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

Kent T. Symons,¹ Phan M. Nguyen, Mark E. Massari,¹ John V. Anzola,² Lena M. Staszewski,³ Li Wang, Nahid Yazdani, Steven Dorow, Jerry Muhammad,⁴ Marciano Sablad,⁵ Natasha Rozenkrants,⁵ Celine Bonefous,⁶ Joseph E. Payne,⁷ Peter J. Rix,⁹ Andrew K. Shiau,⁵ Stewart A. Noble, Nicholas D. Smith,⁶ Christian A. Hassig,⁸ Yan Zhang,⁵ and Tadimeti S. Rao⁵


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ABSTRACT
Nitric oxide (NO) derived from neuronal nitric-oxide synthase (nNOS) and inducible nitric-oxide synthase (iNOS) plays a key role in various pain and inflammatory states. KLYP961 (4-((2-cyclobutyl-1H-imidazo[4,5-b]pyrazin-1-yl)methyl)-7,8-difluoroquinolin-2(1H)-one) inhibits the dimerization, and hence the enzymatic activity of human, primate, and murine iNOS and nNOS (IC₅₀ values 50–400 nM), with marked selectivity against endothelial nitric-oxide synthase (IC₅₀ >15,000 nM). It has ideal drug-like properties, including excellent solubility and stability in human plasma, and potent anti-inflammatory and neuroprotective activity. In mice, KLYP961 attenuates endotoxin-evoked increases in plasma nitrites, a surrogate marker of iNOS activity in vivo, in a sustained manner (ED₅₀ 1 mg/kg p.o.). KLYP961 attenuated pain behaviors in a mouse formalin model (ED₅₀ 13 mg/kg p.o.), cold allodynia in the chronic constriction injury model (ED₅₀ 25 mg/kg p.o.), or tactile allodynia in the spinal nerve ligation model (ED₅₀ 30 mg/kg p.o.) with similar efficacy, but superior potency relative to gabapentin, pregabalin, or duloxetine. Unlike morphine, the antiallodynic activity of KLYP961 did not diminish upon repeated dosing. KLYP961 also attenuated carrageenan-induced edema and inflammatory hyperalgesia and writhing response elicited by phenylbenzoquinone with efficacy and potency similar to those of celecoxib. In contrast to gabapentin, KLYP961 did not impair motor coordination at doses as high as 1000 mg/kg p.o. KLYP961 also attenuated capsaicin-induced thermal allodynia in rhesus primates in a dose-related manner with a minimal effective dose (≤10 mg/kg p.o.) 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 (NOSs), 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 NO has been implicated in multiple human pathologies such as pain, inflammation, arthritis, asthma, chronic obstructive pulmonary disease, migraine, and neurodegenerative disorders (Vallance and Leiper, 2002). Of particular relevance to pain and inflammation

¹Current affiliation: Dart Neuroscience, San Diego, California.
²Current affiliation: Ludwig Institute for Cancer Research, La Jolla, California.
³Current affiliation: Alphavera, Georgia.
⁴Current affiliation: Johnson and Johnson Pharmaceutical Research and Development, San Diego, California.
⁵Current affiliation: Aragon Pharmaceuticals, San Diego, California.
⁶Current affiliation: Carlsbad, California.
⁷Current affiliation: Carlsbad, California.
⁸Current affiliation: San Diego, California.
⁹Current affiliation: San Diego, California.

ABBREVIATIONS: NOS, nitric-oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; CCI, chronic constrictive nerve injury; PBQ, phenylbenzoquinone; BBS-4, (R)-1-(2-(1H-imidazol-1-yl)-6-methylpyrimidin-4-yl)-N-[2-(benzo[d][1,3]dioxol-5-yl)ethyl]pyrrolidine-2-carboxamide; HEK, human embryonic kidney; SEITU, 2-ethyl-2-thiopseudourea hydrobromide; LPS, lipopolysaccharide; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; ANOVA, analysis of variance; AUC, areas under the curve; DMSO, dimethyl sulfoxide; KLYP961, N-(3-chlorophenyl)-N-((8-fluoro-2-oxo-1,2-dihydroquinolin-4-yl)methyl)-4-methylthiazole-5-carboxamide; HA, hemagglutinin; CI, confidence interval; KLYP961 (4-((2-cyclobutyl-1H-imidazo[4,5-b]pyrazin-1-yl)methyl)-7,8-difluoroquinolin-2(1H)-one; GW274150, (2-[(1-minoethyl)aminomethyl]L-homocysteine).
tion, 1) NO is involved in the transmission and modulation of nociceptive information at peripheral, spinal, and supraspinal levels (Yamamoto et al., 1993; Goettl and Larson, 1996; Wu et al., 2001), 2) NO contributes to the development and maintenance of central sensitization (Haley et al., 1992; Malnberg and Yaksh, 1993; Meller and Gebhart, 1993; Wu et al., 2001) and peripheral neuropathic pain (Levy and Zochodne, 1998; Levy et al., 1999), 3) NO is a pronociceptive 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 nonhuman primates and rodents (Holthusen and Arndt, 1994; Kawabata et al., 1994; Aley et al., 1998; Lin et al., 1999). Antinociceptive activity of structurally diverse iNOS-selective inhibitors, as well as nonselective NOS inhibitors (Tao et al., 2003; De Alba et al., 2006; LaBuda et al., 2006; Tang et al., 2007), provides further rationale for the pursuit of NOS inhibitors as therapeutics for pain and inflammation. Currently, one iNOS active-site inhibitor, GW274150, (2-((1-iminoethyl) amino)ethyl)amide (KLYP956), a nonimidazolylpyrimidine, quinolone in- 

Materials and Methods

**Materials**

KLP961 HCl salt, KLYP322, KLYP775, and celecoxib were synthesized at Kalypsys Inc (Bonefous et al., 2009; Payne et al., 2010). AZ102222C (LaBuda et al., 2006), a substrate competitive inhibitor, and (R)-1-(2-(1H-imidazol-1-yl)-6-methylpyrimidin-4-

![Fig. 1. Chemical structures of KLP961 (top left), KLYP322 (top center), KLYP775 (top right), and AZ102222C (bottom).](image-url)
were conducted per institutionally approved animal care and use
varium for a minimum of 5 days before use in the experiments that
weighing 19–25 g) were obtained from Charles River Breeding Lab-
knockout mice and appropriate age-/sex-matched wild-type controls;
In Vivo Studies
bovine serum, 100 U/ml penicillin, and 100

In Vitro Studies
Molecular Cloning. Human and murine iNOS, nNOS, and eNOS
were cloned as described previously (Symons et al., 2009).
Cell Culture. RAW 264.7 (murine macrophage) and HEK293
cells (American Type Culture Collection, Manassas, VA) were cul-
tured in Dulbecco’s modified Eagle’s medium containing 10%

NOS assay in Transiently Transfected HEK293 Cells. HEK293
cells were transiently transfected with a cytomegalovirus-
driven plasmid expressing a specific NOS isozyme. For iNOS, 19 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
cy nomoglus eNOS expression plasmids and 45 µl of Fugene 6 were
used. For nNOS, 10 µg of human, murine, rat, or cyclomolgus 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 diamin-
onaphthalene assay as described previously (Symons et al., 2009).

Cytchrome P450 Inhibition Assay. The potential for
KLYP961 to inhibit human cytochrome P450 enzymes was assessed
in pooled human liver microsomes using isofrom-specific substrates
as summarized in Supplemental Methods S1.

Gel-Based Dimer Assay (Low-Temperature SDS-Polyacryl-
amide Gel Electrophoresis) for iNOS and nNOS. The impact of
other NOS inhibitors on murine iNOS dimer stability was
assessed in the murine macrophage cell line (RAW 264.7) as
described previously (Symons et al., 2009). Additional details are
provided in Supplemental Methods S2.

In Vivo Studies
Rodents. Male BALB/c and C57BL/6j mice (iNOS and nNOS
dudoxin knockout mice and appropriate age-/sex-matched wild-type controls;
weighing 19–25 g) were obtained from Charles River Breeding Lab-
laboratories (Portage, MI) and The Jackson Laboratory (Bar Harbor,
ME), respectively. All animals were acclimated to the Kalypsys
vivarium for a minimum of 5 days before use in the experiments that
were conducted per institutionally approved animal care and use
protocols.

Primates. Male Rhesus monkeys (Macaca mulatta; 3–5 kg) were
used in pharmacology studies, and pharmacokinetic studies used
both male rhesus and cylnomolgus nonhuman primates. All primate
studies were conducted under a collaborative agreement between
Kalypsys and Yunnan Laboratory Primate Laboratory Inc. (Kun-
ming City, China) per guidelines for primate health/welfare and use
in animal experimentation implemented by relevant regulatory au-
thorities. Additional studies were conducted at the Biological Re-
course Laboratory of the University of Illinois (Chicago, IL) under
a collaborative research agreement between Kalypsys and CoDynam-
ics (Chicago, IL), and the study was governed by the U.S. Drug
Administration Animal Welfare Act and the Institute of Laboratory
Animal Research 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 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. The effects of KLYP961 were examined in
the mouse LPS model for the inhibition of iNOS enzyme activity in
vivo. Effects on pain processing were determined in a mouse formalin
model, the chronic constriction nerve injury (CCI) model, the Chung
model (Kim and Chung, 1992), and a primate model of capsaicin-
induced thermal hyperalgesia. The effects on inflammation were
assessed in carrageenan-induced paw edema and hyperalgesia and in
PBQ-induced peritoneal writhing models. Detailed methods for
these models are summarized in Supplemental Methods S4.

