

KLYP956 Is a Non-Imidazole-Based Orally Active Inhibitor of Nitric-Oxide Synthase Dimerization

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ABSTRACT

Nitric-oxide synthases (NOS) generate nitric oxide (NO) through the oxidation of L-arginine. Inappropriate or excessive production of NO by NOS is associated with the pathophysiology of various disease states. Efforts to treat these disorders by developing arginine mimetic, substrate-competitive NOS inhibitors as drugs have met with little success. Small-molecule-mediated inhibition of NOS dimerization represents an intriguing alternative to substrate-competitive inhibition. An ultra-high-throughput cell-based screen of 880,000 small molecules identified a novel quinolinone with inducible NOS (iNOS) inhibitory activity. Exploratory chemistry based on this initial screening hit resulted in the synthesis of KLYP956, which inhibits iNOS at low nanomolar concentrations. The iNOS inhibitory potency of KLYP956 is insensitive to changes in

concentrations of the substrate arginine, or the cofactor tetrahydrobiopterin. Mechanistic analysis suggests that KLYP956 binds the oxygenase domain in the vicinity of the active site heme and inhibits iNOS and neuronal NOS (nNOS) by preventing the formation of enzymatically active dimers. Oral administration of KLYP956 [*N*-(3-chlorophenyl)-*N*-((8-fluoro-2-oxo-1,2-dihydroquinolin-4-yl)methyl)-4-methylthiazole-5-carboxamide] inhibits iNOS activity in a murine model of endotoxemia and blocks pain behaviors in a formalin model of nociception. KLYP956 thus represents the first nonimidazole-based inhibitor of iNOS and nNOS dimerization and provides a novel pharmaceutical alternative to previously described substrate competitive inhibitors.

The overproduction of nitric oxide (NO) has been implicated in the pathophysiology of a broad range of human diseases including pain, inflammation, migraine and neurodegenerative disorders. Three nitric-oxide synthase (NOS) isoforms have been described, including endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Data support the notion that the inhibition of iNOS and nNOS may have therapeutic utility in the treatment of a variety of disease states, including inflammation and pain. However, inhibition

of eNOS is considered detrimental because it results in elevated systemic blood pressure. Among the three isoforms, iNOS generates stoichiometrically higher amounts of NO and is expressed at sites of inflammation. Irrespective of the source, excessive NO can generate peroxynitrite and other reactive species that can trigger protein nitrosylation, leading to tissue damage (Vallance and Leiper, 2002).

NOS isoforms catalyze the NADPH- and O₂-dependent oxidation of L-arginine to NO and citrulline, with *N*-hydroxy-L-arginine formed as an intermediate. NOS isoforms are flavoheme enzymes that are only active as homodimers. Each monomer has a carboxyl-terminal diflavin-reductase domain

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ABBREVIATIONS: NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; SEITU, S-ethyl isothiourea; 1400W, *N*-(3-(aminoethyl)benzyl)-acetamidine hydrochloride; BBS-4, (*R*)-1-(2-(1*H*-imidazol-1-yl)-6-methylpyrimidin-4-yl)-*N*-(2-(benzo[d][1,3]dioxol-5-yl)ethyl)pyrrolidine-2-carboxamide; BH4, tetrahydrobiopterin; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; DAN, 2,3-diaminonaphthalene; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; LPS, lipopolysaccharide; HA, hemagglutinin; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; Blotto, bovine lacto transfer optimizer; UT, untagged; LT SDS-PAGE, low-temperature SDS-polyacrylamide gel electrophoresis; AR-C102222, 5-(4'-amino-5',8'-difluoro-1',2'-dihydrospiro[piperidine-4,2'-quinazoline]-1-ylcarbonyl)pyridine-2-carbonitrile; DI, dimerization inhibitor; m, mouse; h, human; IFN, interferon.

and an amino-terminal oxygenase domain. Extensive studies have defined many biochemical and structural requirements for NOS dimerization. Dimerization is thought to activate the enzyme by sequestering iron, generating high-affinity binding sites for arginine and the essential cofactor tetrahydrobiopterin (BH₄), and allowing electron transfer from the reductase-domain flavins of one monomer to the oxygenase-domain heme of the other monomer (Siddhanta et al., 1998). Several reports have suggested that both iNOS and nNOS activity is regulated by controlling the timing and extent of dimer formation and/or stability via protein interactions and NO itself (Kone et al., 2003; Li et al., 2006).

