JPET Fast Forward. Published on October 29, 2010 as DOI:10.1124/jpet.110.172817

Title Page

Pharmacological Characterization of KLYP961, A Dual Inhibitor of Inducible and Neuronal Nitric Oxide Synthases

Kent T Symons, Phan M Nguyen, Mark E Massari, John V Anzola, Lena M. Staszewski, Li Wang, Nahid Yazdani, Steven Dorow, Jerry Muhammad, Marciano Sablad, Natasha Rozenkrants, Celine Bonefous , Joesph E Payne, Peter J. Rix, Andrew K Shiau, Stewart A. Noble, Nicholas D. Smith, Christian A Hassig, Yan Zhang and Tadimeti S. Rao*

Departments of Biology (KTS, PMN, MEM, JVA, LMS, AKS, CAH), Drug Metabolism & Pharmacokinetics (LW, PJR), Pharmaceutical Sciences (NY, SD, JM), Chemistry (CB, JEP, SAA, NDS) and Pharmacology (MS, NR, YZ, TSR)

Kalypsys Inc

10420 Wateridge Circle, San Diego, CA 92121

Running Title page

"KLP961- iNOS and nNOS dimerization Inhibitor"

Corresponding Author: Dr.Tadimeti S. Rao, Ph.D., Johnson and Johnson, 3210 Merryfield Row, San Diego, CA 92121. E-mail: trao1@its.jnj.com

Number of Text pages:39 (including title page)

Number of Tables: 1

Number of Figures:7

Number of References:36

Number words in Abstract: 250

Number words in Introduction: 728

Number words in Discussion: 1153

List of non-standard abbreviations:

NOS: Nitric oxide synthase; NO: Nitric oxide; iNOS, inducible NOS, e-NOS, endothelial NOS, nNOS, neuronal NOS; CCI, chronic constrictive nerve injury; PBQ, phenyl-benzoquinone; BBS-4, (*R*)-1-(2-(1*H*-imidazol-1- yl)-6-methylpyrimidin-4-yl)-*N*-(2-(benzo[*d*][1,3]dioxol-5-yl)ethyl)pyrrolidine-2-carboxamide; HEK (human embryonic kidney); COPD (chronic obstructive pulmonary disease); SEITU (2-ethyl-2-thiopseudourea hydrobromide); LPS, lipopolysaccharide.

Recommended Section Assignment:

Option 1: Inflammation, Immunopharmacology &Asthma

Option 2: Neuropharmacology

Abstract

NO derived from nNOS and iNOS plays a key role in various pain and inflammatory states. KLYP961 inhibits the dimerization, and hence the enzymatic activity of human, primate and murine iNOS and nNOS (IC_{50} values: 50-400 nM), with marked selectivity against eNOS (IC_{50} >15000 nM). It has ideal drug like-properties, including excellent rodent and primate pharmacokinetics coupled with a minimal off-target activity profile. In mice, KLYP961 attenuated endotoxin-evoked increases in plasma nitrates, a surrogate marker of iNOS activity *in viv,* in a sustained manner (ED_{50} of 1 mg/kg, po.). KLYP961 attenuated pain behaviors in a mouse formalin model (ED_{50}: 13 mg/kg, po), cold allodynia in the chronic constriction injury model (ED_{50}: 25 mg/kg, po) or tactile allodynia in the spinal nerve ligation model (ED_{50}: 30 mg/kg, po) with similar efficacy, but superior potency relative to gabapentin, pregabalin or duloxetine. Unlike morphine, the anti-allodynic activity of KLYP961 did not diminish upon repeated dosing. KLYP961 also attenuated carrageenin-induced edema and inflammatory hyperalgesia, and writhing response elicited by PBQ with efficacy and potency similar to those of celecoxib. In contrast to gabapentin, KLYP961 did not impair motor coordination at doses as high as up to 1000 mg/kg, po. KLYP961 also attenuated capsaicin-induced thermal allodynia in rhesus primates in a dose-related manner with a minimal effective dose $\leq$ 10 mg/kg, po and a greater potency than gabapentin. In summary, KLYP961 represents an ideal tool with which to probe the physiological role of NO derived from iNOS and nNOS in human pain and inflammatory states.

Introduction

Three mammalian nitric oxide synthases (NOS), neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (eNOS, NOS-3) are involved in the generation of NO, a diffusible second messenger molecule with diverse pharmacological actions. All three isoforms are active only as homodimers and use L-arginine as the sole, common substrate. The overproduction of nitric oxide (NO) has been implicated in multiple human pathologies such as pain, inflammation, arthritis, asthma, chronic obstructive pulmonary disease (COPD), migraine and neurodegenerative disorders (Vallance and Leiper, 2002). Of particular relevance to pain and inflammation, NO: (1) is involved in the transmission and modulation of nociceptive information at peripheral, spinal and supraspinal levels (Goettel and Larson, 1996; Yamamoto et al., 1993; Wu et al., 2001), (2) contributes to the development and maintenance of central sensitization (Wu et al., 2001; Haley et al., 1992; Malmberg and Yaksh, 1993; Meller and Gebhardt, 1993) and peripheral neuropathic pain (Levy et al., 1998, 1999), (3) is a pro-nociceptive mediator that synergizes with hyperalgesic prostaglandins in nociceptor sensitization (Aley et al., 1998), and importantly, (4) NO donating compounds induce hyperalgesia and migraine in humans, and hyperalgesia in non-human primates (NHP), and rodents (Aley et al., 1998; Holthusen and Arndt, 1995; Kawabata et al., 1994; Lin et al., 1999). Antinociceptive activity of structurally diverse iNOS-selective, as well as non-selective NOS inhibitors (Tang *et al*., 2007; Labuda *et al.*, 2005; Tao *et al*., 2003; DeAlba et al., 2006) provides further rationale for the pursuit of NOS inhibitors as therapeutics for pain and inflammation. Currently, one iNOS active site inhibitor, GW274150 is in clinical trials.

Of the three isoforms, iNOS and nNOS are the most intimately associated with inflammation and pain. Stoichiometrically, iNOS generates the highest amount of NO and an important role for iNOS in the pathophysiology of inflammatory and neuropathic pain is supported by its

expression in glia and perineural Schwann cells following peripheral nerve injury and at sites associated with axonal degeneration (Levy and Zochedne, 1998; 1999) and by a low, constitutive expression in the spinal cord of naïve animals with rapid upregulation upon injury (Tang et al., 2007). Chronic pain is often associated with persistent activation of N-methyl-D-aspartate (NMDA) receptor and downstream nNOS-derived production of NO, which in turn, augments further glutamate release, thus setting a stage for multisynaptic nociceptive processing in the spinal cord. NO derived from eNOS is critical for the maintenance of blood pressure and its inhibition leads to hypertension. Therefore, NOS inhibitor-based therapeutics must selectively inhibit iNOS and/or nNOS isoforms, while sparing eNOS.

Drug discovery efforts to identify inhibitors of iNOS and nNOS have largely focused on mimicry of arginine, the common substrate for all three isoforms and the efficacy of selective or non-selective inhibitors of iNOS that act via such mechanisms has been extensively examined in preclinical models of pain and inflammation (*vide supra*). However, biosynthesis of NO via NOS enzymes is a highly regulated process involving dimerization of enzymes, substrate and co-factor dependence (eg., tetrahydrobiopterin and calmodulin), the requirement of calcium for eNOS and nNOS, but not for iNOS, and regulation of expression of iNOS by inflammatory cytokines. Another level of post-translational regulation occurs via turnover of the dimer. While all NOS isoforms are active only as functional homodimers, the three isoforms appear to differ in the cellular turnover rates of their respective dimers. For example, in primary human bronchial epithelial cells, the cellular half-life for iNOS dimers was reported to be ~1.6 hr, which contrasts with reported values of 28 hr for eNOS and 20 hr for nNOS (Kolodziejski et al., 2004). The feasibility of inhibition or destabilization of, the iNOS dimerization process, a protein-protein interaction process, by small molecule ligands has been successfully demonstrated (Daway et al.,

2007; Symons et al., 2009). Rapid cellular turnover of the iNOS dimer suggests the possibility of achieving superior isoform selectivity by targeting the dimerization process. In vivo, the potential also exists for this novel mechanism of inhibition of NOS to engender a differential profile relative to substrate competitive inhibitors. A cell-based, high-throughput screen of recombinant human iNOS transiently expressed in human embryonic kidney (HEK) cells led to the identification of KLYP956, a non-imidazolylpyrimidine, quinolone inhibitor of iNOS that acts via inhibition or destabilization of, dimerization (Symons et al., 2009). Optimization of KLYP956 for desirable drug-like properties, culminated in the identification of KLYP961(Bonefous et al., 2009; Payne et al., 2010;Figure 1) and the present manuscript describes its pharmacological profile.

Methods

Materials

KLYP961 HCl salt, KLYP322, KLYP775, and celecoxib were synthesized at Kalypsys Inc (Bonefous et al., 2009; Payne et al., in press). AZ102222C (Labuda et al., 2005), a substrate competitive inhibitor and BBS-4 (Davey et al., 2007), a reference *N*-substituted imidazole-based iNOS dimerization inhibitor, were synthesized at Kalypsys as per published methods. The sources of other pharmacological agents are as follows: gabapentin (Sigma #126K1121), capsaicin (Sigma; # 045K7031), pregabalin (Batch #01/06), duloxetine HCl (NT009/05; both from QV Chemicals, St, Louis, MO), MK-801 (Sigma; #026K4625), and PBQ (Acros Organics, A0083952). All other laboratory reagents were of the highest quality commercially available.

In Vitro Studies

Molecular Cloning

Human and murine iNOS, nNOS, eNOS were cloned as previously described (Symons et. al 2009).

Cell Culture

RAW 264.7 (murine macrophage), and HEK293 cells (ATCC) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. RAW 264.7 cells were stimulated in media with lipopolysacharride (1 µg/ml) (Sigma) and mIFN-γ (100 U/ml) (Roche Diagnostics).

NOS Assay in Transiently-Transfected HEK293 Cells

HEK 293 cells were transiently transfected with a CMV-driven plasmid expressing a specific NOS isozyme. For iNOS, 10 µg of human, murine, rat, or rhesus iNOS expression plasmids and 30 µl of Fugene 6 were used. For eNOS, 15 µg of human, murine, or cynomolgus eNOS

expression plasmids and 45 µl of Fugene 6 were used. For nNOS, 10 µg of human, murine, rat, or cynomolgus nNOS expression plasmids and 30 µl of Fugene 6 were used. The effect of test compounds on NOS activity was assessed by measurement of accumulated nitrates in the tissue culture media using the diaminonaphthalene (DAN) assay as described earlier (Symons et al., 2009).