Mouse LPS Test. Injection of LPS activates a cascade of inflam-
atory pathways, leading to production of cytokines such as tumor
necrosis factor α, interleukin-1, and interleukin-6, and induces en-
zymes such as iNOS. The latter is reflected in time-dependent in-
creases 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, and
100 mg/kg). In both cases, all treatments were given orally 15 min
before intraplantar injection of formalin. The third experiment de-

erved thermal allodynia. Fifteen
MK-801 (1 ml/kg, saline solution;

Effects of KLYP961 were examined. In addition, studies were con-
ducted to examine whether antinociceptive effects of KLYP961 in
nerve injury tolerate upon repeated dosing. In these assays, the
effects of KLYP961 were compared with selected reference com-
ounds such as gabapentin, pregabalin, and duloxetine.

Carrageenin-Induced Paw Inflammation and Phenylben-
ziquinone-Induced Peritoneal Wring Assays. Anti-inflam-

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

Primate Efficacy Studies
Capsaicin-Induced Thermal Hyperalgesia in Rhesus Non-
human Primates. The effects of KLYP961, gabapentin, and MK-
801 on capsacin-induced thermal hyperalgesia were examined in
rhesus nonhuman primates based on the methodology developed by
Butelman et al. (2003).

Effects of Dizocilipine (MK-801). Efficacy of MK-801 (0.06
mg/kg s.c.) was evaluated on established thermal allodynia. Fifteen
minutes after the removal of capsacin 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 6 h.

Effects of Gabapentin. Efficacy of gabapentin was evaluated in
prophylactic mode of administration. In this paradigm, animals were
given an oral dose of gabapentin (dissolved in distilled water; 60
mg/kg/day; n = 4 animals) or vehicle (distilled water; n = 2 animals)
for 3 consecutive days, via nasogastric tube. Baseline thermal with-

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drawal latencies were recorded on days 2 and 3, and the average latency was used in defining treatment effect. On day 3, 45 min 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 p.o.) was also evaluated. For this study, animals received either gabapentin (n = 4) or water (n = 3) 45 min before 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. The 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 four cycles with two animals in the control group and four animals in each of the KLYP961 treatment groups, with at least 7 days of washout between cycles. 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) were 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 0). Thermal allodynia was assessed 45 min after patch removal. Fifteen minutes later, i.e., 60 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 after 1.5, 2, 3, and 4 h (all times relative to time 0, i.e., capsaicin patch removal).

**Side Effect Profile Studies.** Potential side effects of KLYP961 on gastrointestinal transit and motor coordination were explored in mouse models, whereas 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 b.i.d. (total daily doses of 30, 100 and 300 mg/kg p.o.) 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 s.c.).

**Motor Coordination: Mouse Rotorod.** Effects of KLYP961 on motor coordination were assessed in the rotorod assay (Dunham and Miya, 1957).

**Primate Safety Studies: Cardiovascular Studies in Telemetered Cynomolgus Primates.**

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

**Data Analyses.**

Data represent mean ± S.D. or S.E. 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 to either control or other treatments was inferred at p ≤ 0.05. The number of replicates is indicated, and the time points for sampling that were used in areas under the curve (AUC) estimates (Prism; GraphPad Software Inc., San Diego, CA) are identified in relevant figure legends.

**Results**

**NOS Selectivity Profile.**

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

KLYP961 inhibits iNOS and nNOS with superior selectivity against eNOS. The iNOS to eNOS selectivity ratios in human, primate, and mouse enzymes were 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 were 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 versus 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, whereas 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-polyacrylamide gel electrophoresis 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 2-ethyl-2-thiopeudourea hydrobromide (SEITU) (substrate competitive inhibitor), during the induction of iNOS in the murine RAW264.7 cells resulted in the appearance of

<table>
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<tr>
<th>Human NOS</th>
<th>Nonhuman Primate NOS</th>
<th>Rat NOS</th>
<th>Mouse NOS</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>eNOS</td>
<td>nNOS</td>
<td>iNOS</td>
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<tr>
<td>(n = 28)</td>
<td>(n = 20)</td>
<td>(n = 20)</td>
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<td>0.09 ± 0.04</td>
<td>16.6 ± 6.0</td>
<td>0.30 ± 0.14</td>
<td>0.05 ± 0.02</td>
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* Rhesus.
* Cynomolgus.

**TABLE 1**

Inhibitory activity of KLYP961 on NOS isoforms

Numbers of experiments run in triplicate/quadruplicate are shown in parentheses.
higher-order multimers in the KLYP961-treated samples accompanied by a reduction in dimeric enzyme (Fig. 2A). These findings parallel results obtained with its parent molecule, KLYP956 (Symons et al., 2009). As expected, all three inhibitors block NO production (Fig. 2B). KLYP961 and BBS-4 also destabilize human iNOS protein-protein interactions under more native conditions. The experiment involved transient cotransfection of HEK293 cells with FLAG-tagged and HA-tagged native human iNOS enzymes. Lysates prepared from cotransfected cells allows coimmunoprecipitation of the HA-tagged enzyme using anti-FLAG antibodies provided the enzyme is capable of dimerization (Fig. 2A). Neither HA-tagged nor untagged native human iNOS coimmunoprecipitated with anti-FLAG antibodies in the absence of coexpressed FLAG-tagged human iNOS (Fig. 2C). Whereas SEITU increases the amount of coimmunoprecipitated HA-iNOS, both KLYP961 and BBS-4 have 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) all were reduced by >95%, indicating that residual human iNOS-tagged heterodimer/multimers were inactive (Fig. 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 its interactions at a test concentration of 10 μM with a panel of 50 targets comprised of G protein-coupled receptors, ion channels, and transporters of biogenic amines and enzymes such as monoamine oxidases A and B and cytochrome P450s, CYPs (Table S1). A murine iNOS western blot (Fig. 2A) indicates that KLYP961 is remarkably selective. In addition, in the concentration range of 10 to 30 μM KLYP961 was devoid of agonist or antagonist activity at the vanilloid receptor.

CYP Inhibition and Interaction with Pregnan X Receptor

KLYP961 did not exhibit any appreciable inhibitory activity against the six human isofoms examined, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A4. The estimated IC₅₀ value for all targets exceeded 30 μM. Furthermore, KLYP961, at concentrations as high as 30 μM, did not bind to human pregnane X receptor and constitutive androstane receptor in a biochemical assay (data not shown).

Microsomal Stability

KLYP961 showed species-dependent in vitro microsomal stability. The half-lives (minutes, mean ± S.D., n = 3) were 345 ± 3 (mouse), 151 ± 16 (rat), 63 ± 13 (dog), 160 ± 56 (cynomolgus monkey), 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% (cynomolgus monkey), and 73.5% (human).

Pharmacokinetic Parameters in Mice and Rhesus Nonhuman Primates

Mice and rhesus nonhuman primates were used as preclinical species to define antinociceptive effects of KLYP961. As such, pharmacokinetic profiles of KLYP961 after single-dose administration were evaluated in these two species under fasting conditions. The results summarized in Supplemental Table S2 and Supplemental Fig. S1, A and B indicate that KLYP961 is orally bioavailable, with approximately 60% oral bioavailability in both species. KLYP961 exhibits low systemic clearance in both mice and nonhuman 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 $V_{ss} \sim 4$ L/kg) versus mice (calculated $V_{ss} \sim 0.4$ L/kg), and it is anticipated that relative differences in the plasma-free fractions in these two species (62 versus 16% in cynomolgus monkey and mouse plasma, respectively). KLYP961 has a robust pharmacokinetic profile in all preclinical species examined. Although the oral bioavailability was fairly similar, KLYP961 exhibited a longer half-life and larger volume of distribution in primates versus 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 to 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 Fig. 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 Fig. S2A). KLYP961 (30 mg/kg p.o.) inhibited the LPS plasma nitrate response by $\approx 50\%$ for up to 12 h. At doses as high as 100 mg/kg, KLYP961 did not affect LPS-induced inflammatory cytokine production (Supplemental Fig. S2C).