Small-molecule inhibitors of NOS have been described and seem to function through two predominant modes of action. Many classic substrate-competitive inhibitors share structural similarities with L-arginine and are thus sensitive to local arginine concentrations. These compounds often have isoform selectivity limitations and may also interfere with physiological processes intimately dependent on arginine transport and metabolism (Feldman et al., 1993; Robertson et al., 1993; Olken et al., 1994; Edwards et al., 1998; Paige and Jaffrey, 2007). Several groups have described small molecules capable of interfering with the formation and enzymatic activity of NOS dimers. With the exception of a peptide-derived compound (Chida et al., 2005), all iNOS dimerization inhibitors described to date contain an *N*-substituted imidazole moiety that is thought to directly coordinate the heme iron in the active site of the enzyme (Sennequier et al., 1999; McMillan et al., 2000; Ohtsuka et al., 2002; Sohn et al., 2008). Given that nonsubstrate mimetic inhibitors, such as those that act by blocking formation of the enzymatically active dimer, are likely to have distinct *in vivo* profiles, we conducted a cell-based high-throughput screen of approximately 880,000 small molecules to discover novel, nonarginine, non-imidazole-based inhibitors of recombinant human iNOS. These efforts resulted in the identification of a quinolinone scaffold that was subsequently optimized for potency and selectivity to generate KLYP956, a low nanomolar inhibitor of iNOS with greater than 1900-fold selectivity over eNOS. Mechanistic analysis suggests that KLYP956 acts by preventing the formation of the stable enzymatically active form of iNOS and nNOS. Spectral studies suggest that the compound binds to the enzyme in a mode distinct from that of the *N*-substituted imidazole-based inhibitors.

Materials and Methods

Compounds. *S*-Ethyl isothiurea (SEITU) and 1400W were obtained from Sigma (St. Louis, MO); BBS-4 and KLYP956 were synthesized internally at Kalypsys, Inc.

Molecular Cloning. Using standard polymerase chain reaction techniques, full-length coding sequences were generated to match the following published GenBank accession numbers: human iNOS (NM_000625), human nNOS (NM_000620), human eNOS (NM_000603), murine iNOS (NM_010927), murine nNOS (NM_008712), and murine eNOS (NM_008713). All clones were sequenced to confirm identity. Full-length coding sequences were subcloned into pCS2 mammalian expression constructs (described at <http://sitemaker.umich.edu/dltturner.vectors>).

Cell Culture. A172 human glioblastoma, RAW 264.7 murine macrophage, and HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HEK293

nNOS-stable cells were grown in this media supplemented with 2 μ g/ml puromycin to retain selection. A172 cells were stimulated to express iNOS by adding a cocktail containing hIFN- γ (4000 U/ml), hTNF- α (40 ng/ml), hIL-1 β (4 ng/ml) (Roche Diagnostics, Indianapolis, IN). RAW 264.7 cells were stimulated in media with lipopolysaccharide (1 μ g/ml; Sigma) and mIFN- γ (100 U/ml; Roche Diagnostics). HEK 293 cells were transiently transfected with a cytomegalovirus-driven plasmid expressing murine iNOS, murine nNOS, murine eNOS, human iNOS, human nNOS, or human eNOS.

Nitric Oxide Detection (DAN Assay). HEK293 cells were plated into 15-cm dishes and grown to 70% confluence. Cells were transiently transfected with 30 μ l of Fugene and 10 μ g/dish of the appropriate NOS plasmid for 4 h. Transfected cells were plated in 384- or 1536-well plates at a density of 5×10^5 cells/ml, and compounds were added immediately using a proprietary pin-transfer technology. Cells for the iNOS assay were incubated for 18 h for nitrite accumulation. NO production was determined indirectly by measuring nitrite accumulation in cell culture supernatants using the 2,3-diaminonaphthalene (DAN) assay. Supernatants were mixed with (final concentration, 10 μ g/ml DAN; Invitrogen, Carlsbad, CA) diluted in DMEM supplemented with hydrochloric acid (final concentration, 0.1 N) and incubated for 20 min at room temperature. Fluorescence (excitation 360 nm/emission 425 nm) was measured after increasing pH with sodium hydroxide (final concentration, 0.12 N) on an Acquest plate reader (Molecular Devices, Sunnyvale, CA). Cells for eNOS and nNOS were incubated 24 h with compound and then activated by ionophore (for eNOS: A23187, final concentration, 0.8 μ M; for nNOS: ionomycin, final concentration, 30 μ M) and incubated an additional 18 h for nitrite accumulation followed by the DAN assay.