Cytochrome P450 Inhibition Assay

The potential for KLYP961 to inhibit human cytochrome P450 (CYP) enzymes was assessed in pooled human liver microsomes using isoform specific substrates as per methods summarized in *Supplemental Methods S1*.

Gel-based dimer assay (Low Temperature SDS-PAGE) for iNOS and nNOS

The impact of KLYP961 or other NOS inhibitors on murine iNOS dimer stability was assessed in the murine macrophage cell line (RAW 264.7) as per methods described earlier (Symons et al., 2009). Additional details are provided in the *Supplemental Methods S2*.

In Vivo Studies

Rodents

Male Balb/C and C57Bl6/j mice (iNOS and nNOS knock-out mice and appropriate age/sed matched wild type controls; weighing 19-25g were obtained from Charles River (Portage MI) and Jackson Labs (Ban Harbor, ME), respectively. All animals were acclimated to the Kalypsys vivarium for a minimum 5 days prior to use in the experiments that were conducted as per institutionally-approved animal care and use protocols.

Primates

Male Rhesus monkeys (*macaca mulatta* – 3 to 5 kg) were used in pharmacology studies and pharmacokinetic studies employed both male rhesus and cynomolgus non-human primates. All primate studies were conducted under a collaborative agreement between Kalypsys and Yunnan Laboratory Primate Laboratory Inc. (Kunming City, China) as per guidelines for primate health/welfare and use in animal experimentation implemented by relevant regulatory authorities. Additional studies were conducted at Biological Resources Laboratory of the University of Illinois-Chicago (BRL) under a collaborative research agreement between Kalypsys and CorDynamics and the study conduct was governed by USDA Animal Welfare Act and the ILAR Guide for the Care and Use of Laboratory Animals.

Drug Substance, Dose Formulations and Pharmacokinetic Studies

A HCl salt form of KLYP961 was used in all the studies described herein and doses refer to its neutral form. Details of oral and intravenous formulations, and methodology for pharmacokinetic profiling , are summarized in *Supplemental Methods S3*.

Pharmacology Studies

Effects of KLYP961 were examined in the mouse LPS model for inhibition of iNOS enzyme activty in vivo. Effects on pain processing were determined in a mouse formalin model, the CCI and Chung models, and in a primate model of capsaicin-induced thermal hyperalgesia. Effects on inflammation were assessed in carrageenan-inducd paw edema and hyperalgesia, and in PBQ-induced peritoneal writhing models. Detailed methods for these models are summarized *Supplemental Methods S4*.

Mouse LPS Test

Injection of LPS activates a cascade of inflammatory pathways leading to production of cytokines such as TNFα, Il-1 and IL-6, and induces enzymes such as iNOS. The latter is reflected in time-dependent increases in plasma nitrates.

Mouse Pain Studies

Formalin Assay: Three experiments were conducted in the mouse formalin model. The first experiment defined the potency of gabapentin and pregabalin, two clinically used benchmarks. The second experiment defined the potency of KLYP961 (3, 10, 30, 100 mg/kg). In both cases, all treatments were given orally 15 min prior to intraplantar injection of formalin. The third experiment defined the duration of action of KLYP961 (30 mg/kg) administered 0.25, 4 and 6 hr in advance of the formalin injection.

Nerve Injury Models: Initial experiments in the Bennett model explored mechanical allodynia and cold allodynia as the indices of nerve injury-evoked neuropathic pain state. As the acetone-induced cold allodynia was found to be more robust and consistent response than the mechanical allodynia, all efficacy studies used cold allodynia as the end point. In the Chung model, nerve ligation-induced tactile allodynia was assessed as the end point. In both models, dose-related effects of KLYP961were examined. In addition, studies were also conducted to examine if anti-nociceptive effects of KLYP961 tolerate upon repeated dosing. In these assays, the effects of KLYP961 were compared to selected reference compounds such as gabapentin, pregabalin and duloxetine.

Carrageenin-induced Paw Inflammation and Phenylbenzoquinone (PBQ)-induced Peritoneal Writhing Assays

Anti-inflammatory activity of KLYP961 (dose range: 10-300 mg/kg, po) was assessed in two pharmacological models: (1) λ-carrageenan-induced paw edema and thermal hyperalgesia, and (2) PBQ-induced peritoneal writhing. Celecoxib (dose range: 3-300 mg/kg, po) was used as the reference compound.

Primate Efficacy Studies

Capsaicin-induced Thermal Hyperalgesia in Rhesus non-human Primates

Effects of KLYP961, gabapentin and MK-801 on capsaicin-induced thermal hyperalgesia were examined in rhesus non-human primates based on the methodology developed by Butelman et al. (1992).

Effects of dizocilipine (MK-801)

Efficacy of MK-801 (0.06 mg/kg, sc) was evaluated on established thermal allodynia. Fifteen minutes after the removal of capsaicin, thermal allodynia was rated animals were randomized to receive either vehicle (N=2 animals) or MK-801 (1 mL/kg, saline solution; n=4 animals). The treatment-related effects were monitored over a 6 hr period.

Effects of gabapentin

Efficacy of gabapentin was evaluated in prophylactic mode of administration. In this paradigm, animals were given an oral dose of gabapentin (60 mg/kg/day; n=4 animals; dissolved in distilled water) or vehicle (distilled water; n=2 animals) for three consecutive days, via nasogastric tube. Baseline thermal withdrawal latencies were recorded on Days 2 and 3 and the average latency was used in defining treatment effect. On Day 3, forty-five minutes after the oral dose of either gabapentin or water, all animals received topical application of capsaicin and changes in

withdrawal latencies were determined. In a separate study, efficacy of a single dose of gabapentin (60 mg/kg, oral) was also evaluated. For this study, animals received either gabapentin (n=4) or water (n=3) 45 min prior to topical application of capsaicin.

Effects of KLYP961

Efficacy of KLYP961 was examined under two experimental conditions. In the first instance, four different doses (3, 10, 30 and 100 mg/kg; suspension) were orally administered via a nasogastric tube.. Control group of animals received appropriate vehicle (*Supplemental Section S3)*. The treatments were given 45 min before the capsaicin patch was applied. The entire experiment was completed in 4 cycles with two animals in the control group and four animals in each of the KLYP961-treatment groups, with at least 7days washout between cycles and in each cycle, animals were randomized and rotated between control and KLYP961 treatment groups. Pooled data from the control group of animals in the entire experiment (n=8) was used to determine relative effects of KLYP961 on allodynia.

In the second experiment, the efficacy of KLYP961 was evaluated on established thermal allodynia at a dose of 30 mg/kg, given orally. On the study day, the capsaicin patch was applied for 15 min and then removed (time zero). Thermal allodynia was assessed 45 min post-patch removal. Fifteen minutes later, ie., sixty min after capsaicin removal, animals received either vehicle (5 mL/kg, n=4 animals) or KLYP961 (30 mg/kg as a suspension; 5 mL/kg, n=7 animals) and allodynia was measured 1.5, 2, 3 and 4 hr (all times relative to time zero, ie., capsaicin patch removal).

Side Effect Profile Studies

Potential side effects of KLYP961 on gastrointestinal transit and motor coordination were explored in mouse models where as the impact on cardiovascular function was assessed in telemetered cynomolgus primates. The relevant methods are summarized in *Supplemental Methods S5*.

Gastrointestinal Transit Test in Mice

The impact of repeated administration of KLYP961 on gastric motility was assessed using the charcoal meal transit assay. KLYP961 was dosed at 15, 50 and 150 mg/kg, BID (total daily doses of 30, 100 and 300 mg/kg, po) for 6 days followed by one additional dose on day 7. Morphine sulfate was used as the reference compound and was dosed once (5.5 mg/kg, sc).

Motor Coordination: Mouse Rotorod

Effects of KLYP961 on motor coordination were assessed in the rotarod assay (Dunham and Miya, 1957).

Primate Safety Studies: Cardiovascular Studies in Telemetered Cynomolgus Primates

NO derived via e-NOS is essential to maintain vascular tone and its inhibition leads to dose-limiting increases in blood pressure. As eNOS enzyme has slow turnover, repeat dose, dose-escalation studies were conducted in telemetered cynomolgus non-human primates. The dose levels were 0 (vehicle), 23, 72 and 96 mg/kg/po/day of KLYP961, po (4 days at each level). The systemic hemodynamic variables of mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), and heart rate (HR) and several electrocardiographic parameters such PR interval, QRS duration, QT/QTc interval and arrhythmogenesis were examined continuously throughout the doing phase of this study.

Data Analyses

Data represent mean ± SD or SE and were analyzed by appropriate statistical tests (one-way or two-way analysis of variance [ANOVA] followed by post-hoc tests (Dunnett's/ Bonferroni's or t-test). Statistical significance relative either control or other treatments was inferred at a $p \leq 0.05$. The number of replicates is indicated and the time points for sampling that were used in AUC estimates (GraphPad Prism; GraphPad Inc, San Diego, CA) were identified in relevant figure legends.

Results

NOS Selectivity Profile

The inhibitory activity of KLYP961 against iNOS and nNOS and selectivity against e-NOS in various species was examined using nitrite measurements as surrogate for NOS activity and the results are summarized Table 1.

KLYP961 inhibits iNOS and nNOS with superior selectivity against e-NOS. The iNOS to eNOS selectivity ratios in human, primate and mouse enzymes are 184, 290 and 37, respectively. The iNOS to eNOS selectivity ratio in rat enzymes has not been determined. The iNOS to nNOS selectivity ratios in human, primate, rat and mouse enzymes are 3, 1.4, 0.04 and 0.07, respectively. KLYP961 exhibits species-dependent differences in iNOS/nNOS selectivity and potency; KLYP961 is a more potent murine nNOS vs iNOS inhibitor whereas the selectivity is reversed in humans or in primates.

Mechanism of NOS Inhibition: Inhibition of Dimerization

The inhibitory potencies for KLYP961 in cell-based assays were strongly influenced by the timing of its addition relative to NOS expression, i.e., its inclusion with cell lines during expression of NOS provides robust inhibition of NOS enzyme activity while incubation after NOS expression results in significantly reduced inhibition (e.g., 100% inhibition versus 15% inhibition at 100 μM, respectively). This inhibition signature is similar to that seen for the pyrimidine imidazole dimerization inhibitors and contrasts with substrate competitive inhibitors that show little change in potency or efficacy under these conditions (Symons et al., 2009).

