectives or hypotheses	20
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	14-15
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	13-14
	4b	Settings and locations where the data were collected	17
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	15-16
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	20-21
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	21
	7b	When applicable, explanation of any interim analyses and stopping guidelines	30
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	15
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	15
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	15
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	15

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	20-21
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	20-21
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	
	13b	For each group, losses and exclusions after randomisation, together with reasons	
Recruitment	14a	Dates defining the periods of recruitment and follow-up	
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	
Other information			
Registration	23	Registration number and name of trial registry	1, 30
Protocol	24	Where the full trial protocol can be accessed, if available	19
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

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Certificate of Approval

THE FOLLOWING WERE APPROVED

INVESTIGATOR: Wayne Drevets M.D.
6655 South Yale Avenue
Tulsa, Oklahoma 74136

BOARD ACTION DATE: 08/22/2011
PANEL: 6
STUDY APPROVAL EXPIRES: 08/05/2012
STUDY NUM: 1126576
WIRB PRO NUM: 20111159
INVEST NUM: 160921
WO NUM: 1-683377-1
CONTINUING REVIEW: Annually
SITE STATUS REPORTING: Semi-Annual

SPONSOR: Laureate Institute for Brain Research (LIBR)
PROTOCOL NUM: 2011-002-00
AMD. PRO. NUM:
TITLE:
MINOCYCLINE AND ASPIRIN IN THE TREATMENT OF BIPOLAR DEPRESSION

APPROVAL INCLUDES:
Subject Information Sheet - Visit Five #9212383.0 - As Submitted
Subject Information Sheet - Visit Four #9212382.0 - As Submitted
Subject Information Sheet - Visit One #9212379.0 - As Submitted
Subject Information Sheet - Visit Six #9212384.0 - As Submitted
Subject Information Sheet - Visit Three #9212381.0 - As Submitted
Subject Information Sheet - Visit Two #9212380.0 - As Submitted
Consent Form [IN1]

WIRB APPROVAL IS GRANTED SUBJECT TO:
RE-CONSENTING INSTRUCTIONS: Subjects currently enrolled are not required to sign the enclosed version(s) of the consent form(s). All subjects who will be enrolled in the future for this study must sign the most current WIRB-approved consent form(s).

WIRB HAS APPROVED THE FOLLOWING LOCATIONS TO BE USED IN THE RESEARCH:
Laureate Institute for Brain Research, 6655 South Yale Avenue, Tulsa, Oklahoma 74136
University of Kansas Medical Center Research Institute, 8911 East Orme, Wichita, Kansas 67207

If the PI has an obligation to use another IRB for any site listed above and has not submitted a written statement from the other IRB acknowledging WIRB's review of this research, please contact WIRB's Client Services department.

Robert A Taylor DO for

Theodore D. Schultz, J.D., Chairman

8/23/2011
(Date)

ALL WIRB APPROVED INVESTIGATORS MUST COMPLY WITH THE FOLLOWING:

1. Conduct the research in accordance with the protocol, applicable laws and regulations, and the principles of research ethics as set forth in the Belmont Report.

2. Although a participant is not obliged to give his or her reasons for withdrawing prematurely from the clinical trial, the investigator should make a reasonable effort to ascertain the reason, while fully respecting the participant's rights.

3. Unless consent has been waived, conduct the informed consent process without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate. (Due to the unique circumstances of research conducted at international sites outside the United States and Canada where WIRB approved materials are translated into the local language, the following requirements regarding consent forms bearing the WIRB approval stamp and regarding certification of translations are not applicable.)
   a. Use only the most current consent form bearing the WIRB "APPROVED" stamp.
   b. Provide non-English speaking subjects with a certified translation of the approved consent form in the subject's first language. The translation must be approved by WIRB.
   c. Obtain pre-approval from WIRB for use of recruitment materials and other materials provided to subjects.

4. Obtain pre-approval from WIRB for changes in research.

5. Obtain pre-approval from WIRB for planned deviations and changes in research activity as follows:

   If this research is federally funded or conducted under an FWA, obtain pre-approval from WIRB for all planned deviations and changes in research activity, except where necessary to eliminate apparent immediate hazards to the human subjects. OHRP considers all planned protocol deviations to be changes in research that need prior IRB review and approval.

   If this research is **not** federally funded and **not** conducted under an FWA, obtain pre-approval from WIRB for any planned deviations that could adversely affect the rights, safety or welfare of subjects, or the integrity of the research data and any changes in the research activity, except where necessary to eliminate apparent immediate hazards to the human subjects. FDA has not adopted the policy that all planned protocol deviations are changes in research that need prior IRB review and approval.

   Deviations necessary to eliminate apparent immediate hazards to the human subjects should be reported within 10 days.

6. Promptly report to WIRB all unanticipated problems (adverse events, protocol deviations and violations and other problems) that meet all of the following criteria:
   a. Unexpected (in terms of nature, severity or frequency);
   b. Related or possibly related to participation in the research; and
   c. Suggests that the research places subjects or others at a greater risk of harm than was previously known or recognized.

   Please go to www.wirb.com for complete definitions and forms for reporting.

7. Provide reports to WIRB concerning the progress of the research, when requested.

8. Ensure that prior to performing study-related duties, each member of the research study team has had training in the protection of human subjects appropriate to the processes required in the approved protocol.

Federal regulations require that WIRB conduct continuing review of approved research. You will receive Continuing Review Report forms from WIRB. These reports must be returned even though your study may not have started.

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