**Formalin Model**

Orally administered KLYP961 attenuated formalin-induced nocifensive behaviors in a dose-related manner (Fig. 3, A and B). KLYP961 was more potent than gabapentin or pregabalin at attenuating both phases of nocifensive behaviors. The ED$_{50}$ values (mg/kg p.o.) 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 p.o.) 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 dependence (Fig. 3C); a 30 mg/kg dose significantly inhibited phase II behaviors for up to 4 h, whereas inhibition of phase I behaviors was more transient with only the 15-min pretreatment being effective (two-way ANOVA: phases I and 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 toward understanding the causal relationship between NOS inhibition and efficacy of KLYP961 in the formalin model, two approaches were used. The first used the chemical approach, and the second used a genetic approach. Two structurally related analogs, KLYP322 and KLYP775

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**Fig. 3.** A and B, KLYP961, gabapentin, and pregabalin dose-dependently attenuate formalin-induced pain behaviors [phase I (A) and phase II (B)]. C, KLYP961 (30 mg/kg p.o.) attenuates formalin-induced phase II behaviors for up to 4 h with a transient effect on phase I. D, inhibitory potency of KLYP322 (IC$_{50}$ 3600 nM, murine iNOS; 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. Both compounds were dosed orally 30 min in advance of formalin. Data represent phase II behavior and mean ± S.E. ($n = 5–6$ all groups; except KLYP961, 100 mg/kg, $n = 3$). * $p < 0.05$ versus vehicle (Veh).
(Fig. 1), differing only in their NOS inhibitory profiles, were evaluated in the formalin assay. The IC50 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 and 1 h after dose, KLYP322, but not KLYP775, attenuated both phases of formalin-induced pain behaviors (p < 0.05; Fig. 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 knockout 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 endpoint. In time-course experiments, KLYP961 (30 mg/kg p.o.), gabapentin (300 mg/kg p.o.), duloxetine (100 mg/kg p.o.), pregabalin (100 mg/kg p.o.), and morphine sulfate (3 mg/kg s.c.) attenuated cold allodynia with a robust reduction in allodynia seen at 60 to 90 min after dose (data not shown). Therefore, dose-response curves for KLYP961, gabapentin, and duloxetine were generated with allodynia measurements conducted 60 min after dose, whereas a 90-min time point after dose was selected for pregabalin. KLYP961 and benchmark compounds, gabapentin, pregabalin, and duloxetine, attenuated cold allodynia in a dose-related manner (Fig. 4) with similar magnitudes of efficacy. KLYP961 was more potent than benchmarks: ED50 values in mg/kg (with confidence intervals) 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 the Chung model of neuropathic pain induced by spinal nerve ligation (Kim and Chung, 1992). The experimental control positive, gabapentin, attenuated tactile allodynia at the highest dose of 300 mg/kg p.o. (Fig. 5A; dose, F2,45 = 13.9, p < 0.0001; time, F5,45 = 18, p < 0.001; interaction, F10,45 = 14.1, p < 0.05). KLYP961 attenuated tactile allodynia at 30 and 100 mg/kg doses with a peak effect at 30 min (Fig. 5B; dose, F3,104 = 16.3, p < 0.0001; time, F3,104 = 15, p < 0.0001; interaction, F3,104 = 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 understanding whether repeated administration of KLYP961 would lead to development of tolerance to its antiallodynic actions, mice that underwent CCI surgery were repeatedly dosed with either KLYP961 (30 mg/kg b.i.d., 3 days), gabapentin (300 mg/kg b.i.d., 3 days), or morphine sulfate (2 mg/kg s.c., b.i.d., 3 days). Animals received one additional dose approximately 16 h after the last dose, i.e., on day 4 for KLYP961 and morphine sulfate groups or day 3 for gabapentin group, and changes in alldynia were measured at preselected time points. Whereas morphine-induced antiallodynic effects tolerated quickly, the effects of KLYP961 or gabapentin were not tolerated. Likewise, the antiallodynic effects of KLYP961 also did not show tolerance in the Chung model (Kim and Chung, 1992) (Supplemental Results S6.3; Supplemental Figs. S3 and S4).

Anti-Inflammatory Activity

Carrageenan Model. Orally administered KLYP961 and celecoxib attenuated carrageenan-induced edema and tactile allodynia in a dose-related manner (Supplemental Fig. S5, A and B) with comparable efficacy and potency. The calculated ED50 value for KLYP961 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 with that of celecoxib (Supplemental Fig. 5C, D).
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. After capsaicin application for 15 min (referred to as time 0), 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%. AUC data were calculated using the trapezoidal method (GraphPad Prism). Two animals received vehicle and four animals received KLYP961 at each dose level. Animals were given a minimum of 7 days washout before inclusion in the next dose-level testing. Animals were assigned to vehicle or KLYP961 treatments with a crossover between treatments. Withdrawal responses in vehicle-treated animals were pooled. *p < 0.05; **p < 0.01; ***p < 0.001 versus vehicle group [two-way ANOVA for time course (A) or one-way ANOVA (AUC, B)].

Side Effect Profile Studies

Gastrointestinal Transit in Mice. The phenotype of nNOS knockout mice (Mashimo et al., 2000) combined with impairments in gastric motility reported with nonselective inhibitors of all three isofoms of NOS (Orihata and Sarna, 1994) implicate a role for NO derived from NOS isofoms 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 subchronic conditions with suprapharmacological doses, KLYP961 did not affect charcoal transit, whereas the experimental positive control, morphine, markedly reduced charcoal transit (Supplemental Results S6.5 and Supplemental Fig. S7).
hibition 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 the 2-imidazol-1-ylpyrimidine chemical scaffold, is also a potent inhibitor CYP3A4, 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 article (Bonefous et al., 2009; Payne et al., 2010).

KLYP961 is a dual iNOS/nNOS inhibitor with selectivity against eNOS 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 the mechanism of action of KLYP956, a structurally related compound (Symons et al., 2009).

Endotoxin injection in wild-type, but not iNOS knockout, 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 ED50 value of 1 mg/kg. Despite its pharmacokinetic half-life of ~2 h, a dose of 30 mg/kg inhibited nitrate response by 50% for ~12 h, suggesting a pharmacokinetic-pharmacodynamic dichotomy. Changes in plasma nitrates reflect systemic iNOS activity, therefore the time course of changes in plasma nitrates is less likely to mirror the 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 latter, 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 h, 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. Although the precise contribution of inhibition of each isoform to its efficacy is unclear, similar levels 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 (Bennett and Xie, 1988)) 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 (Kim and Chung, 1992). The antiallodynic 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 ED50 values (mg/kg) in endotoxin, formalin, the Bennett model (Bennett and Xie, 1988), and the Chung model (Kim and Chung, 1992) are 1, 13, 25, and 29 mg/kg, respectively. In addition to the shift in potency, the duration of action of KLYP961(30 mg/kg i.p) was different in these assays: plasma nitrate (~12 h), formalin (~4 h), and Chung and Bennett (~60–90 min) models. 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 (e.g., NOS inhibition in liver, spleen, and lungs driving the inhibition of nitrate response versus inhibition of NOS in pain pathways along the neuroaxis, 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 versus efficacy in pain models. Despite its limited central nervous system penetration, KLYP961 has demonstrable activity in neuropathic pain models, suggesting a peripheral component in such models. The clinical utility of topically applied lidocaine attests 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 with that of celecoxib. The precise source 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 to 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 nonhuman primates, a model that is sensitive to opioid and N-methyl-D-aspartate 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 versus single pretreatment, 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, experiencing detectable allodynia only at the I-h 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 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, and CYP450 and hERG activities. Acute or subchronic administration of (supra)pharmacological doses of KLYP961 was well tolerated in both mice and primates with acceptable side effect profile in both gastrointestinal motility and cardiovascular function. In addition, KLYP961 has efficacy in a range of pain/inflammation models in both rodents and nonhuman primates. These attributes suggest that KLYP961 has a unique profile relative to known NOS 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:** Massari, Rix, Shiu, Noble, Smith, Hassig, Zhang, and Rao.

**Conducted experiments:** Symons, Nguyen, Anzola, Staszewski, Wang, Yazdani, Dorow, Muhammad, Sablad, Rozenkrafts, Bonefous, and Payne.

**Performed data analysis:** Smith, Hassig, and Zhang.

Wrote or contributed to the writing and review of the manuscript: Shiu, Noble, Smith, Hassig, and Rao.

**References**


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**Address correspondence to:** Dr. Tadimeti S. Rao, Johnson and Johnson, 3210 Merryfield Row, San Diego, CA 92121. E-mail: trao1@its.jnj.com
Figure S1. Pharmacokinetic profile of KLYP961 following oral or intravenous administration in male Balb/c mice and male non-human rhesus primates (Panels A & B). Data represents mean ± SD (n=3-4). Rat brain penetration of KLYP961 following intravenous infusion. Sprague Dawley rats were given a loading dose of 4.48 mg over 2 min followed by a 98 min infusion at a rate of 0.128 µg/min. Levels of KLYP961 in plasma and hippocampal microdialysate samples were measured every 20 min and concentrations of KLYP961 in microdialysates were corrected for 10% recover from probes in vitro. Data represent mean ± SE (n=3).
Pharmacological Characterization of KLYP61, 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, Joseph E Payne, Peter J. Rix, Andrew K Shiau, Stewart A. Noble, Nicholas D. Smith, Christian A Hassig, Yan Zhang and Tadimeti S. Rao, JPET #172817. Figure S2. KLYP61 attenuates endotoxin-induced increases in plasma nitrates in a dose-dependent manner. Mice were orally administered vehicle (Veh) or inhibitors (AZ, AZ102222c or KLYP61; values in parenthesis refer to doses in mg/kg) and immediately injected with LPS via intraperitoneal injection. Nitrate levels were measured from plasma samples collected 6 hr post-LPS injection (Panel A) and cytokine levels were measured in plasma samples collected 1.5 hr post-LPS injection (Panel C). KLYP61 (30 mg/kg, po) inhibits nitrate response with long duration of action (Panel B). Mice were given KLYP61 either concurrent with (0 hr) or several hours (-4, -12 and -16 hr) in advance of LPS injection and nitrate levels were measured 6 hr post-LPS. Data represent mean ± SE (n=4-6) ***; p<0.0001 vs vehicle, #, p<0.05 vs LPS (ANOVA followed by appropriate post-hoc test).
Pharmacological Characterization of KLYP961, A Dual Inhibitor of Inducible and Neuronal Nitric Oxide Synthases


**Figure S3.** Repeated administration of morphine sulfate (3 mg/kg, sc, BID), but not gabapentin (300 mg/kg, po, BID) or KLYP961 (30 mg/kg, BID) results in loss of efficacy in the 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 ± SE (n=4-6). *, p<0.05, **, p<0.01 and ***, p<0.001 all relative to vehicle (2-Way ANOVA followed by appropriate post-hoc test).
Repeated administration of KLYP961 (30 mg/kg, BID) does not lead to loss of efficacy in the 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).