Low temperature SDS PAGE provides a more direct means of investigating compound effects on the quaternary structure of NOS enzymes. Treatment with KLYP961 or the pyrimidine imidazole, BBS-4, but not SEITU (substrate competitive inhibitor) during the induction of iNOS in the murine RAW264.7 cells results in the appearance of higher order multimers in the KLYP961 treated samples accompanied by a reduction in dimeric enzyme (Figure 2A). These findings parallel results obtained with its parent molecule, KLYP956 (Symons et al.2009). As expected, all three inhibitors block NO production (Figure 2B). KLYP961 and BBS-4 also destabilize human iNOS protein-protein interactions under more native conditions. The experiment involved transient co-transfection of HEK293 cells with FLAG-tagged and HA-tagged human iNOS enzymes. Lysates prepared from cotransfected cells allows co-immunoprecipitation of the HA-tagged enzyme using anti-FLAG antibodies provided the enzyme is capable of dimerization (Figure 2A). Neither HA-tagged, nor untagged native human iNOS (UT), co-immunoprecipitated with anti-FLAG antibodies in the absence of coexpressed FLAG-tagged human iNOS (Figure 2C). While SEITU increases the amount of co-immunoprecipitated HA-iNOS, both KLYP961 and BBS-4 has substantially reduced levels of anti-HA immunoreactivity, consistent with a reduction in dimeric human iNOS. Enzymatic

activity from cell culture supernatants treated with SEITU (50 μM), BBS-4 (0.5 μM) and KLYP961 (0.5 μM) were all reduced by >95%, indicating that residual human iNOS-tagged heterodimer/multimers were inactive (Figure 2D). Collectively, these data indicate that KLYP961 interferes with iNOS dimer formation and/or destabilizes a dimer leading to preferential accumulation of functionally inactive iNOS.

Off-Target Activity Profile

The selectivity profile of KLYP961 was examined by determining it's interactions at a test concentration of 10 μM with a panel of fifty targets comprised of G-protein coupled receptors, ion channels, transporters of biogenic amines and enzymes such as monoamine oxidases A and B (MAO-A and MAO-B) and cyclooxygenases 1 and 2 (COX-1 and COX-2; CEREP Screen). Under these conditions, KLYP961 did not show any measurable interaction at any of the targets examined. These results summarized in *Supplemental Table S1*, indicate that KLYP961 is remarkably selective. In addition, in the concentration range of 10-30 μM, KLYP 961 was devoid of agonist or antagonist activity at the vanilloid receptor (VR1).

CYP Inhibition and Interaction with PXR

KLYP961 did not exhibit any appreciable inhibitory activity against the 6 human isoforms examined, CYP1A2, 2C8, 2C9, 2C19, 2D6 or 3A4. The estimated IC_{50} value for all targets exceeded 30 μM. Further, KLYP96, at concentrations as high as 30 μM, did not bind to human PXR /CAR in a biochemical assay (data not shown).

Microsomal Stability

KLYP961 showed species-dependent in vitro micosomal stability. The half-lives (min, mean ± SD, n=3) 345 ± 3 (mouse), 151 ± 16 (rat), 63 ±13 (dog), 160 ± 56 (cyno) and 277 ± 77 (human), respectively.

Plasma Protein Binding

KLYP961 showed species-dependent plasma protein binding differences. The binding was moderate: 84.4 % (mouse), 78% (rat), 37.8% (Cyno) and 75.3 % (human).

Pharmacokinetic Parameters in Mice and Rhesus Non-human Primates

Mice and rhesus non-human primates were employed as preclinical species to define antinociceptive effects of KLYP961. As such, pharmacokinetic profiles of KLYP961 following single dose administration were evaluated in these two species under fasting conditions. The results summarized in *Supplemental Table S2 and Supplemental Figure S1A/B)* indicate that KLYP961 is orally bioavailable, with approximately 60% oral bioavailability in both species. KLYP961 exhibits low systemic clearance (CL) both in mice and non-human primates, with systemic clearance generally less than 20% of hepatic blood flow in both species. KLYP961 exhibits substantially higher volume of distribution in primates (calculated Vss~4L/kg) vs mice (calculated Vss ~0.4 L/kg) and it is anticipated that relative differences in the plasma free fractions in these two species (fu = 62% vs. 16% in Cynomolgus monkey and mouse plasma, respectively). KLYP961 has a robust pharmacokinetic profile in all preclinical species examined. While the oral bioavailability was fairly similar, KLYP961 exhibited a longer half-life and larger volume of distribution in primates *vs* mice. The pharmacokinetic profile of KLYP961 in rats was fairly similar to that in mice (data not shown).

Based on brain to plasma level ratio of KLYP961 in mice, the brain penetration was estimated to be in the range of 1-2% (data not shown). Using a more refined technique of intravenous infusion of KLYP961 to achieve steady state levels and microdialysis of hippocampal parenchyma, brain penetration in rats was determined to be 1% (*Supplemental Results S6.1; Supplemental Figure S1C)* These results are also consistent with a lower volume of distribution and smaller plasma free fraction in rodents. The higher volume of distribution and larger plasma free fraction in primates suggests the possibility that KLYP961 may be more brain penetrant in this species.

Mouse Pharmacology Studies: LPS Assay

Orally administered KLYP961 attenuated LPS-induced increases in plasma nitrates in a dose-dependent manner with an ED_{50} value of 0.98 mg/kg (*Supplemental Results S6.2; Supplemental Figure S2A*). KLYP961 (30 mg/kg, po) inhibited the LPs plasma nitrate response by $\geq$ 50% for up to 12 hr. At doses as high as 100 mg/kg, KLYP961 did not affect LPS-induced inflammatory cytokine production (*Supplemental Figure S2C*).

Formalin Model

Orally administered KLYP961 attenuated formalin-induced nocifensive behaviors in a dose-related manner (Figure 3A and 3B). KLYP961 was more potent than gabapentin or pregabalin at attenuating both phases of nocifensive behaviors. The ED_{50} values (mg/kg, po) for inhibition of phase I behaviors by KLYP961, gabapentin and pregabalin respectively were 28 ([95% confidence intervals, CI]: 19-43), 142 (CI: 124-162) and 72 mg/kg (CI: 63-82), respectively. The corresponding ED_{50} values (mg/kg, po) for inhibition of phase II behaviors were 12.6 (CI: 9.7-16), 116 (CI: 105-128) and 72 (CI: 63-83), respectively.

The inhibitory effects of KLYP961 on formalin-induced pain behaviors showed time-dependency (Figure 3C); a 30 mg/kg dose significantly inhibited phase II behaviors for up to 4 hr whereas inhibition of phase I behaviors was more transient with only the 15 min pre-treatment being effective (two-way ANOVA: phases I & II, $F[1,26]=8.8$, $p < 0.0001$; time: $F [3,26]=72$, $p< 0.0001$; interaction: $F(3,26)=12$, $p < 0.0001$).

With a view to understand the causal relationship between NOS inhibition and efficacy of KLYP961in the formalin model, two approaches were used. The first used the chemical approach and the second, a genetic approach. Two structurally related analogs, KLYP322 and KLYP775 (Figure 1), differing only in their NOS inhibitory profiles, were evaluated in the formalin assay. The IC_{50} values to inhibit murine nNOS and iNOS for KLYP322 were 160 and 3600 nM, respectively. The corresponding values for KLYP775 were >10,000 and > 10,000 nM, respectively. At an oral dose of 50 mg/kg that resulted in plasma levels in excess of 20 μM at between 0.5-1 hr post dose, KLYP322, but not KLYP775, attenuated both phases of formalin-induced pain behaviors ($p<0.05$; Figure 3D).

In the second instance, effects of KLYP961 in the formalin assay were compared in iNOS or nNOS knockout mice on C57Bl6/j background relative to its profile in appropriate age- and gender- matched, C57Bl6/j wild type mice (data not shown). The degree of inhibition of phase II behaviors in both iNOS and nNOS knock-out animals was approximately 50% of that seen in C57Bl6/j wild-type control mice suggesting that inhibition of both isoforms contributes to the attenuation of formalin response by KLYP961(data not shown).

Neuropathic Pain Models in Mice

Chronic constrictive injury of sciatic nerve induces neuropathic pain state and acetone-induced cold allodynia was used as the end point. In time course experiments, KLYP961 (30 mg/kg, po), gabapentin (300 mg/kg), duloxetine (100 mg/kg, po), pregabalin (100 mg/kg, po) and morphine sulfate (3 mg/kg, sc) attenuated cold allodynia with a robust reduction in allodynia seen at 60-90 min post-dose (data not shown). Therefore, dose response curves for KLYP961, gabapentin, duloxetine were generated with allodynia measurements conducted 60 min post-dose whereas a 90 min time point post-dose was selected for pregabalin. KLYP961 and benchmark compounds, gabapentin, pregabalin and duloxetine, attenuated cold allodynia in a dose-related manner (Figure 4) with similar magnitudes of efficacy. KLYP961 was more potent than benchmarks: ED_{50} values in mg/kg (with confidence intervals, CI) for KLYP961, gabapentin, pregabalin and duloxetine were: 25 (CI:15-38), 254 (CI:117-519), 72 (CI:44-118) and 53 (CI:28-106), respectively.

Efficacy of KLYP961 was also assessed in Chung model of neuropathic pain induced by spinal nerve ligation. The experimental positive control, gabapentin attenuated tactile allodynia at the highest dose of 300 mg/kg, po (Figure 5A; dose, $F[2,48]=13.9$, $p<0.0001$; time, $F[3,48]=18$, $p<0.001$, interaction, $F[6,48]=14.1$, $p<0.05$). KLYP961 attenuated tactile allodynia at 30 and 100 mg/kg doses with a peak effect at 30 min (Figure 5B; dose, $F[3,104]=16.3$, $p<0.0001$; time, $F[3,104]=14$, $p<0.0001$, interaction, $F[9,104]=19$, $p<0.0001$). The estimated ED50 value for this effect was 30 mg/g (confidence interval: 11-37).

In preclinical models, analgesic actions of opiates show tolerance upon repeated dosing. With a view to understand if repeated administration of KLYP961 would lead to development of tolerance to its anti-allodynic actions, mice that underwent CCI surgery were repeatedly dosed with either KLYP961 (30 mg/kg, BID, 3d), gabapentin (300 mg/kg, BID, 3 days) or morphine

sulfate (2 mg/kg, sc, BID,3d). Animals received one additional dose approximately 16 hr after the last dose, ie., on day 4 for KLYP961 and morphine sulfate groups or day 3 for gabapentin group, and changes in allodynia were measured at pre-selected time points. While morphine-induced anti-allodynic effects tolerated quickly, the effects of KLYP961 or gabapentin did not tolerate. Similarly, the anti-allodynic effects of KLYP961 also did not show tolerance in the Chung model (*Supplemental Results S6.3, Supplemental Figures S3 and S4*).

