A Selective Nociceptin Receptor Antagonist to Treat Depression:

Evidence from Preclinical and Clinical Studies

Running title: A nociceptin receptor antagonist to treat depression

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ABSTRACT

Nociceptin/Orphanin FQ (N/OFQ) is an endogenous ligand of the N/OFQ peptide receptor (NOP), which is a G protein-coupled receptor in brain regions associated with mood disorders. We used a novel, potent, and selective orally-bioavailable antagonist, LY2940094, to test the hypothesis that blockade of NOP receptors would induce antidepressant effects. In this paper, we demonstrate that targeting NOP receptors with LY2940094 translates to antidepressant-like effects in rodent models, and importantly to antidepressant efficacy in patients with major depressive disorder (MDD). The proof-of-concept study was an 8-week, double-blind, placebo-controlled trial that evaluated LY2940094 as a novel oral medication for the treatment of patients with MDD. Once daily oral dosing of LY2940094 at 40 mg for 8 weeks versus placebo provided evidence for an antidepressant effect based on the change from baseline to week 8 in the GRID-Hamilton Depression Rating Scale-17 item total score. LY2940094 also had an early effect on the processing of emotional stimuli at Week 1 as shown by an increased recognition of positive relative to negative facial expressions in an emotional test battery. LY2940094 was safe and well tolerated. Overall, these are the first human data providing evidence that the blockade of NOP receptor signaling represents a promising strategy for the treatment of MDD.

INTRODUCTION

Nociceptin/Orphanin FQ (N/OFQ) is a 17 amino acid peptide that binds to the N/OFQ peptide (NOP) receptor (Reinscheid *et al*, 1995; Meunier *et al*, 1995). The NOP receptor is expressed in widespread areas of the central nervous system (CNS) in rodents (Neal *et al*,1999), monkeys (Kimura *et al*, 2011), and humans (Lohith *et al*, 2012; Lambert, 2008). These regions include the cortex, hippocampus, amygdala, thalamus, hypothalamus, and dorsal raphe nucleus, which are associated with mood disorders. The NOP receptor is also expressed in the peripheral nervous system as well as in the gastrointestinal tract, smooth muscles, and in cells of the immune system. The NOP receptor is functionally coupled to inhibition of adenylate cyclase, activation of mitogen-activated protein kinases, activation of K+ conductance, and inhibition of Ca+2 conductance (Mogil and Pasternak, 2001; New and Wong, 2002). N/OFQ modulates numerous physiological processes in the brain including feeding behavior, learning and memory functions, pain, and pathways associated with stress, depression and anxiety, and alcohol abuse, (New and Wong, 2002; Gavioli and Calo', 2013; Witkin *et al*, 2014). In preclinical studies, either knockout of the NOP receptor gene or blockade of NOP receptors using selective NOP antagonists has produced antidepressant-like behavioral effects in rodents (Neal *et al*, 1999; Gavioli *et al*, 2003). NOP receptor agonism and chronic stress decreases monoamine levels in specific brain areas (Vitale *et al*, 2009; Gavioli and Calo, 2013). Consistent with the effects of classical antidepressants, NOP receptor antagonists have been shown to restore stress-induced monoamine level alterations in rodent brains (Vitale *et al*., 2009; Gavioli and Calo, 2013). Other recent studies in rodents have demonstrated that the NOP receptor system plays an important role in stress-related behaviors and activation of the hypothalamic pituitary adrenal (HPA) axis and in restoring stress-induced neurogenesis. (Devine *et al*, 2003; Fernandez *et al*, 2004; Gavioli and Calo', 2006). In humans, N/OFQ levels in the plasma of patients with post-partum depression, bipolar depression and MDD were reported to be significantly elevated as compared with healthy subjects (Gu *et al*, 2003; Wang *et al*, 2009, Zhang *et al*, 2009). These findings combined with the profound impact of the N/OFQ system on stress-driven biology and behavior suggests the possibility that antagonism of NOP receptors might be associated with antidepressant effects in MDD patients.

In order to test this hypothesis, we designed LY2940094 ([2-[4-[(2-chloro-4,4-difluoro-spiro[5H-thieno[2,3-c]pyran-7,4'-piperidine]-1'-yl)methyl]-3-methyl-pyrazol-1-yl]-3-pyridyl]methanol), a potent and selective antagonist of NOP receptors in rodents and humans (Toledo *et al*, 2014). LY2940094 fully occupied NOP receptors in rat brain when dosed orally at 30 mg/kg 60 minutes post administration (Raddad *et al* submitted). A single 40 mg dose of

LY2940094 in healthy volunteers resulted in NOP receptor occupancies of >80% (2.5 hours) and >70% (26.5 hours) across different regions of interest providing evidence of high and sustained antagonism of the NOP receptor (Raddad *et al* submitted).

Here, we report the results of preclinical studies of LY2940094 in which we further document an antidepressant-like phenotype induced in rats and the results of the first clinical trial with this novel mechanism in patients with MDD. Taken as a whole, results of these studies indicate that occupancy of NOP receptors results in antidepressant-like but not anxiolytic behavioral effects in rodents and provides evidence for an antidepressant effect in MDD patients.

METHODS

Pre-clinical Rodent Studies

All experiments were conducted in accordance with the NIH regulations of animal care covered in "Principles of Laboratory Animal Care", NIH publication 85-23, and were approved by the Institutional Animal Care and Use Committee. Compounds were dosed in a volume of 1 ml/kg in rats and 10 ml/kg in mice. LY2940094 was dissolved in captisol (20%, pH=2) and dosed either orally 60 min prior to testing. Chlordiazepoxide HCl (Sigma Chemical Co.) and imipramine HCl (Sigma) were dissolved in 0.9% NaCl and dosed i.p. 30 minutes prior to testing.

Effects of LY2940094 on Brain Neurochemistry

Male Sprague-Dawley rats (260 - 300 g, Taconic Farms, Germantown, NY) were implanted with a cannula (BASi, West Lafayette, IN) in the prefrontal cortex (PFC) 5-7 days before the experiment and in vivo microdialysis conducted generally as described by Rorick-Kehn *et al*, (2007). A concentric type probe (BASi, West Lafayette, IN) with a 4 mm (PFC) or 2 mm (NAC) membrane tip was inserted into the guide cannula 18-24 houra before the experiment. The probe inlet tubing was connected to a syringe pump that delivered a CSF (in mM: 145 NaCl, 2.7 KCl, 1.0 MgCl2, 2.5 CaCl2, 2.0 Na2HPO4, pH 7.2-7.4) at a flow rate of 1.5 uL/min. Following this equilibration, 20 uL samples were collected every 30 minutes, and maintained at 4^{o}C in a fraction collector. The sensitivity for norepinephrine, dopamine, and serotonin was 0.1 pmol/ml dialysate or 2 fmol/sample (20 µl). Duloxetine (1µM) was perfused continuously through the probe in order to increase detection levels of the monoamines. All values for

microdialysis studies were calculated as percent of baseline at each time point using the average of the first three baseline values as 100%. All data points are reported as the mean plus or minus the standard error of 3-4 rats per time point. Comparisons between post treatment and baseline values were done with a repeated measures analysis on the percent change from baseline with fixed time effects using the software SAS 9.2.