*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, Josephs E Payne, Peter J. Rix, Andrew K Shiau, Stewart A. Noble, Nicholas D. Smith, Christian A Hassig, Yan Zhang and Tadimeti S. Rao, JPET #172817. **Figure S4.**
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, Joseph E Payne, Peter J. Rix, Andrew K Shiah, Stewart A. Noble, Nicholas D. Smith, Christian A Hassig, Yan Zhang and Tadimeti S. Rao, JPET #172817. Figure S5. Orally administered KLYP961 or celecoxib attenuate carrageenan-induced edema (Panel A) and tactile allodynia (Panel B), and PBQ-evoked writhing response (Panel C) in a dose-related manner. In the carrageenan assay, oral dose of treatments (vehicle or test compounds) were given 15 min before intraplantar injection of carrageenan. In the PBQ assay, test compounds or vehicle were given by oral route 1 hr prior to intraperitoneal injection of PBQ. Writhing responses were measured over 20 min post PBQ. Data represent mean ± SE (n=7-8). **, p<0.05 and ***, p<0.001 all relative to vehicle (2-Way ANOVA).
Pharmacological Characterization of KLYP961, A Dual Inhibitor of Inducible and Neuronal Nitric Oxide Synthases


Figure S6. Topical application of capsaicin induced thermal hyperalgesia is responsive to single dose of MK-801 (60 μg/kg, sc; Panel A) or repeated administration of gabapentin (60 mg/kg, po, once a day for 3 days, Panel B), but not to single oral dose of gabapentin (60 mg/kg, po; Panel C). 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 15 min. MK-801 was given as a therapeutic treatment to animals, ie., 15 min after the removal of the capsaicin patch. There was a significant overall treatment effect (F[1,20]=17.8, p < 0.05; time, F[4,20]=11.7, p > 0.05; interaction, F[4,20]=7.9, p > 0.05); however, post-hoc analyses did not reveal significant time-related differences relative to vehicle control. Gabapentin or vehicle was given orally once a day for 3 days (Panel B). Baseline withdrawal responses were recorded on days 1 and 2. The last dose of vehicle or gabapentin was given 60 min before the application of capsaicin patch. Baseline withdrawal latency of each animal was normalized to 100 %. Gabapentin significantly altered thermal allodynia (treatment, F[1,20]=34, p < 0.0001; time, F[4,20]=39.5; p<0.0001; interaction, F[4,20]=19.6; p<0.0001). The altered time course of allodynia manifested in increased AUC (t=11.3, df= 4; p =0.0003). Single oral dose of gabapentin does not affect thermal allodynia (treatment, F[1,20]=3.16, p > 0.05; time, F[3,20]=28, p>0.05; interaction, F[3,20]=4.3, p>0.05). Areas under the curve (AUC) data were calculated using trapezoidal method (GraphPad Prizm). N=2 (vehicle), n=4 (gabapentin or MK-801). ***, p<0.001 vs vehicle group (Two-way ANOVA for time course or t-test for AUC).
Sub-chronic administration of KLYP961 does not affect charcoal transit in mice. Mice received KLYP961 (15, 50 and 150 mg/kg, po) or vehicle twice daily for 6 days. All animals were fasted overnight on Day 6 and on the morning of day 7, and received vehicle, the last dose of KLYP961 or the single dose of morphine sulfate. Two hours later, mice received of charcoal meal (10% Charcoal in Gum Arabic 10% w/v suspension in distilled H₂O; 0.1 ml per mouse) orally, euthanized 15 min later and the length of the charcoal transit from the pyloric sphincter to ileocecal junction in the GI was measured in each animal. ***, p<0.001 vs vehicle (one-way ANOVA followed by Dunnett’s test). Data represent mean ± SE (n=7-8).
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Supplementary Methods

S1. 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 (Phenacetin for CYP1A2, Amodiaquine for CYP2C8, Diclofenac for CYP2C9, (S)-Mephenytoin for CYP2C19, Dextromethorphan for CYP2D6, Midazolam and Testosterone for CYP3A4). KLYP961 was evaluated at seven different concentrations (0, 0.03, 0.1, 0.3, 1, 3, 10, 30 µM) in duplicate, while the substrates were tested near their apparent Km values. In an additional set of experiments to test for time-dependent (mechanism based) CYP inhibition, KLYP961 was pre-incubated with microsomes and NADPH for 30 minutes before addition of substrates. Product formation for all reactions was monitored by LC-MS/MS. Assay suitability was confirmed for all enzyme substrate pairs by use of known isoform specific inhibitors (α-Naphthaflavone, Montelukast, Sulfaphenazole, S-Benzylnirvanol, Quinidine, Ketoconazole, respectively for reversible inhibition and Furafylline, Gemfibrozel glucuronide, Tienillic acid, S-Fluoxetine, Paroxetine and Azamulin, respectively for time-dependent inhibition). All human CYP inhibition experiments were performed by GenTest (Woburn, MA).

S2. 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 below.

Briefly, the mouse RAW264.7 cells were seeded into 6-well dishes at a density of 1.5x10^6 cells/well, incubated for 5 hours and 1 mL of medium was removed from each well and replaced
with 1 mL of a 2X cocktail containing mIFN-γ (final concentration, 100 U/mL), LPS (final concentration, 2 µg/mL) and compound or vehicle (final concentration, 0.1% DMSO) diluted in DMEM with 10% serum. Cells were incubated overnight at 37 °C, 5% CO₂. Cells were washed once with ice cold PBS and then 200 µL of ice-cold lysis buffer (250 mM sucrose, 10 mM Tris pH 7.5, 1 mM EDTA) containing protease inhibitors was added to each well. Cells were scraped from the dish and transferred to micro-centrifuge tubes on ice. Samples were sonicated for 5 seconds at setting 4 (Branson) and centrifuged at 16,000g for 10 minutes at 4 °C. The concentration of protein was normalized using a Bradford assay (Advanced). An equal volume of ice-cold 2X loading buffer was added to each sample and loaded onto a 4-20% Tris-glycine polyacrylamide gel (Invitrogen). The gel was run in pre-chilled 1X SDS running buffer in a cold room for 2.5 hours at 125 V. Proteins were transferred to nitrocellulose for 2.5 hours at 70 V in 1X transfer buffer and detected as per methods described below (Western blotting).

S2.1 Protein extraction and Immunoprecipitation

In order to evaluate effects of KLYP961 on the dimeric state of iNOS under more native conditions, two iNOS constructs designed to contain a FLAG-tag (FLAG) or hemaglutinin tag (HA) were co-transfected into HEK293 cells. Following incubation with KLYP961 or other iNOS inhibitors during induction of iNOS, cells were harvested with 1 mL of ice cold PBS, centrifuged for 2 min at 400xg, washed once with 1 mL of ice-cold PBS, lysed with 500 µl lysis buffer (25 mM Tris pH 8.0, 10% glycerol, 150 mM NaCl and 0.5% Triton-X-100) containing protease inhibitors, centrifuged at 16,000xg for 10 min at 4 °C and supernatants transferred to clean tubes and kept on ice. The protein levels in cell extracts were determined using a Bradford assay with a BSA standard curve as described by the manufacturer and protein levels were normalized by dilution in lysis buffer. Approximately 200 to 400 µg of protein was
immunoprecipitated for 1 hour at 4°C using anti-FLAG M2 (Sigma) beads in a total volume of 1 mL. Extracts were then washed 3x with 1 mL of lysis buffer followed by elution with 50 µl of 2x NuPage loading buffer. DTT was added to each sample at a final concentration of 0.1M. Bis-Tris polyacrylamide gels (4-12%) loaded with 12.5 µl of sample per well were run in pre-chilled 1X MES running buffer for 1 hr at 160 V and proteins were transferred to nitrocellulose for 2 hr at 80 V in 1X transfer buffer. The gels were then probed for anti-HA (see western blot).

S2.2 Western blot

Membranes were blocked overnight in Blotto (20 mM Tris pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20 (TBST) containing 3% (w/v) nonfat milk) at 4 ºC and incubated in 1:2,500 of mouse anti-iNOS (BD Biosciences) or 1:1,000 of mouse anti-HA 16B12 (Covance) in Blotto for 1 hour at room temperature followed by 3 x 5 minute washes in TBST. The blot was then incubated in a 1:2,000 dilution of goat anti-mouse (BioRad) HRP-conjugated secondary antibody in Blotto for 1 hour at room temperature. Following 4 x 5 minute washes, the proteins were visualized by chemiluminescence using SuperSignal West Dura (Pierce) detection reagent and captured on a CCD-based imaging device (Alpha Inotech).

S3. Pharmacokinetic Studies

S3.1 Drug Substance

A HCl salt form of KLYP961 was used in all the studies described herein and doses refer to its neutral form.