Anti-inflammatory Activity

Carrageenan Model

Orally administered KLYP961and celecoxib attenuated carrageenan-induced edema and tactile allodynia in a dose-related manner (*Supplemental Figures S5A and S5B*) with comparable efficacy and potency. The calculated ED_{50} value for KLYP and celecoxib at inhibiting edema were 30 and 45 mg/kg, respectively. The corresponding values for tactile allodynia were 30 mg/kg, for both compounds.

Peritoneal Writhing

Intraperitoneal injection of PBQ, an irritant, produces writhing response. Orally administered KLYP961 attenuated writhing response with efficacy and potency comparable to that of celecoxib (*Supplemental Figure S5C*). The calculated ED_{50} value for both compounds in this assay was ~100 mg/kg.

Primate Efficacy Studies- Capsaicin-induced Thermal Allodynia

Chair-trained rhesus primates were used in these experiments; these primates, in the absence of capsaicin treatment, withdrew their tails from a 38°C water bath with a latency of 80-120 sec.

However, after topical application of capsaicin for 15 min, these animals show marked thermal allodynia, reflected in reduced tail withdrawal latencies of < 10 sec, typically 1-5 sec. The allodynic responses showed a time course with peak pain responses approximately 1 hr after the removal of the capsaicin patch. The animals recover from the allodynia over a period of 3-6 hr.

In this model, subcutaneous administration of MK-801 (0.06 mg/kg) led to marked attenuation of established allodynia (*Supplemental Results S6.4; Supplemental Figure S6A*). Repeated oral administration of gabapentin at a dose of 60 mg/kg, once a day for 2 days and a 3rd dose on day 3, prior to application of capsaicin patch, led to a significantly altered the time course of allodynia, whereas as single dose of gabapentin showed a trend for attenuation (*Supplemental Figure S6B/C*).

The dose-related effects of KLYP961 were examined as a pre-treatment to capsaicin in the thermal allodynia model. The lowest dose of 3 mg/kg was a no effect dose; as the dose was increased from 10-100 mg/kg, there was a dose-related attenuation of allodynia. At 10 and 30 mg/kg, KLYP961 treatment enhanced the recovery from thermal allodynia and at the highest dose of 100 mg/kg, thermal allodynia was nearly completely abolished (Figure 6A; treatment, $F[4,95]=28$, $p<0.0001$; time $[4,95]=28.7$, $p<0.0001$, interaction, $[16,95]=10.9$, $p=0.02$). The attenuation of thermal allodynia also reflected in significant improvements in AUC (Figure 6B; $F[4,19]=10.3$, $p<0.0001$).

The effects of KLYP961 on established thermal allodynia were examined at a test dose of 30 mg/kg (po). Thermal allodynia was assessed 45 min post-capsaicin patch removal, animals were given KLYP961 or vehicle 15 min later and allodynic responses were measured 1.5, 2, 3 and 4 hr post-capsaicin patch removal (all times relative to removal of the capsaicin patch). Compared to

vehicle treatment, KLYP961 markedly enhanced the rate of recovery of allodynia (Figure 7A; treatment, $F[1,64]=12.4$, $p<0.0001$; time $[5,64]=63.4$, $p<0.0001$, interaction, $[5,64]=10.2$, $p<0.0001$) and increased AUC ($t=4.3$, $df=10$, $p=0.0017$; Figure 7B). These results indicate the ability of KLYP961 to ameliorate established pain states.

Side Effect Profile Studies

Gastrointestinal Transit in Mice

The phenotype of neuronal nitric oxide synthase (nNOS) knockout mice (Mishimo 2000) combined with impairments in gastric motility reported with non-selective inhibitors of all three isoforms of NOS (Orihita and Sarna, 1994), implicate a role for No derived from NOS isoforms in the regulation of gastric motility. Given that KLYP961 is a dual inhibitor of nNOS and iNOS, the potential impact on gastric motility in a mouse model of charcoal transit was explored. Under sub-chronic conditions with supra-pharmacological doses, KLYP961 did not impact charcoal transit while the experimental positive control, morphine, markedly reduced charcoal transit (*Supplemental Results S6.5 and Supplemental Figure S7*).

Motor Coordination in Mice

Effects of single oral dose administration of KLYP961 on motor coordination were assessed using the rotarod assay with gabapentin as the reference compound. Gabapentin produced dose- and time-dependent impairments in motor coordination. The calculated ED_{50} value for gabapentin, based on its effects at 2 hr post-dose, was ~140 mg/kg. KLYP961 over the dose range of 100-1000 mg/kg, did not affect motor performance. Therefore, an ED_{50} value for KLYP961 could not be calculated (*Supplemental Results S6.6; Supplemental Figure S8A-C*).

Primate Safety Studies: Cardiovascular Studies in Telemetered Cynomolgus Primates

Under the conditions of the experiment, repeated daily dosing with KLYP961 at doses up to 96 mg/kg q.d. did not adversely affect any systemic cardiovascular hemodynamic or electrocardiographic variables in this model. There were no notable changes in mean arterial pressure (or its associated components, systolic and diastolic arterial pressures), PR interval, or QRS duration (data not shown).

Discussion

Drug discovery efforts to identify selective iNOS inhibitors have initially focused on mimetics of arginine. While many initial substrate mimetics had dose-limiting cardiovascular signals such as increased blood pressure via inhibition of e-NOS, substrate-competitive, time-dependent iNOS inhibitors such as GW274150 with optimum selectivity against e-NOS have been identified. The preclinical profile support a role for NO derived from iNOS both in inflammatory pain and neuropathic pain states (DeAlba et al., 2006). In addition to serving as a substrate for NOS isoforms, arginine also participates in physiologically important amino acid metabolic cycle. Substrate competitive inhibitors such as GW274150 can compete with arginine transport mechanisms (Baydoun et al., 2006) and long term consequences of interference with arginine transport/metabolism are unclear. Inhibition of iNOS dimerization provided another avenue for imparting greater isoform selectivity (Davey et al., 2007). BBS-4, one of the first generation dimerization inhibitors based on 2-imidazol-1-ylpyrimidine chemical scaffold, is also a potent inhibitor CYP 3A4, thus limiting its use as pharmacological probe. Similar limitations were also noted with chemotypes exemplified by AZ102222C. The pursuit for identification of highly selective pharmacological tools with appropriate drug-like properties culminated in the identification of KLYP961, whose profile is summarized in this investigation (Bonefous et al., 2009; Payne et al., 2010).

KLYP961 is a dual iNOS/nNOS inhibitor with selectivity against e-NOS ranging from approximately 40-fold in mice to 290-fold in primates and 180-fold in humans. Mechanistic studies indicate that KLYP961 affects dimerization of NOS and the results are consistent with mechanism of action of KLYP956, a structurally related compound (Symons et al., 2009).

Endotoxin injection in wild-type, but not iNOS knock-out mice, produces time-dependent induction of iNOS mainly in the liver, spleen and kidney accompanied by time-dependent increases in plasma nitrates. KLYP961 attenuated plasma nitrate response with an ED_{50} value of 1 mg/kg. Despite its pharmacokinetic half-life of ~2 hr, a dose of 30 mg/kg inhibited nitrate response by 50% for ~12hr suggesting a PK-pharmacodynamic dichotomy. Changes in plasma nitrates reflect systemic iNOS activity therefore the time course of changes in plasma nitrates is less likely to mirror plasma half-life of KLYP961.

KLYP961 attenuated both the acute inflammatory and the secondary nocifensive behavior driven by central sensitization in the formalin model and the efficacy was both dose- and time-dependent. KLYP961 was more potent at inhibiting Phase II behaviors than phase I behaviors. In the later, KLYP961 was equi-efficacious to, but more potent than, two clinically used agents, gabapentin and pregabalin. A single dose of 30 mg/kg inhibited phase II behavior for up to 4 hr whereas the inhibition of phase I was more transient. With a view to defining the role of NOS inhibition in the formalin response, two structurally-related compounds, KLYP322 and KLYP775 were examined that differed in their NOS inhibitory potency profiles. The concordance of efficacy of these two ligands with their NOS inhibitory profiles establishes a causal relationship between NOS inhibition and efficacy. KLYP961 is a dual inhibitor of iNOS and nNOS; while the precise contribution of inhibition of each isoform to its efficacy is unclear, similar level of reduction of antinociceptive effects of KLYP961 in both iNOS and nNOS knockout mice relative to wild type mice, in the formalin model, suggest that dual inhibition iNOS and nNOS plays a role in its efficacy.

Chronic constriction of the sciatic nerve (the Bennett model), leads to a neuropathic pain state evidenced by marked cold allodynia (Walczak and Beaulieu, 2006). In this model. KLYP961

was equi-efficacious to, but more potent than, three clinically used agents, duloxetine, pregabalin and gabapentin. Consistent with its effects in the Bennett model, KLYP961 also attenuated tactile allodynia in the Chung model. The anti-allodynic efficacy of KLYP961 did not show tolerance as was the case with morphine.

A comparison of potency of KLYP961 in various rodent assays reveals interesting features; the ED_{50} values (mg/kg) in endotoxin, formalin, the Bennett and the Chung models are: 1, 13, 25 and 29 mg/kg, respectively. Besides the shift in potency, the duration of action of KLYP961(30 mg/kg, po) was also different in these assays: plasma nitrates (~12 hr), formalin (~4 hr), Chung and Bennett (~60-90 min). The shift in potency/duration in pain models relative to "plasma nitrate" inhibition assay may reflect the necessity of near complete inhibition target mechanism for engendering efficacy in pain modality and/ or differential sites of action (eg., NOS inhibition in liver, spleen, lungs driving the inhibition of nitrate response *vs.* inhibition of NOS in pain pathways along the neuraxis, both central and peripheral). The limited brain penetration of KLYP961 and smaller volume of distribution in mice may also contribute to the dichotomy between plasma nitrate vs efficacy in pain models. Despite its limited CNS penetration, KLYP961 has demonstrable activity in neuropathic pain models, suggesting that a peripheral component in such models. The clinical utility of topically applied lidocaine, do attest to the role of peripheral mechanisms in human neuropathic pain states.

KLYP961 demonstrated anti-inflammatory activity in intraplantar carrageenin and intraperitoneal PBQ models with potency and efficacy comparable to that of celecoxib. The precise role and contribution of NO in the above acute inflammation models are unknown and the shift in potency for KLYP961 in these acute inflammation models relative its profile in nociception assays summarized above may reflect relative contributions of NO in such models.