Evaluation of an Antidepressant Effect

Forced-Swim Test is a behavioral despair test which predicts the efficacy of antidepressant treatments and was utilized as described by Gleason *et al*, (2015). Immobility time was analyzed with a one-way ANOVA followed by a Dunnett's test.

Evaluation of an Anxiolytic Effect

The Vogel Conflict (Vogel *et al*, 1971) assay is a punishment-based test that rats receive a mild shock after specific licks, leading to suppression behavior that is used to detect anxiolytic drug effects. The test was conducted in experimentally naïve adult male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN, 200 to 300 g). Details of the methods, apparatus used, training and testing sessions, and data analysis are described in Alt *et al*, (2007).

The Marble Burying Assay has been used to model anxiety disorders. CD1 mice were used in these experiments that were conducted according to the protocol of Li *et al*, (2006). Effects were analyzed by ANOVA followed by post-hoc Dunnett's tests with P values ≤ 0.05 considered to be significant.

Effect of LY2940094 on Sleep/Wake EEG

Adult, male Wistar rats were anesthetized and surgically prepared with a cranial implant that permitted chronic electro-encephalogram (EEG) and electromyogram (EMG) recording. Rats were individually housed with vigilance state determined automatically using SCORE-2004™ and verified by trained personnel as previously described (Van Gelder et al, 1991; Seidel et al, 1995; Edgar and Seidel, 1997).

Drug treatment was preceded by at least 24 hours of undisturbed recording to establish a baseline for sleep-wake and physiological measures. On the day of treatment, animals were dosed orally with LY2490094 at zeitgeiber time 5 (ZT-5; 5 hours after lights on, LD 12:12). A between subjects design was used, with 30mg/kg of LY2490094

and vehicle tested in parallel in groups of nine animals. Recording continued for 29 hours after treatment to establish a return to behavioral norms following drug administration.

Analysis of covariance (ANCOVA) was used to estimate drug-effects using the baseline sleep outcome value as a covariate. Least squares mean differences to vehicle were calculated for the 7 hours in the light phase after treatment (ZT5-12). Sleep bout lengths over the same 7 hour-period were calculated using a threshold criterion of 3 consecutive epochs. Kaplan-Meier curves were created in SAS 9.2 (SAS Institute Inc., Cary, NC) using the LIFEREG procedure. Treatment comparisons were evaluated, using the Cox proportional hazards model. To account for multiple events within each subject, the competing risks method developed by Wei, Lin and Weissfeld (1989) was performed with the coxph package in R (https://www.r-project.org) (Therneau and Grambsch, 2000).

Clinical Study in Patients with MDD

Overview

This proof-of-concept (POC) study was conducted under protocol I5J-MC-NOAC (b) (ClinicalTrials.gov identifier: NCT01724112) at 11 sites in the United States. Enrollment began in November 2012, and the study was completed in March 2014. The institutional review boards for each site approved the protocol, and all patients provided written informed consent. This study was conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki, and are consistent with Good Clinical Practices and applicable laws and regulations.

Patient selection

The study included male and female outpatients 18 to 65 years of age, who met criteria for MDD without psychotic features as defined by the Diagnostic and Statistical Manual of Mental Disorders 4th edition Text Revision (DSM-IV-TR) (APA, 2000), presented with a new episode of depression of at least 4 weeks duration, and had at least one other major depressive episode in the prior 10 years. Patients were required to have a total score ≥20 on the GRID-Hamilton Depression Rating Scale, 17 items (GRID-HAMD-17) (Williams *et al*, 2008; Hamilton, 1959); Clinical Global Impression of Severity (CGI-S) (Guy, 1976) score ≥4; Hospital Anxiety and Depression Rating Scale (HADS) (Zigmond and Snaith, 1983) depression subscale score ≥11; and a body mass index (BMI) between 18 and 35 kg/m2.

Patients were excluded if they had: any other previous or current Axis I disorder other than MDD; treatment-resistant depression, history of dysthymia or depressive episodes of mild intensity; any unstable medical condition or clinically significant laboratory abnormality; prior seizures or any condition with increased risk of seizures; electroconvulsive treatment, transcranial magnetic stimulation, or vagus nerve stimulation in the prior six months; hepatitis, severe renal impairment, or end-stage renal disease; abnormal thyroid stimulating hormone levels; clinically significant ECG abnormalities; history of substance or alcohol abuse within the prior six months or dependence within the prior 12 months; a positive urine drug screen for excluded medications; currently taking medication that inhibit or induce CYP3A4; at serious suicidal risk as assessed by the Columbia Suicide Severity Rating Scale (C-SSRS) (Posner *et al*, 2011) and clinical evaluation, or homicidal in the opinion of the investigator.

Study design

This was a multicenter, randomized, double-blind, parallel-group, fixed-dose, placebo-controlled, 8-week study that consisted of 3 study periods: screening/washout period (up to 52 days), 8-week treatment period, and a 1 to 2-week follow-up period. After baseline, study visits were conducted at Weeks 1, 2, 4, 6, and 8.

Eligible patients were randomly assigned in a 1:1 ratio to treatment with 40 mg LY2940094 or placebo taken orally once daily (QD). The dose selected was based on pharmacokinetic and positron emission tomography data from healthy subjects. LY2940094 40 mg was predicted to maintain NOP receptor occupancy above 70% throughout the dosing interval upon once daily oral dosing (data on file). Additionally, this dose had previously been demonstrated as being safe and well tolerated in clinical pharmacology studies in healthy subjects for up to 14 days (data on file).

Patients were discontinued from the study if the investigator decided that the patient was at imminent risk of harm to him/herself or others based on the clinician's assessment of the C-SSRS; the patient experienced a clinically significant adverse event that would be inconsistent with continuation of the investigational product; or had a clinically significant laboratory value. Patients were also discontinued if they were significantly noncompliant with study drug regimen, withdrew their consent, or were lost to follow up.

Efficacy assessments

The primary efficacy measure was the change from baseline to Week 8 in the GRID-HAMD-17 total score. The GRID-HAMD-17 is a modified version of the HAMD that permits the rater to consider the dimensions of intensity and frequency independently for each relevant item in the scale. Response to treatment was defined as a reduction of at least 50% in the baseline GRID-HAMD-17 total score at Week 8. Remission was defined by a total score of ≤7 on the GRID-HAMD-17 (Zimmerman *et al*, 2013) at Week 8. Clinical improvement was defined as a score of 1 (very much improved) or 2 (much improved) on the Clinical Global Impression of Improvement (CGI-I) (Guy, 1976). Secondary efficacy measures included the Maier-Philipp Scale (MPS) (Maier and Philipp, 1985) on the GRID-HAMD-17, the Hamilton Anxiety Rating Scale (HAMA) (Hamilton, 1959); the CGI-I; HADS total, and subscale scores for depression and anxiety; and the CGI-S.

Depressed patients are known to have a bias towards interpreting emotionally neutral information as negative Thus emotional processing was assessed using the P1vital® Oxford Emotional Test Battery (ETB) to determine if treatment with LY2940094 modulated the processing of emotional information contained in facial expressions using the Facial Expression Recognition Task (FERT). The task assesses the ability of the patient to accurately identify emotions from facial stimuli. Two hundred fifty stimuli are presented for 500ms and consisted of faces displaying varying levels of anger, disgust, fear, happiness, sadness, and surprise (n=40 each) as well as 10 neutral examples (Harmer *et al*, 2013). Patients were asked to press a labeled button on a keypad to indicate which facial expression was presented. Accuracy for each facial expression was computed as percentage correct and averaged for positive (happy and surprise) and negative (anger, disgust, fear, sad) emotions. The ETB assessments were implemented at Weeks 1 and 6 of treatment.