S3.2 Dose Formulation for Oral Studies

KLYP961 and other compounds, unless specified, were formulated in the vehicle composed of PEG-400: Tween-80: PVP-K30: CMC in water (0.5% w/v) in the ratio of 9: 0.5: 0.5: 90. The
final vehicle for oral administration was prepared by combination of two components, a solvent portion (90% PEG-400, 5% Tween-80 and 5% PVP-K30 (polyvinyl povidone K value 28 – 32) and the aqueous portion (0.5% w/v carboxymethylcellulose (CMC) Na Salt in distilled water). The formulations were prepared by homogenizing dry powder of test article with 1/10th of the final volume with solvent portion (Polytron™) on ice and diluting to final volume in aqueous portion.

**S3.3 Dose formulation for Mouse and Rhesus non-human Primate Intravenous Studies**

For intravenous dosing, KLYP961 was dissolved in 5% DMSO, 20% PG, 5% Tween-80 and 70% saline.

**S3.4 Dose formulation for Rat IV Infusion Study**

KLYP961 HCl salt was dissolved in dimethylsulfoxide (DMSO) at 70 mg/mL (63.6 mg/mL free-base). In a separate container, a 30% (w/v) solution of beta-hydroxypropyl cyclodextrin (βHPCD) was prepared in water. The DMSO stock was combined 1:8 with polyethylene glycol 400 (PEG-400). The combined DMSO/PEG solution was mixed with the (βHPCD in water for a final composition of 3.5 mg/mL KLYP961 HCl in 5% DMSO, 40% PEG, 55% (βHPCD in water). This formulation was prepared just prior to use and used immediately.

**S3.5 Dosing volumes**: In mice, dosing volumes of 10 mL/kg and 3 mL/kg were used for oral and intravenous routes, respectively. In primates, the corresponding dosing volumes were 5 mL/kg and 2 mL/kg, respectively.

Pharmacokinetics of KLYP961 were defined in rodent (mice) and non-rodent species (rhesus non-human primates) using procedures described below.

**S3.6 Mouse Pharmacokinetics**
Fasted male Balb/C mice received a single nominal dose of 10 mg/kg KLYP961 by oral gavage. For intravenous leg, fasted animals, under temporary restraint, received single nominal dose of 3 mg/kg KLYP961 via tail vein. Blood samples (~150 µL) were collected by retro-orbital puncture into lithium heparin pre-treated tubes at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hr post-dose for oral and 0.08, 0.17, 0.33, 0.67, 1, 2, 4, 8, 12 and 24 hr under isoflurane anesthesia (n=3 mice per time point). Plasma was isolated and stored frozen at -80°C until analysed.

**S3.7 Rhesus non-human Primate Pharmacokinetics**

Overnight fasted animals (male rhesus non-human primates: 3.5-4.5 kg body weight) were dosed 2 mL/kg for intravenous and 5 mL/kg for oral via nasogastric tube, based on a pre-study body weight measurement. Blood sampling time points following oral dosing were: pre-dose (0 hr) and at 15 and 30 minutes and at 1, 2, 3, 4, 8, 12, and 24 hrs post-dose. Plasma was isolated and stored frozen at -80°C until analysed.

**S3.8 CNS Penetration**

Male Balb/C mice were orally dosed with KLYP (30 mg/kg, po). Blood samples were collected from groups of mice (n=3-4) under isoflurane anesthesia. Animals were transcardially perfused with ice-cold saline (~10 mL/ mouse) and brain samples were collected. Brain to plasma levels were determined to calculate brain penetration.

**S3.9 Microdialysis Studies in Rat**

Microdialysis studies in awake rats were conducted to allow simultaneous blood and extracellular brain fluid collection.

Male SD rats (wt: 300-350g) previously implanted with jugular/femoral catheters and microdialysis guide cannulae placed into hippocampus (BASi BR-4 brain microdialysis probes,
Lot #2058.54.1207] extended 4 mm below guide cannula; stereotaxic coordinates: AP -5.2mm, LM +4.5 mm, DV -3.2mm) were used in the study. On the day of the study, animals were placed in suitable apparatus, connected to infusion pumps to infuse an appropriate dosing vehicle or test article solution in an appropriate vehicle into the femoral vein and to collect blood samples from jugular vein and microdialysates from the hippocampus. The loading dose was 4.48 mg of free-base equivalent of KLYP961 (70 μL of 63.6 mg/mL stock solution) delivered over a 2 min period, followed by infusion a rate of 0.128 μg/min for 98 min. Based on an average weight of 300g, rats received a total dose of 51.69 mg of KLYP961 over 100 min. The microdialysis probe was continuously perfused with artificial cerebrospinal fluid (aCSF) at a rate of 2 μL/min. Blood samples and hippocampal dialysates were collected every 20 min over the entire study duration of 4 hr. Recovery of KLYP961 from microdialysis probes was determined in vitro using artificial cerebrospinal fluid containing 1 μM of KLYP961 with the same batch of probes was used both for recovery and in vivo microdialysis studies. The dialysate levels of KLYP961 were corrected for in vitro recovery of approximately 10%.

S3.10 Determination of KLYP961 Levels and Computation of Pharmacokinetic Parameters
KLYP961 levels were quantified by liquid chromatography-mass spectrometric (LC-MS) methodology. Pharmacokinetic parameters were determined by Excel-based templates (Microsoft Corp).

S4. Pharmacology Studies

S4.1 Mouse Lipopolysaccharide (LPS) Test
Male Balb/C mice were given intraperitoneal injection of LPS solution in saline (Sigma, L2880, dose: 10 mg/kg). At pre-determined time points (1.5 hr post-LPS for cytokines and 6 hr post-
LPS for nitrates, blood samples, derived via retro-orbital bleeding, were collected under brief isofluorane anesthesia lasting approximately~2-3 min. Test compounds were administered by oral gavage immediately before the LPS injection. In time course experiments, separate group of animals (n=4-6) were dosed with KLYP961 4-16 hr prior to the LPS injection, and blood samples were collected 6 hr post-LPS injection. Control group of animals received saline (intraperitoneal injection) and appropriate oral vehicle. Blood samples were centrifuged to collect plasma and stored at -80ºC until analysis.

**S4.2 Measurement of Nitrates**

Plasma nitrate levels, a marker of NOS activity, were measured using a fluorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). Plasma samples were allowed to thaw in ice and filtered through a 10 KDa molecular weight cut-off filter (MultiScreen Filter Plate; Ultracel-10 Membrane, Fisher Scientific), at 2000xg for 10 min at 4ºC. The first step in the detection of nitrates is the enzymatic conversion of nitrates to nitrite, utilizing nitrate reductase enzyme. The second step is the formation of a fluorophore by sequential addition of diaminonaphthalne (DAN) and NaOH.

**S4.3 Measurement of TNF-α and IL-6 in plasma**

Effects of KLYP961 on LPS-induced inflammatory cascades were determined by monitoring its effects on inflammatory cytokines, Blood samples collected 1.5 hr post LPS challenge were processed for plasma collection and subsequent cytokine analyses (DuoSet ELISA for TNFα and IL-6; R&D Systems, Minneapolis, MN).

**S4.4 Mouse Formalin Model**
The formalin solution for intraplantar injection was first prepared by diluting a 10% formalin solution to 5% into 0.9% saline. A volume of 20 µL was injected into the left hind paw of each animal. Immediately after injection of formalin into the left hind paw, mice were placed in a see-through observation chambers and a timers were activated. The duration of pain behaviors (hind paw flinches, licking and biting) displayed by treated animal was counted in 5 minute intervals. Phase I was observed from 0-5 minutes post-formalin injection. Phase II was observed from 25-45 minutes post-formalin injection. Data are presented as time spent in nociceptive behaviors during Phase I (0-5 min post-formalin) and Phase II (25-45 min post-formalin). The investigator that rated the animal behavior was blinded to the nature of treatments.

S4.5 Mouse Chronic constriction injury (CCI; Bennett Model)

Mice underwent a chronic constrictive injury as per previously described methods (Bennett and Xie;1988). Briefly, mice were initially anesthetized with 5% isoflurane in medical grade pure oxygen and maintained at 2-3 % isoflurane during the surgical procedure. Using aseptic techniques, the left sciatic nerve at the level of the mid-thigh was exposed and loosely ligated with four ligatures (6-0 chromic gut, Ethicon) with each ligation set 1 mm apart. The thigh muscle incisions were sutured with 6-0 braided black silk and the overlying skin was closed with surgical wound clips. Mice were allowed to recover from peripheral nerve injury for approximately 1-2 weeks. The development of cold allodynia was measured by subjecting the animal’s injured hind paw to acetone (Walczak and Beaulieu, 2006). Briefly, each animal was placed in an acrylic chamber that rested on an elevated wire mesh platform and allowed to acclimate for 1 hr. Using a 1cc syringe attached to a 23g needle, 100 µL of acetone was applied to the plantar surface of the injured hind paw once and duration of pain behaviors (hind foot flinching, licking, and biting) was observed and recorded for 1 minute after acetone application.
More than 90% of animals showed acetone-induced cold allodynia response (11± 2 sec (mean ± SE, n=8-10)) by day 7 post-surgery which remained stable for up to 14 days. Animals typically underwent cold-allodynia testing on day 7 and randomized to receive drug treatments on days 8-10, with all drug treatments replicated on all treatment days. The investigator that rated the animal behavior was blinded to the nature of treatments.