Given the greater translational relevance of primate biology to humans, and in light of similarities between primate and human "challenge" pain models (Petersen and Rowbotham, 1999), we sought to examine the profile of KLYP961 in a capsaicin-induced thermal hyperalgesia model in rhesus non-human primates, a model that is sensitive to opioid and NMDA modulation (Butelman et al., 2003). The effects of MK-801 in the present study replicate earlier findings. The model is also sensitive to intervention by gabapentin with greater efficacy seen with repeated administration vs single pre-treatment, perhaps reflecting its pharmacokinetics. Qualitatively, gabapentin-treated animals showed a distinct time course of thermal allodynia relative to vehicle-treated animals in that later group experienced detectable allodynia only at the 1 hr time point with a relative lack of allodynia at all other time points. The time course profile of gabapentin indicates that it did not abrogate the development of thermal allodynia, but rather delayed the onset and markedly enhanced the recovery. A similar profile was seen for KLYP961; administration to animals with established allodynia resulted in significant enhancement in recovery. These results, *in toto*, suggest that KLYP961 is "anti-hyperalgesic" as opposed to "analgesic", as exemplified by opiate agonist-induced abrogation of allodynia in this model (Butelman et al., 2003).

KLYP961 has minimal off-target activity, desirable drug like properties such as pharmacokinetic profile, CYP-450and hERG activities. Acute or subchronic administration of (supra)pharmacological doses of KLYP961 was well tolerated both in mice and primates with acceptable side effect profile both in GI motility and cardiovascular function. In addition, KLYP961 has efficacy in a range of pain/inflammation models both in rodents and non-human primates. These attributes suggest that KLYP961 has a unique profile relative to known iNOS inhibitors described in the literature (Vallance and Leiper, 2002).The profile of KLYP961 makes

it an ideal tool with which to investigate therapeutic utility of iNOS and nNOS inhibition in humans in a variety of disease states where a causal role of NO has been implicated.

Authorship Contributions

Participated in research design: MEM, PR, AKS, SAN, NDS, CAH, YZ, TSR

Conducted experiments: KTS, PMN, JVA, LMS, LW, NY, SD, JM, MS, NR, CB, JEP

Performed data analysis: CAH, YZ, NDS

Wrote or contributed to the writing & review of the manuscript: NDS, CAH, SAN, AKS and TSR

References

Aley KO, McCarter G, and Levine JD (1998) Nitric oxide signaling in pain and nociceptor sensitization in the rat. *J Neurosci* **18**, 7008-7014.

Baydoun AR, Bertran J, Thakur S, Dawson J, Palacin M, Knowles RG (2006). Y+LaT-1 mediates transport of the potent and selective iNOS inhibitor, GW274150, in control J774 macrophages. *Amino Acids* **31**, 101-109.

Bennett GJ, and Xie YK (1988) A peripheral mononeuropathy in mouse that produces disorders of pain sensation like those seen in man. *Pain* **33,** 87-107.

Bonefous C, Payne JE, Roppe J, Zhuang H, Chen X, Symons KT. Nguyen PM, Sablad M, Rozenkrants N, Zhang Y, Wang L, Sevarence D, Walsh JP, Yazdani N, Shiau AK, Noble SA, Rix P, Rao TS, Hassig CA, Smith ND. Discovery of inducible nitric oxide synthase (iNOS) inhibitor development candidate KLYP961 (2009), Part 1: identification of a novel, potent, and selective series of quinolinone iNOS dimerization inhibitors that are orally active in rodent pain models *J Med Chem* **52**,3047-3062.

Butelman ER, Ball JW, Harris TJ, Kreek MJ (2003) Topical capsaicin-induced allodynia in unanesthetized primates: pharmacological modulation. *J Pharmacol Exp Ther* **306**, 1106-1114.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, and Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Meth* **53**, 55-63.

Davey,D.D. Adler M, Arnaiz D, Eagen K, Erikson S, Guilford W, Kenrikc M, Morrissey MM, Ohlmeyer M, Pan G, Paradkhar VM, Parkinson J, Polokoff M, Saionz K, Santos C, Subramnayam B, Vergona R, Wei RG, Whitlow M, Ye B, Zhao Z, Devlin JJ and Phillips G

(2007) Design, synthesis, and activity of 2-imidazol-1-ylpyrimidine derived inducible nitric oxide synthase dimerization inhibitors.*J Med Chem* **50**, 1146-1157.

DeAlba J, Clayton NM, Collins SD, Colthup P, Chessel I and Knowles RG (2006). GW274150, a novel and highly selective inhibitor of the inducible isoform of nitric oxide synthase (iNOS), shows analgesic effects in rat models of inflammatory and neuropathic pain. *Pain* **120**, 170-181.

Dunham NW and Miya TS (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *J Am Pharm Assoc* **46**, 208-209.

Goettl VM, Larson AA (1996). Nitric oxide mediates long-term hyperalgesic and antinociceptive effects of the terminus of substance P in the formalin assay in mice. *Pain* **67**, 435-41.

Haley JE, Dickenson AH, and Schachter M (1992) Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. *Neuropharmacol* **31**, 251-258.

Hendershot LC and Forsaith J (1958) Antagonism of the frequency of phenylbenzoquinone-induced writhing in the mouse by weak analgesics and non-analgesics., *J. Pharmacol Exp Ther* **125,** 237-240.

HolthusenH, and rndt JO (1994) Nitric oxide evokes pain in humans on intracutaneous injection. *Neurosci Lett* **165**, 71-74.

Jones BJ and Roberts DJ (1968). The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. *J Pharm Pharmacol* **20**, 302-304.

Kawabata A, Manabe S, Manabe Y, Takagi H (1994) Effect of topical administration of L-arginine on formalin-induced nociception in the mouse: a dual role of peripherally formed NO in pain modulation. *Br J Pharmacol* **12**, 547-50.

Kim SH, and Chung JM (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain **50,*** 355-63.

Kolodziejki PJ, Koo JS, and Eissa NT (2004) Regulation of inducible nitric oxide synthase by rapid cellular turnover and co-translational down-regulation by dimerization inhibitors. Proc Natl Acad Sci USA **101**, 18141-18146.

Labuda CJ, Koblish M, Tuthill P, Dolle RE, and Little PJ (2005). Antinociceptive activity of the selective iNOS inhibitor AR-C102222 in rodent models of inflammatory, neuropathic and post-operative pain. *Eur J Pain, **10**, 505-512.*

Levy D, and Zochodne DW (1998) Local nitric oxide synthase activity in a model of neuropathic pain. *Eur J Neurosci* **10**, 1846-1855.

Levy D, Hoke A, and Zochodne DW (1999) Local expression of inducible nitric oxide synthase in an animal model of neuropathic pain. *Neurosci Lett* **206**, 207-9.

Lin Q, Wu J, Peng YB, cui M and Willis WD (1999) Nitric oxide mediates the central sensitization of primate spinothalamic tract neurons. *J Neurophysiol* **81**, 1075-1085.

Malmberg AB, and Yaksh TL (1993) Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. *Pain* **54**, 291–300.

Mashimo H, Kjellin A, and Goyal R (2002) Gastric stasis in neuronal nitric oxide synthase deficient mice. *Gastroenterol* **119**, 766-773.

Meller ST, Gebhart GF (1993) Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain* **521**,127–36.

Orihata M, Sarna SK (1994) Inhibition of nitric oxide synthase delays gastric emptying of solid meals. *J Pharmacol Exp Ther* **271**, 660-670.

Payne JE, Bonnefous C, Symons KT, Nguyen PM, Sablad M, Rozenkrants N, Zhang Y, Wang L, Yazdani N, Shiau AK, Noble SA, Rix P, Rao TS, Hassig CA and Smith ND. Discovery of Dual inducible/neuronal Nitric Oxide Synthase (iNOS/nNOS) inhibitor Development Candidate KD7332 (Part 2): Identification of a Novel, Potent and Selective series of Benzimidazole-Quinolinone iNOS/nNOS Dimerization Inhibitors that are Orally Active in Pain Models. J. Med Chem. 2010 (in press).

Petersen KL, and Rowbotham MC (1999) A new human experimental pain model: the heat/capsaicin sensitization model. *Neuroreport* **10**, 1511-1516.

Symons KT, Massari ME, Nguyen PM, Roppe J, Bonfous C, Payne JE, Smith ND, Noble SA, Sablad M, Rozenkrants N, Zhang Y, Rao TS, Shiau AK and Hassig CA. KLYP956 is a non-imidazole-based orally active inhibitor of Nitric oxide synthase dimerization (2009). *Mol Pharmacol* **76**, 1-10.

Tang Q, Svensson CI, Fitzsimmons B, Webb M, Yaksh TL, and Hua XY (2007) Inhibition of spinal constitutive NOS-2 by 1400W attenuates tissue injury and inflammation-induced hyperalgesia and spinal p38 activation (2007) *Eur J Neurosci* **25**, 2964-2972.

Tao F, Tao YX, Mao P, Zhao C, Li D, Liaw WJ, Raja SN, and Johns RA.Intact carrageenan-induced thermal hyperalgesia in mice lacking inducible nitric oxide synthase (2003) *Neuroscience* **120**, 847-854

Vallance P and Leiper J (2002). Blocking NO synthesis: how, where and why? *Nat Rev Drug Discov* **1**, 939-950.

Walczak JS, and Beaulieu P (2006) Comparison of three models of neuropathic pain in mice using a new method to assess cold allodynia: The double plate technique. *Neurosci Lett* **399**, 240-244.

Wu J, Fang L, Lin Q, and Willis WD (2001). Nitric oxide synthase in spinal cord central sensitization following intradermal injection of capsaicin. *Pain* **94**, 47-58.

Yamamoto T, Shimoyama N, and Mizuguchi T (1993) Nitric oxide synthase inhibitor blocks spinal sensitization induced by formalin injection into the rat paw. *Anesth Analg* **77,** 886-90.

Footnotes

Current Affiliations:

Aragon Pharmaceuticals, San Diego, CA 92121 (CB, PR, NDS)

Dart Neuroscience, San Diego, CA 92121 (EM, LMS, KTS)

Conrad Prebys Chemical Genomics Center, Sanford Burnham Research Center, San Diego, CA92121

Ludwig Institute for Cancer Research, La Jolla, CA 92093 (JVA, AKS)

Johnson and Johnson PRD, San Diego, CA 92121-1126 (MS, NR, YZ and TSR)

Nitto Denko Technical Corporation, Carlsbad, CA 92058 (JEP)

Traversa Therapeutics, 10480 Wateridge Circle, San Diego, CA 92121-5773 (LMS)

Legends for Figures

Figure 1. Chemical Structure of KLYP961, KLYP722

Figure 2. KLYP961 affects murine or human iNOS dimer stability (Panels A and C, respectively). Enzyme activity was measured by quantittion of nitrates in the medium (panels C and D, respectively). Co-transfected FLAG/Flu samples were treated with either 0.5% DMSO (Veh), 50 μM SEITU, 0.5 μM BBS-4 or 10μM KLYP961. Western blot analysis with a Flu-specific monoclonal antibody reveals dimeric human iNOS after FLAG immunoprecipitation (panel C). The crude lysate input is included as a control for the amount of Flu tagged iNOS that was present prior to immunoprecipitation (indicated in the bottom western blot). Untagged and Flu tagged alone were treated with 0.5% DMSO and are included as controls. Molecular mass is indicated in kilodaltons (kDa). The expected molecular mass of human iNOS is ~130 kDa.