Safety and tolerability

Safety and tolerability were assessed through collection and monitoring of discontinuation rates, treatment-emergent adverse events (TEAEs), serious adverse events (SAEs), vital signs, laboratory analyses, and electrocardiograms (ECGs). In addition, a solicited assessment of suicide-related behavior and ideations was also conducted at every visit using the C-SSRS.

Statistical Analyses

Sample size was determined via computer simulations that assumed a between-patient standard deviation of 7 and a 25% discontinuation rate Given these assumptions, a sample size of 60 patients per arm was selected to give a 90%

probability of meeting a pre-defined POC criterion if the true treatment reduction was 3.5 points relative to placebo on the GRID-HAMD-17 total score, (equivalent to an effect size of 0.5). The POC criterion was defined as having at least an 88% probability that the reduction from baseline in GRID-HAMD-17 total score at Week 8 is greater when treated with LY2940094 compared to placebo.

The efficacy analyses (including POC) were conducted on the full analysis set (FAS) that included all data from all randomized patients who received at least one dose of study medication and had at least one post-dose efficacy assessment. Analyses were performed according to the treatment the patient actually received. Efficacy analyses were repeated on the per-protocol analysis set that included only those patients who completed the study with no major protocol deviations. Safety analyses were conducted on the safety analysis set that included all patients who received at least one dose of study medication.

The primary efficacy analysis of the change from baseline in GRID-HAMD-17 total score utilized mixed model repeated measures analysis (MMRM) comparing LY2940094 and placebo at the last visit of the 8-week double-blind treatment period. Treatment effects were interpreted in a Bayesian framework assuming a non-informative prior. The least-squares (LS) mean for the difference to placebo, corresponding 95% credible interval and posterior probability that the difference relative to placebo at Week 8 was less than or equal to 0 was calculated as the appropriate tail-area in the t-distribution. The MMRM model contained fixed effects for visit, investigative site, treatment, and treatment-by-visit interaction as well as the continuous fixed covariates of baseline measurement and baseline-by-visit interaction.

The statistical analysis of the MPS, HADS total and subscale scores used the same MMRM approach as for the primary endpoint. For CGI-I the baseline and baseline-by-visit terms were removed from the model as CGI-I was not evaluated at baseline. An analysis of covariance (ANCOVA) was used to analyze the change from baseline in HAMA total score at Week 4 with treatment and pooled investigative site as fixed factors and the baseline HAMA total score as a covariate in the model. The change from baseline to Week 8 on CGI-S was analyzed using the same ANCOVA approach.

The proportion of responders (clinical response, remission, and improvement) at Week 8 were analysed using a logistic regression model with fixed effects for treatment and with the addition of the baseline GRID-$HAMD_{17}$ total score as a covariate. Due to the exploratory nature of the study no adjustments for multiple comparisons were made for secondary and exploratory endpoints. Analyses were implemented using SAS Version 9.

The FERT endpoints from the ETB were analyzed using a MMRM with fixed effects for the emotion (happiness, fear, anger, disgust, sadness, and surprise), treatment, the emotion-by-treatment interaction, and patient as a random effect. Covariates of baseline GRID-HAMD-17 and HAMA scores, as well as the patient's age were included in the model. The interaction effect indicated whether there was a treatment difference across emotions. A comparison of the positive emotions (happy and surprise) with the negative emotions (fear, anger, disgust and sadness) was performed.

RESULTS

Pre-clinical Outcomes in Rodent Models

A single oral dose of LY2940094 (30 mg/kg) produced small but statistically significant increases in the extracellular levels of serotonin and a transient, but not significant increase of dopamine levels, and did not change norepinephrine levels in the prefrontal cortex of rats (**Fig. 1A**). Under the same dosing parameters, immobility time of rats in the forced-swim test was decreased by LY2940094 where 30 mg/kg produced comparable maximal efficacy to the tricyclic antidepressant imipramine (30 mg/kg, i.p.) (Cryan *et al*, 2005) (**Fig. 1B**). In the Vogel conflict test, LY2940094 was without anxiogenic- or anxiolytic-like effects whereas the benzodiazepine receptor agonist chlordiazepoxide significantly increased punished licking (**Fig. 1C**) as previously and generally reported (Gleason et al, 2007). Similarly, in the mouse marble-burying assay, LY2940094 did not differ from vehicle in the number of marbles buried; whereas mice that received chlordiazepoxide buried significantly fewer marbles (**Fig. 1D**) as previously and generally reported (Li et al., 2006). In rats, LY2940094 administered orally at 30mg/kg suppressed non-rapid-eye-movement (NREM) sleep (-20.5 ± 8.6 min relative to vehicle over 7 hours post dosing, $P<0.05$, ANCOVA) with no effect on rapid eye movement (REM) sleep (**Fig. 2A, B**). The compound produced shortening of the average sleep bout length and survival analysis of sleep bout data demonstrated a significantly greater likelihood of increased waking during NREM sleep [Cox proportional hazard ratio of 0.769 ± 0.0704, $p<0.001$]. (**Fig. 2C**). LY2940094 had no effect on EEG spectral power.

Clinical Study in Patients with MDD

The overall experimental design and disposition of patients who participated in the study are shown in **Fig. 3**. There were no significant differences between treatment groups for rates of study completion or discontinuation due to adverse events or for any other reason.

Patient characteristics

One hundred and thirty-six patients were randomized to receive LY2940094 (N=70) or placebo (N=66). Patient characteristics and baseline illness severity are summarized in **Table 1**. Patients ranged in age from 18 to 65 years, the majority were Caucasian females; and the average GRID-Hamilton Depression Rating Scale (GRID-HAMD-17) total score was 25 indicating moderate to severe depression (Zimmerman *et al*, 2013). Baseline disease characteristics did not differ between treatment groups.

Efficacy outcomes

The results from the analysis of change from baseline in the GRID-HAMD-17 total score in the FAS revealed an improvement in depressive symptoms in both treatment groups, with a numerically greater reduction from baseline observed in the LY2940094 treatment group (**Fig. 4A**). At Week 8, LS mean changes from baseline in GRID-HAMD-17 total scores were -11.4 and -9.8 for patients in the LY2940094 and placebo treatment groups, respectively. The LS mean difference from placebo was -1.5 (95% credible interval [CI] -4.7, 1.7) and the probability that LY2940094 was better than placebo was 82.9%. In the per-protocol analysis of the change from baseline in GRID-HAMD-17 total score, the probability of LY2940094 being better than placebo was 88.6%. When the analysis of the full analysis set was extended to include the post-study follow up visit (Week 9-10), the difference from placebo in the LS mean change from baseline in GRID-HAMD-17 total score was -2.9 and the probability that treatment with LY2940094 had a greater reduction in GRID-HAMD-17 than placebo was 97.4%.