S4.6 Mouse Model of Spinal nerve ligation-induced tactile allodynia (Chung Model)

Mice underwent a surgical procedure as described in Kim and Chung (1992). Briefly, mice were initially anesthetized with 5% isoflurane in medical-grade pure oxygen and maintained with 2-3% isoflurane during the surgical procedure. Using aseptic techniques, the left Lumbar (L) L5 and L6 spinal nerves were exposed and isolated with a surgical hook, distal to their respective dorsal root ganglion. A tight ligation was made on each nerve using a 6-0 black braided silk thread. The back muscles were sutured (6-0 braided black silk) and the skin laceration was stapled with surgical wound clips. Mice were allowed to recover from spinal nerve injury for approximately 5-10 days. The development of mechanical (tactile) allodynia was assessed using a set of calibrated von Frey filaments (Stoelting Co., IL) with increasing stiffness (0.04, 0.07, 0.16, 0.4, 1.0, 1.4, 2.0 g/force) and exerted increasing force when pushed against the tested paw for a 10 second period. The stimulation with a von Frey filament was completed within a 10 second period and the percent response frequency was calculated using Dixon’s up and down formula (Chaplan et al., 1994). Control animals with no surgery or animals that underwent sham-surgery showed a 50% withdrawal threshold of 1.5 - 2 g (data not shown). Only those animals that showed baseline 50% withdrawal thresholds of ~0.2-0.3 g anytime between 5 - 7 days post-surgery were considered to have achieved tactile allodynia and were randomized for inclusion in all drug treatment studies. The animals that met the inclusion criteria showed stable
baseline for about 2 weeks from the initial day of testing (5-7 d). All drug treatments were replicated on all treatment days. The investigator that rated the animal behavior was blinded to the nature of treatments.

**S4.7 Mouse Carrageenan-induced Paw Inflammation**

A 1% w/v suspension of λ-carrageenan was prepared by dispersing it into saline overnight at 4°C. Mice (C57Bl6/j) were gently anesthetized by induction with isoflurane (3-5% in O₂) and injected with 0.03 mL carrageenan into their left hind paws using 30-gauge needle. After the withdrawal of the needle, gentle pressure was applied for 15-30 sec to the injection site to prevent back flow. Changes in paw thickness at selected time points were measured using Kafer micrometer (0.001” 10mm disc; Precision Graphic Instruments; Spokane, WA). The development of mechanical (tactile) allodynia was assessed using a set of calibrated von Frey filaments (Stoelting Co., IL) with increasing stiffness (0.04, 0.07, 0.16, 0.4, 1.0, 1.4, 2.0 g/force) and exerted increasing force when pushed against the inflamed paw for a 10 second period. The stimulation with a von Frey filament was completed within a 10 second period and the percent response frequency was calculated using Dixon’s up and down formula (Chaplan et al., 1994).

Celecoxib and KLYP961 were evaluated in the dose range of 3-100 mg/kg. The investigator that rated the animal behavior was blinded to the nature of treatments.

**S4.8 Mouse Peritoneal Writhing Assay**

Phenyl-p-benzoquinone (PBQ) administered by the intraperitoneal route induces a characteristic behavior consisting of a wave of constriction and elongation passing caudally along the abdominal wall (Hendershot 1958). A 0.4 mg/ml solution of PBQ was prepared immediately before use by dissolving PBQ in warm ethanol (95% v/v) and dilution with warm distilled water.
at 37°C to a final ethanol concentration of 5% v/v. The solution was placed in an amber colored stoppered vial. C57Bl6/j mice were removed from their home cages and placed in individual observation cages (square plastic enclosures on top of a raised wire mesh platform) and allowed to acclimate for 30 min. Test compounds, except morphine sulfate, were administered orally 60 min prior to intraperitoneal injection of PBQ (4 mg/kg). Morphine sulfate was administered via subcutaneous injection 5 min before the PBQ injection. Immediately after the PBQ injection, animals were returned to observation cages and a hand tally counter was used to record the number of abdominal stretching movements for each mouse over a 20-min period. Control groups of animals were given appropriate vehicle treatments. Data are expressed as percent inhibition of number of writhes over a 20 min period post-PBQ in test compound-treated vs vehicle-treated animals. The doses for KLYP961 and celecoxib were 30, 100 and 300 mg/kg. The investigator that rated the animal behavior was blinded to the nature of treatments.

S4.9 Capsaicin-induced thermal hyperalgesia in rhesus non-human primates

The model was developed as per methods reported earlier (Butelman et al. (1992). Male rhesus primates (3-5 kg), previously chair-trained for several days (2-4 week period) while keeping their tails in ambient water positioned in a container underneath the chairs, were physically inspected for absence of any injuries to their tails. The tails were shaved (~10 cm length from distal end) with standard clippers approximately 24 hr prior to testing and visually inspected to ensure absence of skin damage. Animals were placed in chairs with their tails exposed to lukewarm water (38 °C) and tail withdrawal latencies were recorded using manual timers to the nearest 0.1 sec with a cut off of 120 sec. The baseline measurements were taken one day before the study. On the dosing day, a topical capsaicin patch was applied on the tail (2-6 cm from the distal end) for 15 min and secured by parafilm. The capsaicin patch was prepared by the addition of 0.3 ml
of 4 mM stock solution of capsaicin in 70% ethanol and 30% distilled H₂O onto two layers of 1 cm² surgical gauze. Following the removal of the patches, the tails were cleaned with surgical gauge, allowed to dry and withdrawal latencies from a 38°C water bath were measured at selected time points (baseline, 0.25, 1, 2, 3, 4, 6 hr). Two measurements were taken at each time point and average withdrawal latency was calculated. Animals were screened for their baseline and capsaicin-induced thermal withdrawal latencies initially and animals with suitable profiles were included in the study group. When animals were used repeatedly, they were given a minimum of 7 days rest between testing and rotated between vehicle and drug treatments. In preliminary studies, baseline and post-capsaicin withdrawal thresholds were relatively stable across subjects over several weeks in which animals received cycles of vehicle or capsaicin treatments with intervening washout. Treatment effects on withdrawal latencies were evaluated with each animal’s baseline response serving as its own control and baseline withdrawal latencies were normalized to 100. The areas under the time course of thermal allodynia were calculated using GraphPad Prizm (San Diego, CA). The individuals who rated the allodynia were unaware of the nature of the treatments.

**S5. Side Effect Profile Studies**

**S5.1 Gastrointestinal Transit Test in Mice**

Single dose administration of KLYP961 at doses as high as 300 mg/kg to over-night fasted mice did not affect charcoal transit time assessed 2 hr post dose. With a view to defining effects of sub-chronic dosing on motility, repeat dose studies were undertaken with two studies conducted as described below.
The first study was designed as a general tolerability study over 7 days of repeated dosing of KLYP961 at 15, 50 and 150 mg/kg, BID (total daily doses of 30, 100 and 300 mg/kg; n=6-8 mice/dose group). The tolerability was assessed by monitoring body weight, liver weight, and plasma exposure of KLYP961. All animals had free access to food and water throughout the course of the study. Animals were fasted on overnight for ~16 hr after they received the 2\textsuperscript{nd} dose for day7. On day 8, three mice from each dose level were briefly anesthetized (<2 min) to collect blood (~100 µL; referred to as “time zero”). Animals were allowed to fully recover before receiving their respective KLYP961 dose blood samples were collected at 1,2,4,6 and 8 hr post dose. Three mice from each dose group were used collect blood samples via retro-orbital bleeding method at two time points (ie., 1 and 4hr, 2 and 6 hr; time zero and 8 hr), with the last time point being a terminal sacrifice. Levels of KLYP961 in “time zero” samples allowed determination of its trough plasma levels (plasma levels at 15, 50 and 150 mg/kg BID were 25 nM, 55 nM and 800 nM, respectively; one hr after dosing, these levels respectively increased to ~ 30 µM, 65µM and 100 µM).

Based on the first experiment, a second study was conducted to evaluate effects of KLYP961 at 15, 50 and 150 mg/kg, BID for 6 days (total daily doses of 10, 30, 100 and 300 mg/kg) on charcoal transit. Morphine sulfate (5 mg/kg, sc injection) was used as positive control and was dosed once on Day 7 to a group of animals that received vehicle during the first 6 days. Control group of animals received vehicle for the first 6 days. On day 6, animals received their 2\textsuperscript{nd} daily dose of treatment or vehicle and fasted overnight (~16 hr). On the morning of day 7, animals received appropriate treatments (ie., vehicle, the last dose of KLYP961 or the single dose of morphine sulfate). Two hours later, mice received an oral charcoal meal (10% Charcoal in Gum Arabic 10% w/v suspension in distilled H\textsubscript{2}O; 0.1 ml per mouse), euthanized 15 min later by
cervical dislocation, and the length of the charcoal transit from the pyloric sphincter to ileocecal junction was measured in each animal. Visual observations of stomach size were also undertaken. In a separate experiment, groups of C57Bl6/j and nNOS knockout animals (n=5) were subjected to charcoal transit test as per methods described. In addition, at the time of necropsy, the gross stomach size (in mm) was measured both in length and breadth.

**S5.2 Motor Coordination: Mouse Rotarod Assay**

Male C57Bl/6j mice were acclimated to the testing room for 30 minutes and to the rotarod device for 5 minutes at 4 rpm during which animals were repeatedly returned to the device after falling. Approximately 30 minutes post-acclimation to rotarod device, animals were subjected to two training sessions 30 min apart. Each session involved acclimation to accelerating rotarod (4-40 rpm in 300 sec) with latencies to drop recorded. Only those mice with latencies of >60 sec were included in the assay and randomized to treatment groups. Following oral administration of either gabapentin or KLYP961, latencies to fall on accelerating rotarod were determined at 1, 2 and 3 hr post-dose. Animals unable to stay on the rotarod at 4 rpm were assigned a latency of 0 s; animals completing a trial were assigned a latency of 300 s.