Figure 3. KLYP961, gabapentin and pregabalin dose-dependently attenuate formalin-induced pain behaviors (Phase I [Panel A] and Phase II [Panel B]). KLYP961 (30 mg/kg, po) attenuates formalin-induced phase II behaviors for up to 4 hr with a transient effect on phase I (Panel C). Inhibitory potency of KLYP322 (IC_{50}: 3600 nM [murine iNOS] and IC_{50}: 160 nM [murine nNOS]) and KLYP775 (IC_{50} values of >10,000 nM for both iNOS and nNOS) tracks well with inhibition of pain behaviors (Panel D). Both compounds were dosed orally 30 min in advance of formalin and data represents phase II behavior. Data represent mean $\pm$ SE (n=5-6 all groups except KLYP961 [100 mg/kg], n=3) *, $p<0.05$ vs vehicle (Veh).

Figure 4. Orally administered KLYP961, duloxetine, pregabalin and gabapentin attenuate cold allodynia induced by chronic constrictive nerve injury (CCI model). All compounds were administered 60 min prior to assessment of cold allodynia. Cold allodynia was assessed by monitoring pain behaviors in response to acetone spray. Data represent mean $\pm$ SE (n=5-7).

Figure 5. Orally administered gabapentin (Gbp; Panel A) and KLYP961 (Panel B) attenuate tactile allodynia response following spinal nerve ligation (Chung model). Tactile allodynia was assessed by monitoring paw withdrawal in response to von Frey filaments. Data represent mean ± SE (n=5-6). **, $p<0.01$ and ***, $p<0.001$ all relative to vehicle (2-Way ANOVA followed by appropriate post-hoc test).

Figure 6. Topical application of capsaicin induced thermal hyperalgesia is responsive to KLYP961. Baseline tail withdrawal latencies were measured from 38°C water bath the day before the capsaicin application. Following capsaicin application for 15 min (referred to as time zero), tail withdrawal latencies were measured at various times starting at 30 min. Vehicle or KLYP961 were given 60 min before the application of capsaicin patch. Baseline withdrawal latency of each animal was normalized to 100 %. Areas under the curve (AUC) data were calculated using trapezoidal method (GraphPad Prizm). Two animals received vehicle and 4 animals received KLYP961 at each dose level. Animals were given a minimum of 7 days washout prior to inclusion in next dose level testing. Animals were assigned to vehicle or KLYP961 treatments with a cross-over between treatments. Withdrawal responses in vehicle treated animals were pooled. * $p<0.05$**, $p<0.01$, ***, $p<0.001$ vs vehicle group (Two-way ANOVA for time course(panel A) or one way ANOVA (AUC, Panel B).

Figure 7. KLYP961 attenuates capsaicin-induced thermal allodynia when administered in a therapeutic paradigm. Baseline tail withdrawal latencies were measured from 38°C water bath the day before the capsaicin application. Following the capsaicin application for 15 min (referred to as time zero), tail withdrawal latencies were measured at various times starting at 45 min (0.75 hr). Vehicle or KLYP961 (30 mg/kg) were given at 1 hr post-capsaicin and withdrawal latencies were measured at selected times thereafter. Baseline withdrawal latency of each animal was

normalized to 100 %. Area under the curve (AUC) data was calculated using trapezoidal method (GraphPad Prizm). Four animals received vehicle and 6 animals received KLYP961 at 30 mg/kg. Animals were given a minimum of 7 days washout prior to inclusion in next dose level testing. Animals were assigned to vehicle or KLYP961 treatments with a cross-over between treatments. Withdrawal responses in vehicle treated animals were pooled. * $p<0.05$**, $p<0.01$, ***, $p<0.001$ vs vehicle group (Two-way ANOVA for time course (panel A) or one way ANOVA (AUC, Panel B).

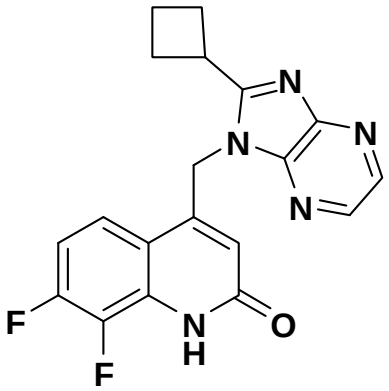
Tables

Table 1. Inhibitory activity of KLYP961 on NOS isoforms

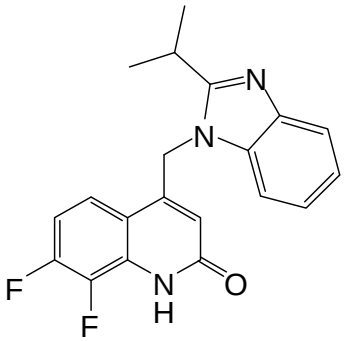
Human NOS (IC_{50}; μM)			Non-human Primate NOS (IC_{50}; μM)			Rat NOS (IC_{50}; μM)		Mouse NOS (IC_{50}; μM)		
iNOS	eNOS	nNOS	iNOS†	eNOS‡	nNOS†	iNOS	nNOS	iNOS	eNOS	nNOS
0.09 ± 0.04	16.6 ± 6.0	0.30 ± 0.14	0.05 ± 0.02	14.5 ± 4.0	0.07 ± 0.06	1.7 ± 0.66	0.06 ± 0.02	0.43 ± 0.36	16.0 ± 9.9	0.03 ± 0.02
N=28*	N=20	N=20	N=5	N=2	N=3	N=4	N=2	N=15	N=3	N=3

*, number of experiments each run in triplicates/quadruplicates

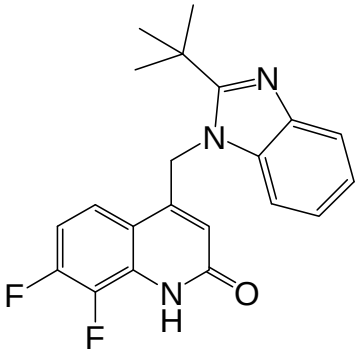
Figure 1



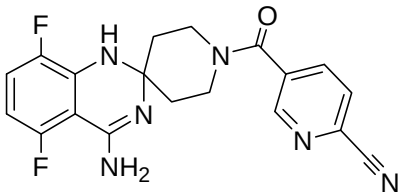
KLYP961



KLYP 322



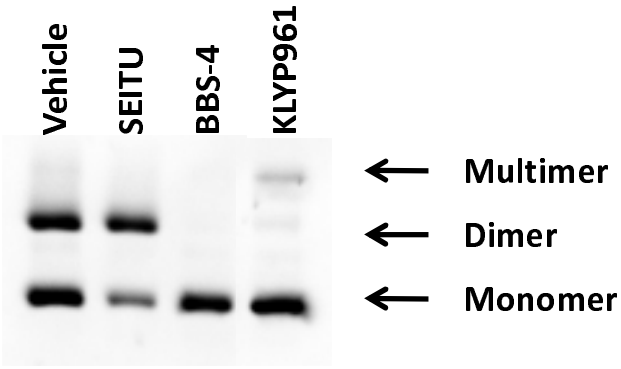
KLYP 775



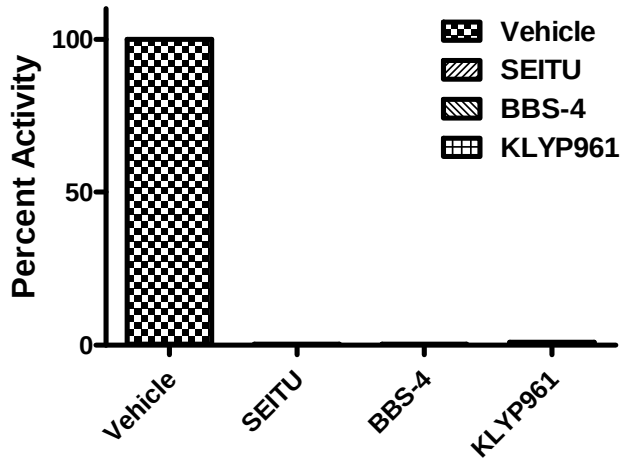
AZ1022222c

Figure 2

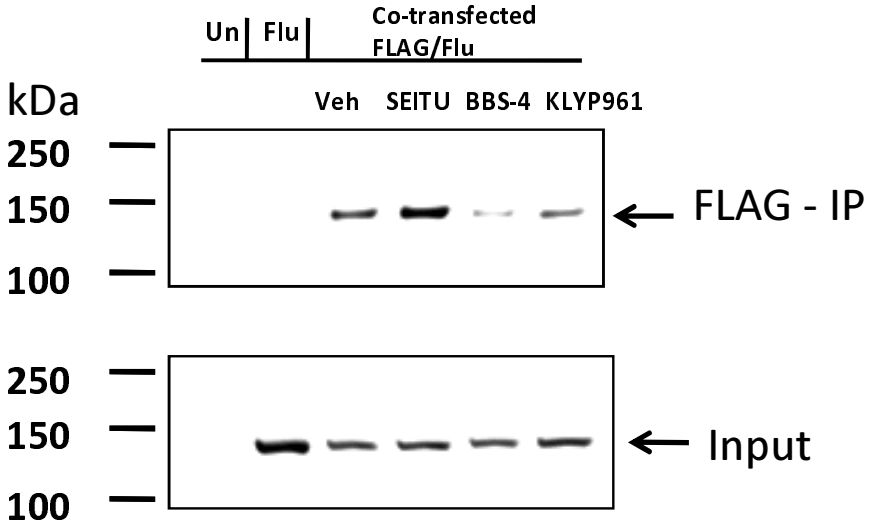
A. Murine iNOS



B.



C. Human iNOS



D.