For the secondary efficacy endpoints, the CGI-I, CGI-S and MPS subscale of the GRID-HAMD-17 (per protocol) all had a greater than 80% probability that patients treated on LY2940094 had a greater reduction then those on placebo. The HADS score showed a greater reduction on placebo than LY2940094 (**Table S1**)

When the individual items on the GRID-HAMD-17 were analyzed in the FAS, LY2940094 had ≥ 90% probability of being better than placebo for changes in mood (99.2%), loss of appetite (98.4%), sexual interest (90.9%), loss of weight (90.1%), and general somatic symptoms (91.2%) (**Table S2**). There was worsening with LY2940094 compared to placebo on sleep-related items, mainly for early and middle insomnia.

The percentage of patients achieving response, remission, and CGI-I scores of ≤2 increased over time in both treatment groups in the FAS. The probability that treatment with LY2940094 had a greater response rate than placebo on the clinical response criterion at Week 8 was 92% with an odds ratio of 1.79 (55% responded on LY2940094 compared to 41% on placebo); 84% for clinical improvement with an odds ratio of 1.51 (57%

responded on LY2940094 compared to 47% on placebo); 59% for remission with an odds ratio of 1.11 (31% responded on LY2940094 compared to 29% on placebo).

Emotional processing after 1 week of treatment was improved in the LY2940094 group with a LS mean of 60.2% accuracy in identifying positive faces as compared with 56.9% for the placebo group (**Fig. 4B**) giving a probability of 92.4% of greater accuracy with LY2940094 than placebo. The LS mean percentage accuracy of identifying negative faces in the LY2940094 group was 42.8% and was 42.4% in the placebo group. The probability that treatment with LY2940094 was more accurate than placebo on the facial recognition of positive versus negative emotions was 88.6%.

Safety and tolerability

Treatment-emergent adverse events were reported by 63.9% of patients in the LY2940094 group and 63.1% in the placebo group. The most common events reported by ≥5% of patients in the LY2940094 group were: headache (23.2%), nausea (10.1%), insomnia (8.7%), upper respiratory tract infection (7.2%), diarrhea (7.2%), dizziness (7.2%), constipation (5.8%), and anxiety (5.8%). Most of the events were of mild to moderate intensity. With the exception of insomnia and dizziness, which were only reported in the LY2940094 group, differences between treatment groups were not statistically significant for any event. No statistically significant differences were observed between LY2940094 and placebo groups but numerically more patients treated with LY2940094 than placebo discontinued early from the study due to an adverse event (n=6, 8.7% in the LY2940094 group; n=2, 3.1% in the placebo group; p=0.276).

Adverse events in the LY2940094 group leading to discontinuation were: nausea (n=2), dizziness (n=1), headache (n=1), derealization (n=1), and insomnia (n=1). Patients in the placebo group discontinued due to blood creatine phosphokinase increased (n=1) and pruritic rash (n=1). Two SAEs were reported by one patient in the placebo group (pericarditis and esophagitis) and there were no SAEs reported in the LY2940094 group. There were no clinically significant findings during treatment with LY2940094 for laboratory assessments, vital signs, ECGs or suicidality based on the C-SSRS.

DISCUSSION

Here, we present the first clinical study demonstrating an antidepressant effect of a potent and selective NOP receptor antagonist, LY2940094, in MDD patients, supported by preclinical data from relevant model systems. An antidepressant-relevant behavioral phenotype for LY2940094 was first shown in rodents, using the forced-swim assay in comparison with an established antidepressant. Our findings were consistent with data from prior preclinical investigations with the NOP receptor antagonists, UFP-101 (Gaviolo *et al*, 2003) and SB612111 (Rizzi *et al*, 2001), as well as data from NOP receptor knockout (NOP-/-) mice and rats (Gavioli *et al*, 2004). The mechanism whereby a NOP receptor antagonist engenders these behavioral effects is not fully understood. Increases in serotonin levels produced by LY2940094 could support in part a serotonergic hypothesis of action (Le Maitre *et al*, 2005; Gavioli and Calo', 2013), however the increases were small compared to other antidepressants (Jordan *et al*, 1994), and not observed with the NOP antagonist UFP-101 (Vitale *et al*, 2009). Collectively, these findings suggest that the antidepressant efficacy produced by LY2940094 results from as yet undefined downstream mechanism(s) that are hypothesized to regulate the positive impact of N/OFQ on multiple pathways of stress biology and their restitution by NOP receptor antagonism (see Witkin *et al*, 2014 for review).

The data from the POC study in MDD patients provides evidence for an antidepressant effect of NOP receptor antagonism. In moderately to severely depressed patients, LY2940094 reduced the severity of depressive symptoms. The evidence for this was more pronounced in the completer's analysis (per protocol set) than in the full analysis set (FAS) and was supported by follow-up study data at Week 9/10. Analysis of the individual elements of GRID-HAMD-17 showed that the greatest effect of LY2940094 versus placebo was on mood (depressed mood, item 1) and other core symptoms of depression as assessed by the MPS. For this first exploration of an antidepressant effect of LY2940094, the HAMD-17 scale was used as a primary out-come measure. The item analysis revealed domains such as depressed mood, to have larger effects in favor of the NOP receptor antagonist comparable to the effect size of those of SSRIs than the HAMD-17 total score; a finding which is consistent with the data of a recent meta-analysis of the effectiveness of established antidepressants (Hieronymus *et al*, 2015). To characterize the clinical profile of this novel antidepressant further, alternative assessment tools may be considered for future trials.

In contrast, LY2940094 was not better than placebo on several non-mood related items, including early and middle insomnia, which correlate with sleep onset and maintenance, respectively. These sleep results are consistent with our preclinical findings in rats where LY2940094 induced modest sleep-impairing effects through selective

suppression of non-REM sleep and reduction of average sleep bout length whereas REM sleep was not disturbed. REM sleep is suppressed by the majority of selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (NRIs) as well as tricyclic antidepressants. Only a few antidepressants improve sleep disruptions associated with major depression and the effects on sleep onset and continuity differ according to the class of drugs used (Thase *et al*, 2010). Sleep disturbances induced by antidepressants, however, are often more pronounced in the first few weeks of initiating antidepressant therapy. The observed effect of LY2940094 on subjective sleep, as assessed by the GRID-HAMD-17 individual scores may diminish over time as follow-up data at Week 9/10 indicated (data on file). However, human sleep EEG data are currently not available to help understand the potential effect of NOP antagonism of LY2940094 on objective sleep measures.

The central expression of the NOP receptor and preclinical studies in rodents suggest that the N/OFQ system may also be implicated in symptoms of anxiety (Gavioli and Calo, 2013). In order to assess the potential of LY2940094 for anxiolytic activity, we studied the molecule in two assays that detect anxiolytic-like effects in rodents. LY2940094 did not have anxiolytic-like activity in either the Vogel conflict or in the marble-burying assays at concentrations associated with 100% occupancy of NOP receptors, nor did LY2940094 induce anxiogenic-like effects in these rodent models. These data are consistent with the general experimental literature indicating that NOP receptor agonists, but not antagonists, can induce anxiolytic-like effects in rodent models (Witkin *et al*, 2014).

The POC study included the HAMA as a secondary measure to assess the effect of LY2940094 on anxiety symptoms in depressed patients. The level of anxiety at baseline was mild overall, and there was no attempt to enrich the population with patients with more severe symptoms of anxiety. Based on this observational approach, there were no anxiolytic effects of LY2940094, as evidenced by the results from the analysis of the change from baseline in HAMA total score at Week 4, the changes from baseline in the scores of the anxiety-related individual items of the GRID-HAMD-17, and the change from baseline in the patient-rated HADS anxiety subscale scores.