**S5.3 Primate Safety Studies: Cardiovascular Studies in Telemetered Cynomolgus Primates**

The studies were conducted at Biological Resources Laboratory of the University of Illinois-Chicago (BRL) and acclimated to BRL for at least 60 days. The study conduct was governed by USDA Animal Welfare Act and the ILAR Guide for the Care and Use of Laboratory Animals. Chronically instrumented cynomolgous non-human primates (3-6 kg, n=4) with stable cardiovascular and hemodynamic parameters were used in the study. Animals were orally dosed with vehicle (Supplemental Section S3) once a day for 4 days to establish a baseline. This was
followed by dose-escalation with dose levels of 23, 72 and 96 mg/kg/po/day of KLYP961 (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 also examined continuously throughout the doing phase of this study.
Supplemental Results

S6.1 CNS Penetration- Microdialysis Study in Rats

With a view to further define CNS penetration, infusion studies were conducted in rats with simultaneous microdialysis from the hippocampus. Loading dose and constant infusion of KLYP961 for 30 min resulted in steady state levels of approximately 40 μM in the plasma. KLYP961 levels in microdialysis samples, corrected for in vitro probe recovery of 10%, demonstrated rapid achievement of steady state levels in the brain. At the end of infusion, levels of KLYP961 both in microdialysates and plasma declined with similar slopes. The calculated CNS penetration of KLYP961 under these conditions was 1% (Supplemental Figure S1C).

S6.2 LPS Assay

Orally administered KLYP961 attenuated LPS-induced increases in plasma nitrates, measured 6 hr post-LPS injection, in a dose-dependent manner with an ED\textsubscript{50} value of 0.98 mg/kg (F[6,28]=55, p<0.0001; Supplemental Figure S3A). The pharmacodynamic duration of inhibition of iNOS by KLYP961 was assessed in a time course study at a single dose of 30 mg/kg. At this dose, iNOS activity was inhibited by ≥ 50% for up to 12 hr (F[5,29]=292, p<0.0001; Supplemental Figure S2B). In contrast to its effects on iNOS pathway, KLYP961 treatment did not significantly affect LPS-induced increases in inflammatory cytokines, TNF\textalpha{} and IL-6, with ED\textsubscript{50} values ≥ 100 mg/kg (Supplemental Figure S2C).

S6.3 Repeat Administration Studies in Neuropathic Pain Models

Mice that underwent CCI surgery were repeatedly dosed with either KLYP961 (30 mg/kg, BID, 3d), gabapentin (300 mg/kg, BID, 2 days) or morphine sulfate (3 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. The time course of inhibition of LPS-induced elevations in plasma nitrates by KLYP961 suggested that a single dose of 30 mg/kg, po, can inhibit iNOS activity by >50% for up to 12 hr. Therefore a twice-a-day regimen was chosen to ensure reasonable target inhibition. Anti-allodynic efficacy of morphine was highly significant after the first dose and the efficacy developed tolerance by day 3 (Supplemental Figure S3A; treatment, F[5,156]=12.3, p<0.0001; days, F[3,156]=3.6, p>0.05; interaction, F[15,156]=8.5, p>0.05]. In contrast, repeated administration of either gabapentin (Figure S3B; treatment, F[3,36]=59, p<0.0001; days, F[2,64]=1.8, p>0.05; interaction, F[6,36]=0.5, p>0.05) or KLYP961 did not lead to loss of efficacy (Figure S3C; treatment, F[6,64]=46, p<0.0001; days, F[3,64]=2.3, p>0.05; interaction, F[9,64]=1.13, p>0.05).

Repeated administration of KLYP961 (30 mg/kg, BID for 3 days) with an additional dose on day 4, did not show tolerance to anti-allodynic actions in the Chung model (Supplemental Figure S4; dose, F[3,64]=64, p<0.0001; time, F[3,64]=1.9, p > 0.05, interaction, F[9,64]=1.5, p>0.05).

S6.4 Capsaicin-induced Thermal Hyperalgesia: Effects of MK-801 and gabapentin

Efficacy of MK-801 (0.06 mg/kg, sc) was evaluated in animals with established allodynic state induced by topical application of capsaicin. While there was a statistically significant overall treatment effect of MK-801 (Supplemental Figure S6A; treatment, F[1,20]=17.8, p < 0.05; time, F[4,20]=11.7, p > 0.05; interaction, F[4,20]=7.9, p > 0.05), post-hoc analyses did not reveal significant time-related differences relative to vehicle control.
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, significantly altered the time course of allodynia (Figure S6B; treatment, $F[1,20]=34$, $p < 0.0001$; time, $F[4,20]=39.5$, $p<0.00001$; interaction, interaction, $F[4,20]=19.6$, $p<0.0001$). The altered time course of allodynia manifested in increased AUC ($t=11.3$, df= 4; $p =0.0003$).

Having established the efficacy of gabapentin under the conditions described above, effects of single pre-treatment were examined. Gabapentin (60 mg/kg, po) given as a pre-treatment to capsaicin did not affect allodynia to any significant effect, although there was a trend towards a higher AUC (Figure S6C; treatment, $F[1,20]=3.16$, $p > 0.05$; time, $F[3,20]=28$, $p>0.05$; interaction, $F[3,20]=4.3$, $p>0.05$).

**S6.5 Gastrointestinal Transit: Charcoal Meal Test**

Genetic ablation of nNOS in mice results in marked changes in physiology of gastric motility (Mashimo et al., 2002; Orhita and Sarna, 1994). To understand if pharmacological inhibition of nNOS in vivo by KLYP961 impacts gastric motility, mice were dosed with KLYP961 (15, 50 and 150 mg/kg, BID, total daily dose of 30, 100 and 300 mg/kg, for 6 days and subjected to charcoal transit test on day 7. Morphine sulfate (5.5 mg/kg, sc) was used as a positive control. Under these conditions, KLYP961 did not impact charcoal transit at any of the doses, while the experimental positive control, morphine, markedly reduced charcoal transit (Supplementary Figure S7; $F(4,25)= 86$, $p < 0.0001$). In a separate group of animals, plasma levels of KLYP961 were measured on day 7 prior to dosing (representing trough levels) and at various times up to 8 hr after the last dose. The trough levels of KLYP961 at 15, 50 and 150 mg/kg doses ranged 25-800 nM; the corresponding levels 8 hr after the last dose ranged from 2000-35000 nM (data not
shown) indicating presence of supra-pharmacological levels of KLYP961 over the study duration. Gross observations indicated that the stomach size was not affected by repeated administration of KLYP961. However, the nNOS knockout mice (C57Bl6/j) background have considerably enlarged stomach size relative to wild-type controls (stomach size, cm²; mean ± SE, n=5; wild type: 1.3 ± 0.12; nNOS knock-out; 3.8± 0.49) and reduced charcoal transit (distance in cm, mean ± SE, n=5; wild type: 16.3 ± 1.5; nNOS knock-out; 10.8± 1.15, p<0.05, t-test).

**S6.6 Motor Coordination: Rotarod Assay**

Effects of single oral dose administration of KLYP961 on motor coordination were assessed using rotarod assay and gabapentin was used as the reference compound. Gabapentin produced dose- and time-dependent impairments in motor coordination (Supplementary Figure S8A; dose, F[3,80]=41, p<0.0001, time, F[3,80]=20, p<0.0001, interaction, F[9,90]=15, p<0.0001). The calculated ED₅₀ value for gabapentin, based on its effects at 2 hr post-dose, was ~140 mg/kg (Supplementary Figure S8C). KLYP961 over the dose range of 100-1000 mg/kg, did not affect motor performance; gabapentin at a single dose of 300 mg/kg, replicated its effects on motor impairment (Supplementary Figure S8B; dose, F[4,140]=31, p<0.0001, time, F[4,140]=3, p<0.05, interaction, F[12,140]=13, p=0.0012). Given the lack of effect, an ED₅₀ value for KLYP961 on motor impairment could not be calculated.
Supplemental Figure Legends

**Figure S1.** Pharmacokinetic profile of KLYP961 following oral or intravenous administration in male Balb/c mice and male non-human rhesus primates ( Panels A & B). Data represents mean ± SD (n=3-4). Rat brain penetration of KLYP961 following intravenous infusion. Sprague Dawley rats were given a loading dose of 4.48 mg over 2 min followed by a 98 min infusion at a rate of 0.128 µg/min. Levels of KLYP961 in plasma and hippocampal microdialysate samples were measured every 20 min and concentrations of KLYP961 in microdialysates were corrected for 10% recover from probes in vitro. Data represent mean ± SE (n=3).

**Figure S2.** KLYP961 attenuates endotoxin-induced increases in plasma nitrates in a dose-dependent manner. Mice were orally administered vehicle (Veh) or inhibitors (AZ, AZ102222c or KLYP961, values in parenthesis refer to doses in mg/kg) and immediately injected with LPS via intraperitoneal injection. Nitrate levels were measured from plasma samples collected 6 hr post-LPS injection (Panel A) and cytokine levels were measured in plasma samples collected 1.5 hr post-LPS injection (Panel C). KLYP961 (30 mg/kg, po) inhibits nitrate response with long duration of action (Panel B). Mice were given KLYP961 either concurrent with (0 hr) or several hours (-4, -12 and -16 hr) in advance of LPS injection and nitrate levels were measured 6 hr post-LPS. Data represent mean ± SE (n=4-6) ***, p<0.0001 vs vehicle, #, p<0.05 vs LPS (ANOVA followed by appropriate post-hoc test).