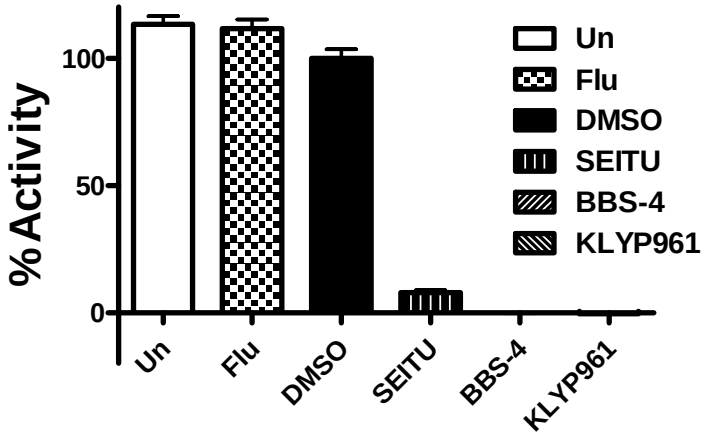


Figure 3

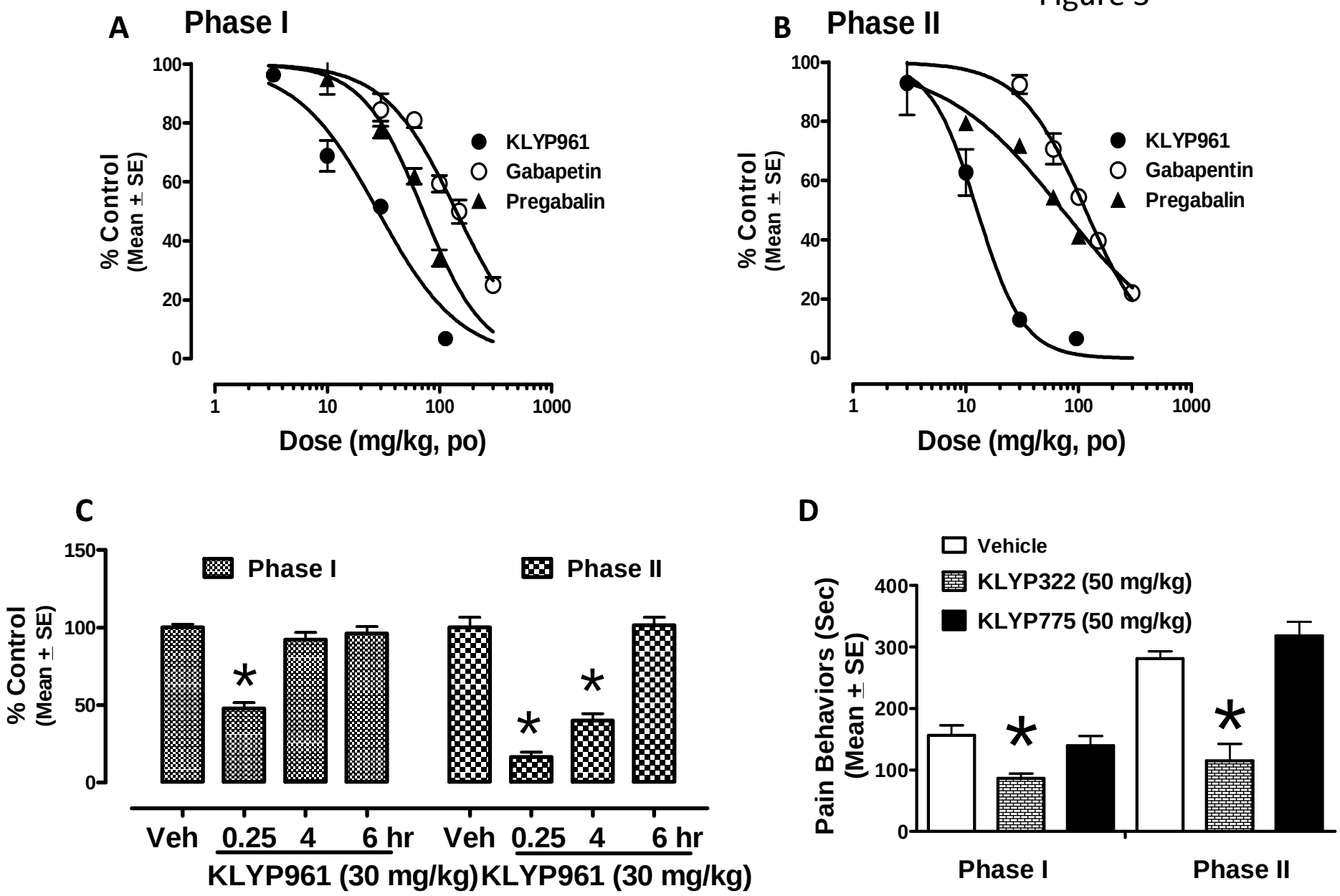

A
Phase I
% Control
(Mean ± SE)
100
80
60
40
20
0
1
10
100
1000
Dose (mg/kg, po)
KLYP961
Gabapetin
Pregabalin
B
Phase II
% Control
(Mean ± SE)
Dose (mg/kg, po)
KLYP961
Gabapentin
Pregabalin
C
% Control
(Mean ± SE)
150
100
50
0
Phase I
Phase II
Veh
0.25
4
6 hr
Veh
0.25
4
6 hr
KLYP961 (30 mg/kg)
KLYP961 (30 mg/kg)
D
Pain Behaviors (Sec)
(Mean ± SE)
400
300
200
100
0
Vehicle
KLYP322 (50 mg/kg)
KLYP775 (50 mg/kg)
Phase I
Phase II

Figure 4

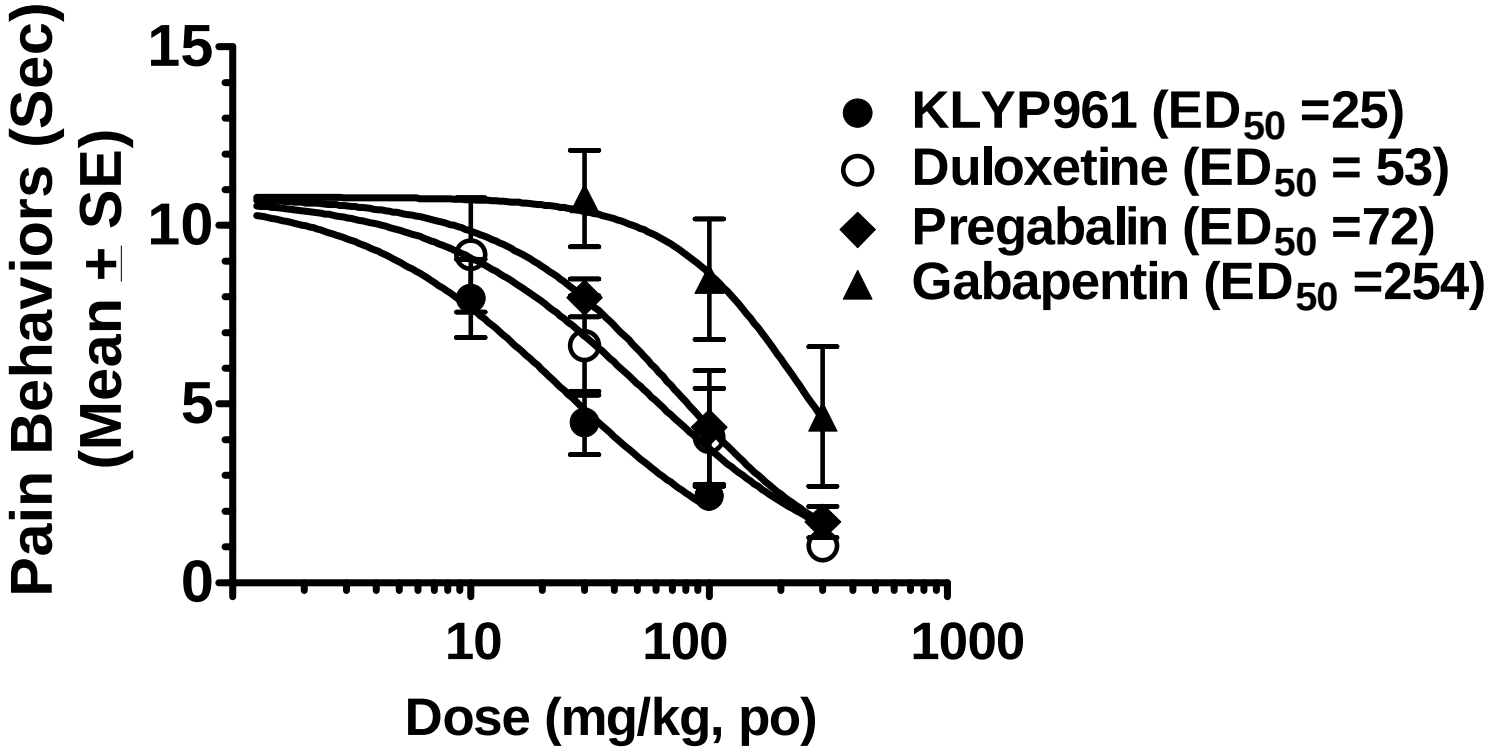

Pain Behaviors (Sec)
(Mean ± SE)
15
10
5
0
10
100
1000
Dose (mg/kg, po)
KLYP961 (ED50 =25)
Duloxetine (ED50 = 53)
Pregabalin (ED50 =72)
Gabapentin (ED50 =254)