The main intent of using the FERT component of the ETB was to determine if it could be used as an early biomarker for eventual antidepressant efficacy of a compound with a novel mechanism of action. Studies have shown that patients with depression are more likely to interpret emotional signals, like facial expressions, as being negative or less positive compared with healthy volunteers (Harmer *et al*, 2011). It has been proposed that antidepressants may work by reversing negative biases in depressed patients prior to the changes in mood (Harmer *et al*, 2011). In this study, treatment with LY2940094 was associated with a greater accuracy of identifying positive

faces as compared with placebo at Week 1 (Day 7). Similar effects were observed by short-term administration (7 days) of an SSRI (citalopram) and an NRI (reboxetine) in healthy volunteers (Harmer *et al*, 2004). Increased accuracy of recognition of happy faces was also shown to be improved in depressed patients treated either with citalopram or reboxetine within the first two weeks of treatment and predicted treatment response (Tranter *et al*, 2009). Thus the results obtained in the present study are consistent with effects observed with established antidepressant drugs. There has been a lack of predictive tools to evaluate pharmacological activity early in clinical development for putative antidepressant drugs. This is a key issue because evidence of efficacy, together with evidence of target engagement, improves the probability of success for early development compounds (Morgan *et al*, 2012). The current effects of LY2940094 on emotional processing bias using the ETB highlights a potential tool which could be utilized to provide an early readout of antidepressant effect in clinical development programs.

LY2940094 was safe and well tolerated. Reported adverse events were mostly mild and resolved on treatment. LY2940094 had no effect on vital signs, laboratory assessments or ECGs. Over 95% of patients in both treatment groups were study drug compliant, and over 70% completed the study.

There are several limitations of the study, one of which is length of treatment. When the MMRM analysis of the change from baseline in GRID-HAMD-17 total score was extended to include the follow-up visit (Week 9/10) in a post-hoc analysis, there was improved efficacy with LY2940094 even though the drug had been discontinued at Week 8. In healthy subjects the mean terminal half-life for LY2940094 was approximately 3 to 4 days after multiple once-daily dosing (data on file). Thus it would be expected that patients would still be exposed to LY2940094 and would have measureable concentrations at the follow up assessment. The observed treatment effect during the follow-up period suggests that any future studies using LY2940094 should include a longer assessment period for the treatment effect to more than 8 weeks to fully evaluate antidepressant efficacy. Dosing could be another limitation. Although the 40 mg dose was associated with predicted receptor occupancy above 70% for the duration of a dosing interval, it is possible that a higher dose could have been more effective. Also, the data from our study may have been limited by its small sample size. The study was powered (90%) for a large effect size of 0.5 for antidepressants; hence there was less chance of meeting the success criterion for POC if the true effect size was less than 0.5. Finally, N/OFQ plasma levels were not used to guide patient selection for this study. N/OFQ levels are reported to be higher in patients with depression and it might be lowered by successful antidepressant treatment (Gu *et al*, 2003; Wang *et al*, 2009; Zhang *et al*, 2009).

In summary, we have provided herein the first report of a clinical study with a NOP receptor antagonist, LY2940094, which was efficacious in alleviating symptoms of depression and influencing emotional biases in MDD patients.

Supplementary information is available at the Neuropsychopharmacology website.

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FIGURE LEGENDS

Figure 1 Effect of LY2940094 on Outcomes in Rodent Models. (**A**) Extracellular levels of the monoamines serotonin (5-HT), dopamine (DA) and norepinephrine (NE) in the medial prefrontal cortex of rat brain before and after a single oral dose of LY2940094 30 mg/kg. (Each point represents the mean ± SE in 3-4 rats) (**B**) Immobility in the rat forced-swim test (n=6-8) when dosed orally 60 minutes prior to testing (*P<0.05 compared to vehicle, Dunnett's test) (**C**) Number of licks of a water spout when licks were either not punished or when they were punished. Each point represents the mean + SE of data in 7-8 rats [*P<0.05 compared to vehicle, Dunnett's test]. (**D**) The number of marbles buried by mice (n=7-8) [*P<0.05, Dunnett's test]. Abbreviations: veh = vehicle, 3, 10, and 30 are doses of LY2940094 in mg/kg, p.o.; IMI = imipramine (positive control) 15 mg/kg, i.p, chlordiazepoxide (20 mg/kg intraperitoneal (i.p.)

Figure 2. Effect of LY2940094 (30 mg/kg dosed orally) on sleep in rats. Drug/vehicle administered at time point indicated. (**A**) non- rapid eye-movement (NREM) sleep, (**B**) rapid eye-movement (REM) [each point or bar represents the mean ± SE of data in 9 rats]. (**C**) Analysis of sleep bout survival time during NREM sleep. Vehicle data are shown as a continuous bar to aid clarity.

Figure 3 Flow of patients through the study.

Figure 4 (**A**) Time course of LS mean change from baseline in GRID-HAMD-17 total score from Week 1 through Week 8 and at Post Study Follow Up at Week 9-10 (Full Analysis Set). Abbreviations: LS = least square; SE = standard error; GRID-HAMD-17 = GRID-Hamilton Depression rating scale. (**B**) Box plot for each treatment of the FERT percentage accuracy for the positive emotions (happy and surprise) measured 7 days after commencing treatment.