**Figure S3.** Repeated administration of morphine sulfate (3 mg/kg, sc, BID), but not gabapentin (300 mg/kg, po, BID) or KLYP961 (30 mg/kg, BID) results in loss of efficacy in the 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 ±
SE (n=4-6). *, p<0.05, **, p<0.01 and ***, p<0.001 all relative to vehicle (2-Way ANOVA followed by appropriate post-hoc test).

**Figure S4.** Repeated administration of KLYP961 (30 mg/kg, BID) does not lead to loss of efficacy in the 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 S5.** Orally administered KLYP961 or celecoxib attenuate carrageenan-induced edema (Panel A) and tactile allodynia (Panel B), and PBQ-evoked writhing response (Panel C) in a dose-related manner. In the carrageenan assay, oral dose of treatments (vehicle or test compounds) were given 15min before intraplantar injection of carrageenan. In the PBQ assay, test compounds or vehicle were given by oral route 1 hr prior to intraperitoneal injection of PBQ. Writhing responses were measured over 20 min post PBQ. Data represent mean ± SE (n=7-8). **, p<0.05 and ***, p<0.001 all relative to vehicle (2-Way ANOVA).

**Figure S6.** Topical application of capsaicin induced thermal hyperalgesia is responsive to single dose of MK-801 (60 μg/kg, sc; Panel A) or repeated administration of gabapentin (60 mg/kg, po, once a day for 3 days, Panel B), but not to single oral dose of gabapentin (60 mg/kg, po; Panel C). 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 at15 min. MK-801 was given as a therapeutic treatment to animals, ie., 15 min after the removal of the capsaicin patch. There was a significant overall treatment effect (F[1,20]=17.8, p < 0.05; time, F[4,20]=11.7, p > 0.05; interaction, F[4,20]=7.9, p > 0.05); however, post-hoc analyses did not reveal significant time-related differences relative to vehicle control. Gabapentin or vehicle was given orally once a day.
for 3 days (Panel B). Baseline withdrawal responses were recorded on days 1 and 2. The last dose of vehicle or gabapentin was given 60 min before the application of capsaicin patch. Baseline withdrawal latency of each animal was normalized to 100%. Gabapentin significantly altered thermal allodynia (treatment, $F[1,20]=34$, $p < 0.0001$; time, $F[4,20]=39.5$, $p < 0.0001$; interaction, interaction, $F[4,20]=19.6$, $p < 0.0001$). The altered time course of allodynia manifested in increased AUC ($t=11.3$, df= 4; $p =0.0003$). Single oral dose of gabapentin does not affect thermal allodynia (treatment, $F[1,20]=3.16$, $p > 0.05$; time, $F[3,20]=28$, $p >0.05$; interaction, $F[3,20]=4.3$, $p >0.05$). Areas under the curve (AUC) data were calculated using trapezoidal method (GraphPad Prizm). N=2 (vehicle), n=4 (gabapentin or MK-801). ***, $p<0.001$ vs vehicle group (Two-way ANOVA for time course or t-test for AUC).

**Figure S7.** Sub-chronic administration of KLYP961 does not affect charcoal transit in mice. Mice received KLYP961 (15, 50 and 150 mg/kg, po) or vehicle twice daily for 6 days. All animals were fasted overnight on Day 6 and on the morning of day 7, animals received vehicle, the last dose of KLYP961 or the single dose of morphine sulfate. Two hours later, mice received of charcoal meal (10% Charcoal in Gum Arabic 10% w/v suspension in distilled H$_2$O; 0.1 ml per mouse) orally, euthanized 15 min later and the length of the charcoal transit from the pyloric sphincter to ileocecal junction in the GI was measured in each animal. ***, $p<0.001$ vs vehicle (one-way ANOVA followed by Dunnett’s test). Data represent mean ± SE (n=7-8).

**Figure S8.** Gabapentin but not KLYP961 impairs motor coordination. Groups of mice (n=6-8) received vehicle, gabapentin or KLYP961 and latency to fall from accelerating rotarod was defined at selected time points. Panel A summarizes dose-related effects of gabapentin. In a separate experiment, effects of KLYP961 were compared to a single dose of gabapentin (300 mg/kg). Panel C summarizes ED$_{50}$ values for effects of gabapentin or KLYP961 on motor
coordination at 2 hr post-oral dose. **, p<0.01 and ***, p<0.001 vs baseline (Two-way ANOVA followed by Bonferroni’s test).
Supplemental Table S1. Off-Target Activity Profile of KLYP961

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<th>Assay Target</th>
<th>% Inhibition (10 μM)*</th>
<th>Reference Compound</th>
<th>Reference compound K&lt;sub&gt;i&lt;/sub&gt; (M)/ [IC&lt;sub&gt;50&lt;/sub&gt;,M]</th>
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<td>CCK-8s</td>
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<td>CCKB (h) (CCK2)</td>
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<td>M1 (h)- Muscarinic</td>
<td>pirenzepine</td>
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<td>M2 (h)-Muscarinic</td>
<td>methoctramine</td>
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<td>M3 (h)-Muscarinic</td>
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<td>3.90E-10</td>
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<td>NK1 (h)- Neurokinin</td>
<td>[Sar&quot;',Met(O2)\textsuperscript{11}]-SP</td>
<td>2.00E-10</td>
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<td>NK2 (h)- Neurokinin</td>
<td>[Nle\textsuperscript{10}]-NKA (4-10)</td>
<td>5.20E-09</td>
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<td>Opioid (non-selective)</td>
<td>naloxone</td>
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<td>delta2 (h) (DOP)-Opiate</td>
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<td>k (KOP)- Opiate</td>
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<td>m (h) (MOP) (agonist site)- Opiate</td>
<td>DAMGO</td>
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<td>5-HT (non-selective)</td>
<td>serotonin</td>
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<td>5-HT1B- Serotonin</td>
<td>serotonin</td>
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<td>5-HT1D -Serotonin</td>
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<td>5HT2A(Human) Serotonin</td>
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<td>5-HT2B (h)- Serotonin</td>
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<td>5-HT2c (h)- Serotonin</td>
<td>RS-102221</td>
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<td>5-HT3 (h)-Serotonin</td>
<td>MDL 72222</td>
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<td>S (non-selective)-Sigma</td>
<td>-9</td>
<td>haloperidol</td>
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<td>Ca+2 Channel (L-DHP site)</td>
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<td>nitrendipine</td>
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<td>Ca2+ channel (L, diltiazem site) (benzothiazepines)</td>
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<td>diltiazem</td>
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<td>Ca2+ channel (L, verapamil site) (phenylalkylamines)</td>
<td>-17</td>
<td>D 600</td>
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<td>Na+ channel (site 2)</td>
<td>9</td>
<td>veratridine</td>
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<td>DA transporter (h)</td>
<td>-3</td>
<td>BTCP</td>
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<td>5-HT transporter (h)</td>
<td>-12</td>
<td>imipramine</td>
<td>2.00E-09</td>
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<td>MAO-A</td>
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<td>clorgyline</td>
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<td>MAO-B</td>
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<td>COX-1</td>
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<td>Diclofenac</td>
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<td>COX-2</td>
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<td>VR1 (Agonism)[^1]</td>
<td>8</td>
<td>Capsaicin</td>
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<tr>
<td>VR1 (Antagonism)[^2]</td>
<td>9</td>
<td>Capsazine</td>
<td>3.8E-08</td>
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</table>

* data represent mean of 2 determinations

VR1 agonist\[^1\] and antagonist\[^2\] assays were conducted at 10 or 50 μM, respectively.
Supplemental Table S2. Summary pharmacokinetics of KLYP961 in mice and rhesus non-human primates

<table>
<thead>
<tr>
<th>Dose</th>
<th>Route</th>
<th>Parameter</th>
<th>Mouse</th>
<th>Rhesus</th>
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<tbody>
<tr>
<td>3 mg/kg</td>
<td>Intravenous</td>
<td>CL(^1)</td>
<td>4.5</td>
<td>6.0</td>
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<td></td>
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<td>V(_{ss})(^2)</td>
<td>0.39</td>
<td>3.0</td>
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<td></td>
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<td>t(_{1/2})(^3)</td>
<td>2.1</td>
<td>12.5</td>
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<tr>
<td>10 mg/kg</td>
<td>Oral</td>
<td>C(_{max})(^4)</td>
<td>15.4</td>
<td>3.28</td>
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<td>AUC(^5)</td>
<td>25</td>
<td>18.2</td>
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<tr>
<td></td>
<td></td>
<td>% Bioavailability</td>
<td>62.1</td>
<td>62.9</td>
</tr>
</tbody>
</table>

\(^1\)CL - Total plasma clearance (mL/min/kg)

\(^2\)V\(_{ss}\) - Volume of distribution at steady-state (L/kg)

\(^3\)t\(_{1/2}\) – terminal elimination half-life (hr)

\(^4\)C\(_{max}\) (µg/mL)

\(^5\)AUC\(_{0-inf}\) (µg*hr/mL)