Figure 5

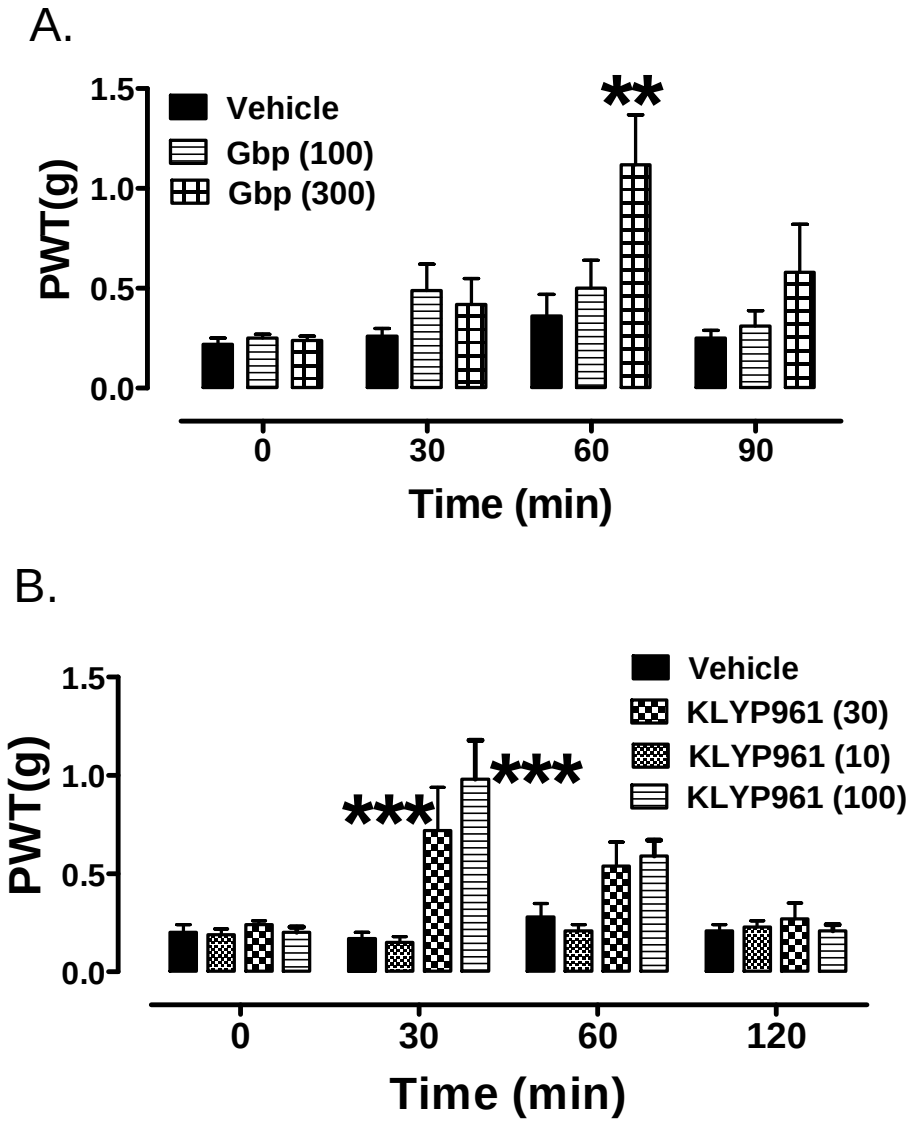

A.
1.5
1.0
0.5
0.0
PWT(g)
Vehicle
Gbp (100)
Gbp (300)
**
0
30
60
90
Time (min)
B.
1.5
1.0
0.5
0.0
PWT(g)
Vehicle
KLYP961 (30)
KLYP961 (10)
KLYP961 (100)
***
***
0
30
60
120
Time (min)

Figure 6

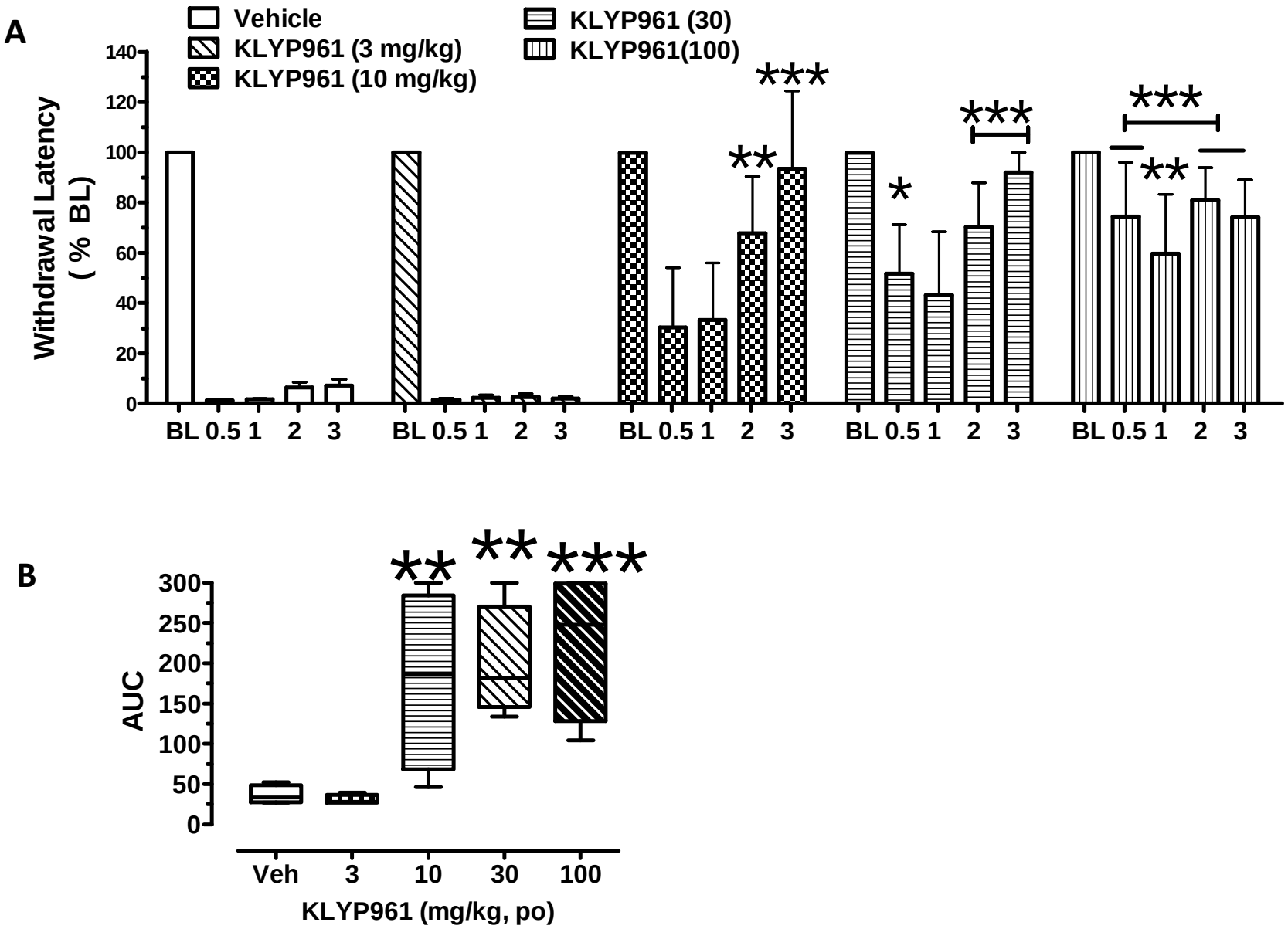
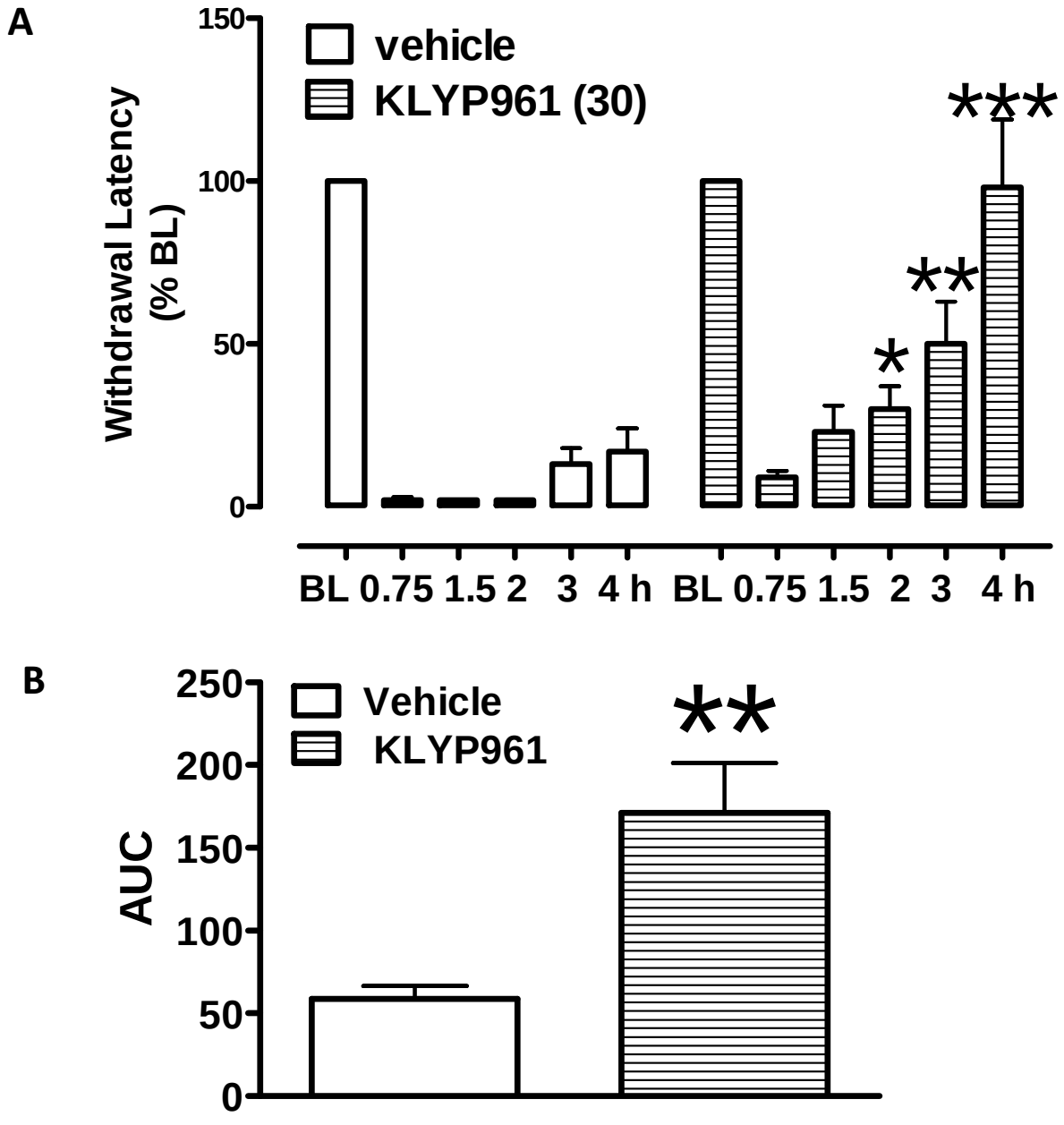

A
Vehicle
KLYP961 (3 mg/kg)
KLYP961 (10 mg/kg)
KLYP961 (30)
KLYP961(100)
Withdrawal Latency
(% BL)
140
120
100
80
60
40
20
0
BL 0.5 1 2 3
BL 0.5 1 2 3
BL 0.5 1 2 3
BL 0.5 1 2 3
BL 0.5 1 2 3
**
***
*
***
**
***
B
AUC
300
250
200
150
100
50
0
**
**
***
Veh
3
10
30
100
KLYP961 (mg/kg, po)

Figure 7


A
150
100
50
0
Withdrawal Latency
(% BL)
vehicle
KLYP961 (30)
*
**
***
BL 0.75 1.5 2 3 4 h BL 0.75 1.5 2 3 4 h
B
250
200
150
100
50
0
AUC
Vehicle
KLYP961
**