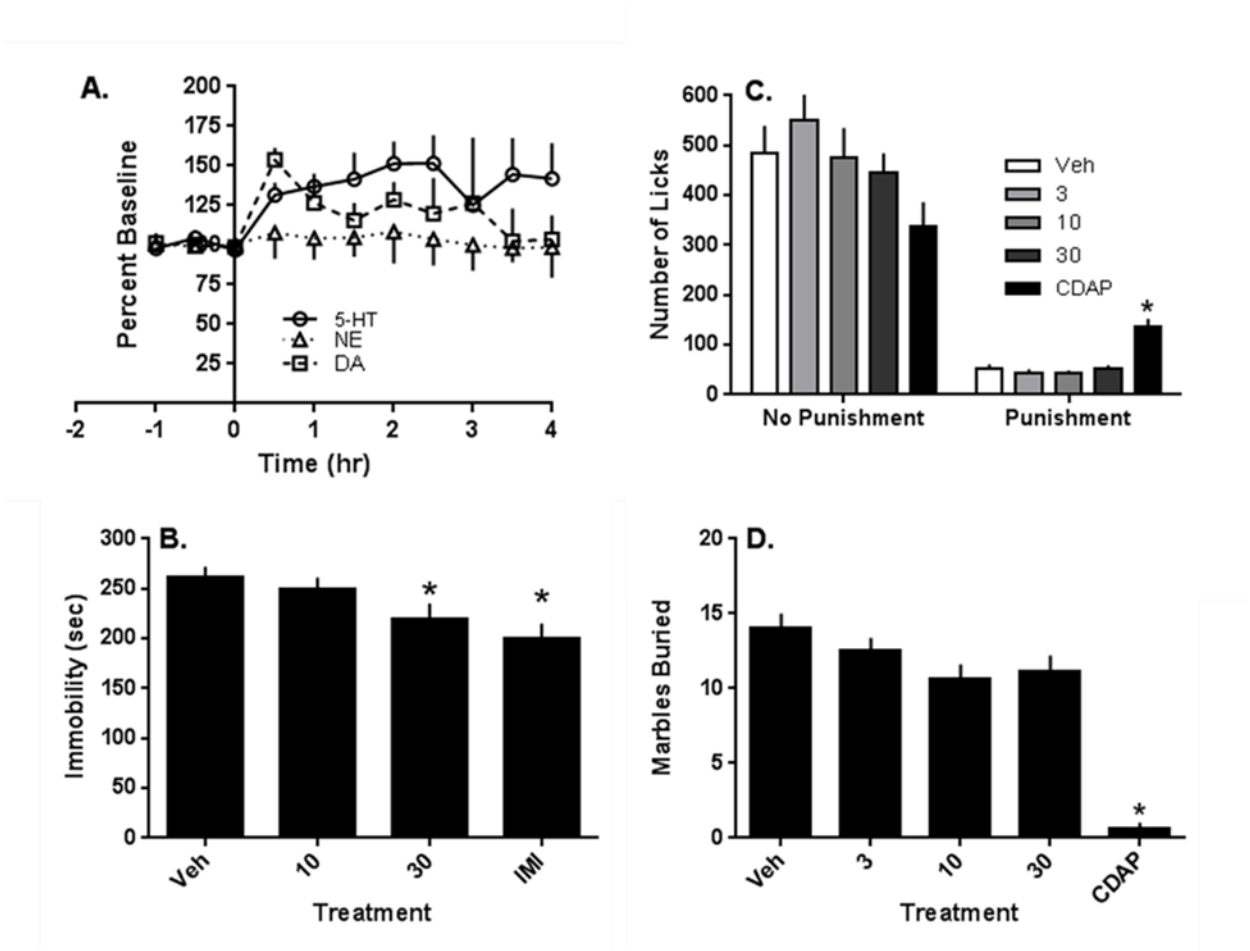
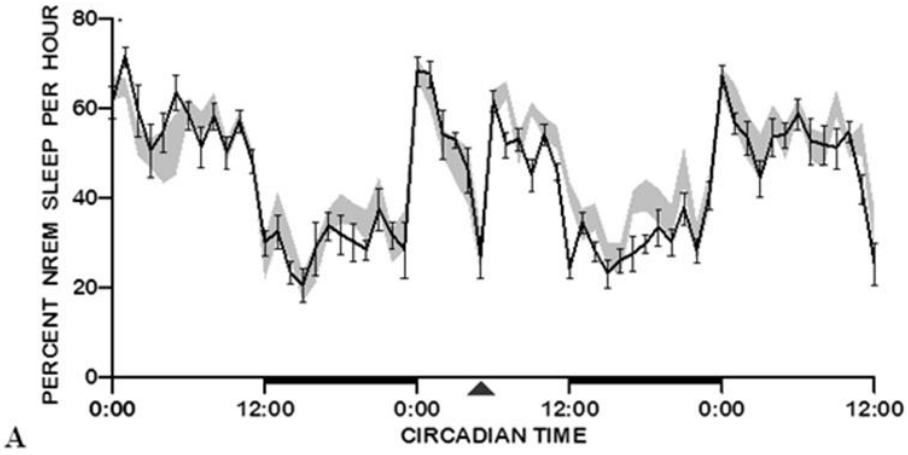
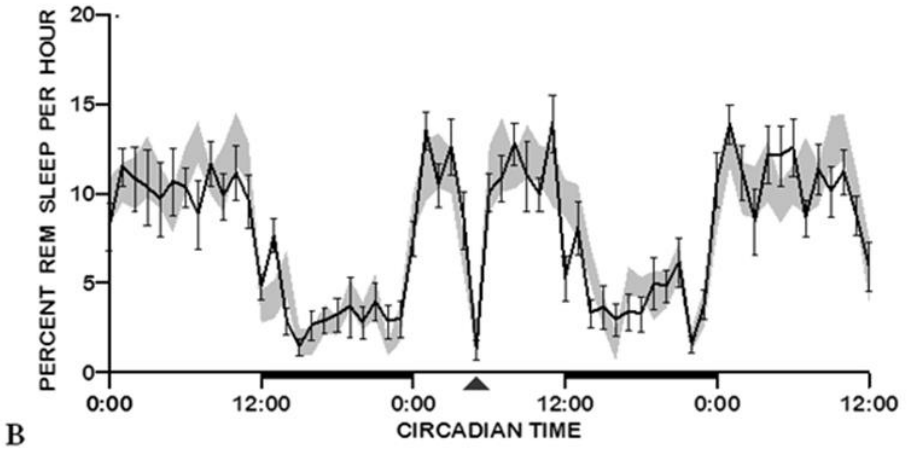

A.
Percent Baseline
200
175
150
125
100
75
50
25
-2
-1
0
1
2
3
4
Time (hr)
5-HT
NE
DA
C.
Number of Licks
600
500
400
300
200
100
0
Veh
3
10
30
CDAP
*
No Punishment
Punishment
B.
Immobility (sec)
300
250
200
150
100
50
0
*
*
Veh
10
30
IMI
Treatment
D.
Marbles Buried
20
15
10
5
0
*
Veh
3
10
30
CDAP
Treatment

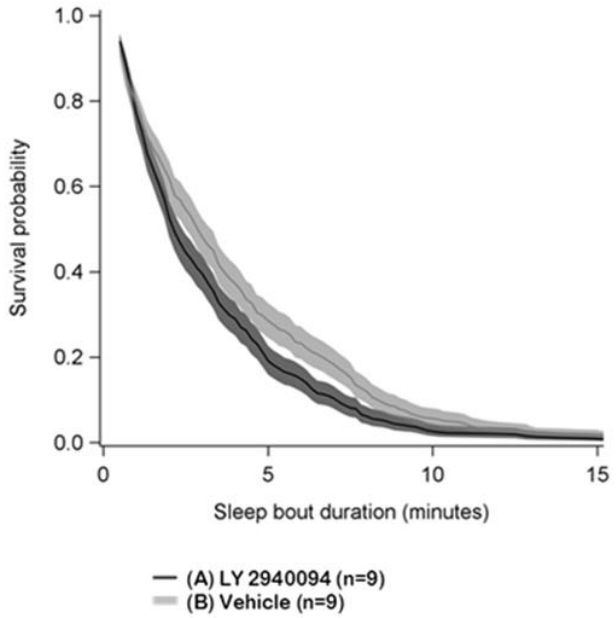
Figure 1


PERCENT NREM SLEEP PER HOUR
80
60
40
20
0
0:00
12:00
0:00
12:00
0:00
12:00
CIRCADIAN TIME

A


PERCENT REM SLEEP PER HOUR
20
15
10
5
0
0:00
12:00
0:00
12:00
0:00
12:00
CIRCADIAN TIME

B


Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Sleep bout duration (minutes)
(A) LY 2940094 (n=9)
(B) Vehicle (n=9)

C

Figure 2

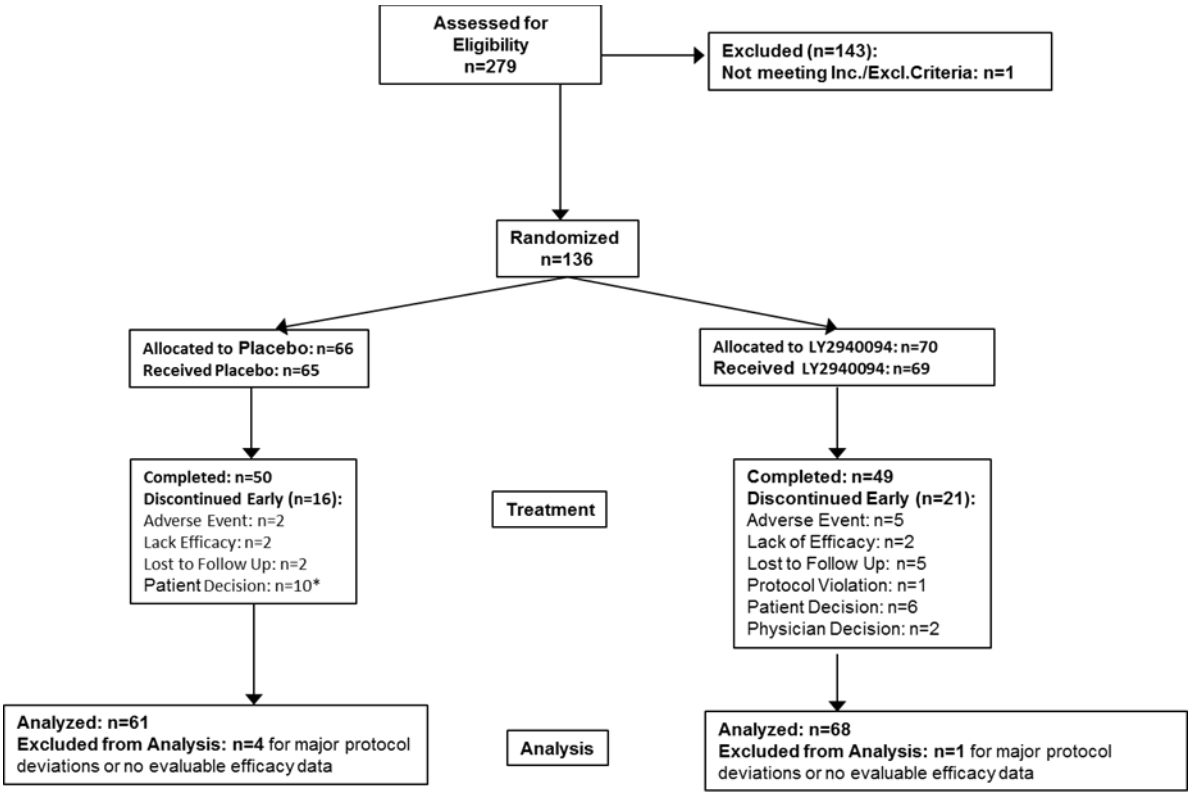
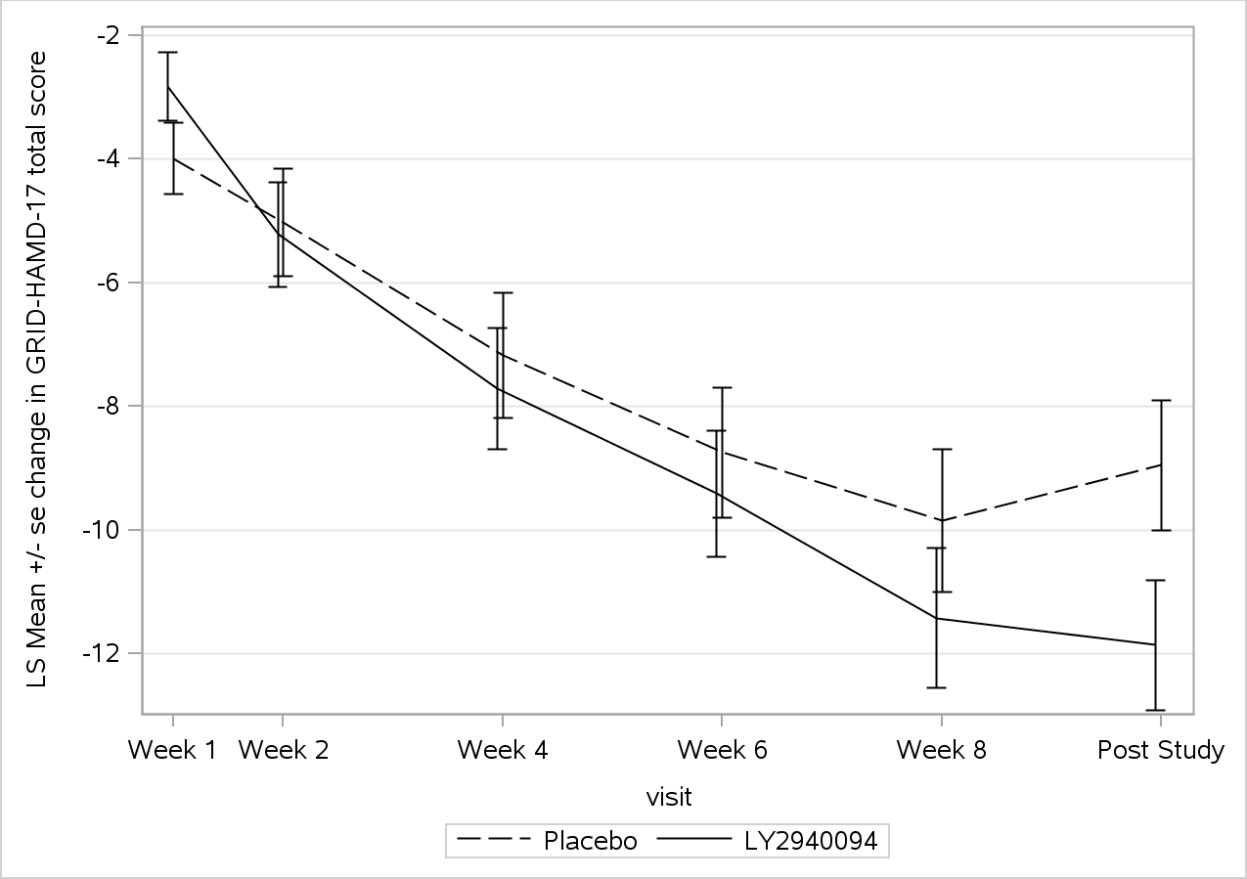

Assessed for Eligibility n=279
Excluded (n=143): Not meeting Inc./Excl.Criteria: n=1
Randomized n=136
Allocated to Placebo: n=66 Received Placebo: n=65
Allocated to LY2940094: n=70 Received LY2940094: n=69
Completed: n=50
Discontinued Early (n=16):
Adverse Event: n=2
Lack Efficacy: n=2
Lost to Follow Up: n=2
Patient Decision: n=10*
Treatment
Completed: n=49
Discontinued Early (n=21):
Adverse Event: n=5
Lack of Efficacy: n=2
Lost to Follow Up: n=5
Protocol Violation: n=1
Patient Decision: n=6
Physician Decision: n=2
Analyzed: n=61
Excluded from Analysis: n=4 for major protocol deviations or no evaluable efficacy data
Analysis
Analyzed: n=68
Excluded from Analysis: n=1 for major protocol deviations or no evaluable efficacy data

Figure 3

A


LS Mean +/- se change in GRID-HAMD-17 total score
-2
-4
-6
-8
-10
-12
Week 1
Week 2
Week 4
Week 6
Week 8
Post Study
visit
Placebo
LY2940094

B

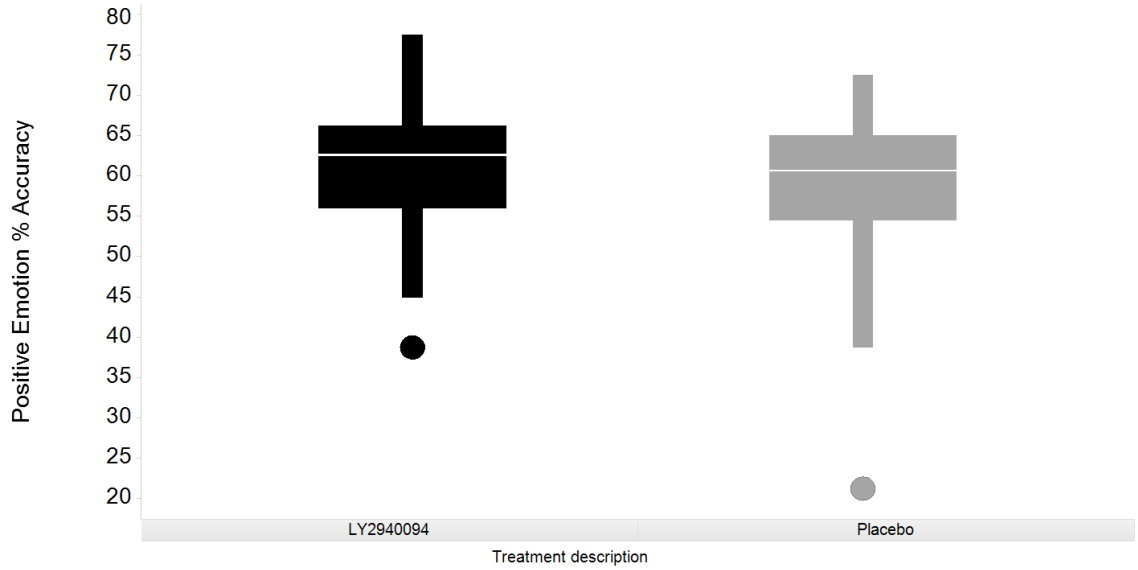

Positive Emotion % Accuracy
80
75
70
65
60
55
50
45
40
35
30
25
20
LY2940094
Placebo
Treatment description

Figure 4

Table 1 Patient Demographics and Baseline Severity of Illness.

Variable	**LY2940094** **N=69**	**Placebo** **N=65**	***P* value**
Age, mean (SD) years	40.2 (13.2)	39.0 (12.3)	0.544
Range	17.8-65.4	18.1-65.0	
Female, n (%)	41 (59)	39 (60)	1.000
Male, n (%)	28 (41)	26 (40)	
Race, n (%)			0.755
White	48 (70)	49 (75)	
Black	18 (26)	14 (22)	
Asian	3 (4)	2 (3)	
GRID-HAMD-17 total score, mean (SD)	25.0 (4.5)	25.0 (5.0)	0.988
MPS total score (SD)	12.4 (2.2)	12.3 (2.5)	0.810
CGI-S, mean (SD)	4.3 (0.5)	4.3 (0.5)	0.947
HAMA total score, mean (SD)	17.9 (6.2)	17.5 (6.0)	0.647
HADS total score, mean (SD)	26.9 (4.4)	27.3 (5.5)	0.696
HADS Depression subscale score, mean (SD)	13.8 (2.0)	14.7 (2.8)	0.041
HADS Anxiety subscale score, mean (SD)	13.1 (3.9)	12.6 (4.1)	0.422
Disease duration in years, median (range)	8.4 (0-33)	8.9 (0-39)	0.980

Abbreviations: SD, standard deviation; GRID-HAMD-17, grid format of the Hamilton Depression Rating Scale, 17 items; MPS, Maier-Philipp sub-scale of the GRID-HAMD-17; CGI-S, Clinical Global Impression of Illness Severity; HAMA, Hamilton Anxiety Rating Scale; HADS, Hospital Anxiety and Depression Scale;

SUPPLEMENTAL INFORMATION

Table S1 provides the results from the analysis of the continuous secondary endpoints including: LS mean change from baseline for each treatment group, the difference from placebo and the probability that this difference was ≤ 0 (a greater reduction on LY2940094 than placebo).

Table S2 provides the results from the analysis of the individual items of the GRID-HAMD-17 including: LS mean change from baseline for each treatment group, the difference from placebo and the probability that this difference was ≤ 0 (a greater reduction on LY2940094 than placebo).

Table S1 Continuous Secondary Efficacy Endpoints

Assessment	**LY2940094 LS Mean Change from Baseline**	**Placebo LS Mean Change from Baseline**	**Mean Difference from Placebo**	**Probability that Difference ≤0**
HAMA (Week 4)	-5.38	-5.20	-0.18	56.5%
CGI-S (Week 8)	-1.64	-1.33	-0.31	87.6%
MPS (Full analysis set) (Week 8)	-5.83	-5.17	-0.66	76.3%
MPS (Per protocol set) (Week 8)	-6.32	-5.17	-1.15	88.1%
HADS total score (Week 8)	-8.11	-10.0	1.89	10.8%
HADS depression sub-scale (Week 8)	-4.73	-5.43	0.70	22.3%
HADS anxiety sub-scale (Week 8)	-3.32	-4.76	1.44	3.2%
	LY 2940094 Mean Score at Week 8	Placebo Mean Score at Week 8	Mean Difference from Placebo	Probability that Difference ≤0
CGI-I score	2.53	2.72	-0.19	80.2%

Abbreviations: HAMA, Hamilton Anxiety Rating Scale; CGI-I, Clinical Global Impression of illness improvement; CGI-S, Clinical Global Impression of illness severity; MPS, Maier-Philipp sub-scale of the grid format of the Hamilton Depression Rating Scale, 17 items; HADS, Hospital Anxiety and Depression Scale; LS, Least squares

Table S2 Change from Baseline in Individual items of the GRID-HAMD-17 at 8 weeks of Treatment.

GRID-HAMD-17 Item	LY2940094 LS mean change	Placebo LS Mean Change	Mean Difference from Placebo	Probability that Difference ≤0
Mood	-1.7	-1.1	-0.6	99%
Guilt	-1.1	-1.2	0.1	42%
Suicide	-0.3	-0.2	-0.1	75%
Insomnia early	-0.5	-0.8	0.4	2%
Insomnia middle	-0.4	-0.6	0.2	11%
Insomnia late	-0.5	-0.5	-0.1	71%
Work/Activities	-1.4	-1.2	-0.2	81%
Psychomotor retardation	-0.4	-0.4	-0.1	65%
Psychomotor agitation	-0.2	-0.2	0.0	47%
Psychic anxiety	-1.0	-1.1	0.0	44%
Somatic Anxiety	-1.0	-0.8	-0.2	87%
Loss of appetite	-0.7	-0.4	-0.3	98%
General somatic symptoms	-0.9	-0.7	-0.2	91%
Sexual interest	-0.5	-0.3	-0.2	91%
Hypochondriasis	-0.4	-0.3	-0.0	59%
Weight loss	-0.4	-0.2	-0.2	90%

Abbreviations: GRID-HAMD, GRID-Hamilton Depression Rating Scale; LS, Least squares