Biochemical Inhibition of iNOS and nNOS in the Arginine-to-Citrulline Conversion Assay. HEK293 cells were plated into 15-cm dishes and grown to 70% confluence. Cells were transiently transfected with 10 μ g/dish of human iNOS or nNOS expression plasmid for 24 h. Proteins were harvested with 1 ml of 1 mM EDTA, centrifuged 2 min at 400g, and lysed with 0.1% Triton-X-100 in 50 mM potassium phosphate buffer containing protease inhibitors (Roche). Lysates were then incubated with compound for 20 min. Reaction mix (Stratagene NOS Detect) was added containing ¹⁴C-labeled arginine (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Conversion of arginine to citrulline was determined by ion-exchange separation and scintillation counting.

Arginine Competition. HEK293 cells were plated into 15-cm dishes and grown to 70% confluence. Cells were transiently transfected with 30 μ l of Fugene and 10 μ g/dish human iNOS expression plasmid for 3 h. Media was replaced with arginine-free DMEM containing 0.3% bovine serum albumin for 1 h. Cells were trypsinized, diluted to 5×10^5 cells/ml in either 10 μ M or 1000 μ M arginine in DMEM containing 0.3% bovine serum albumin, and 5 μ l/well was plated into a black 1536-well plate. Compounds were added and cells were incubated for 18 h followed by the DAN assay as described above.

BH₄ Competition. A172 cells (4×10^6 cells/well) were seeded in a 384-well plate for 4 h. Sepiapterin (Cayman Chemical, Ann Arbor, MI) was added to yield a final concentration of 0, 1, or 10 μ M diluted in induction cocktail (see *Cell Culture*) and incubated for 22 h followed by the DAN assay as described above.

iNOS_{oxy} Spectral Shift. The sequence encoding human iNOS oxygenase (iNOS_{oxy}; amino acids 74–504) was inserted into pET29a(+). The resulting construct carrying a C-terminal 6 \times His tag was expressed in *Escherichia coli* and purified by nickel-nitrilotriacetic acid immobilized-metal affinity chromatography followed by ion exchange chromatography with a Mono Q column. To generate monomeric human iNOS_{oxy}, a protocol was used as described previously (Sennequier et al., 1999). In brief, protein was diluted in 5 M urea for 1.5 h, then dialyzed against 2 M urea for 4 h, followed by 0.1 M urea overnight. Protein was diluted in spectral shift buffer (25 mM HEPES, 5% glycerol, and 1 mM DTT) during urea treatment and

during the spectral shift assay. All incubations were at 4°C. For spectral scans, 50 µl of 0.3 mg/ml of urea-treated protein was dispensed into a 384-well assay dish in 25 mM HEPES, 5% glycerol, and 1 mM DTT. 500 nl of compound was added via the proprietary Kalypsys pin-based transfer technology and incubated for 30 min at room temperature. A spectral scan (UV-Vis) was measured using a Spectramax Plus spectrophotometer (Molecular Devices).

Gel-Based Dimer Assay (Low-Temperature SDS-PAGE) for iNOS and nNOS. RAW 264.7 cells were seeded into six-well dishes at a density of 1.5×10^6 cells/well. Cells were incubated for 5 h, and 1 ml of media was removed from each well and replaced with 1 ml of a 2 C 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, and 1 mM EDTA) containing protease inhibitors was added to each well. Cells were scraped from the dish and transferred to microcentrifuge tubes on ice. Samples were sonicated for 5 s at setting 4 (Branson) and centrifuged at 16,000g for 10 min at 4°C. The concentration of protein was normalized using an advanced protein assay (Cytoskeleton, Inc., Denver, CO). An equal volume of ice-cold 2× loading buffer (63 mM Tris, pH 6.8, 10% glycerol, 3% SDS, 1% β -mercaptoethanol, and 0.002% bromophenol blue) was added to each sample and loaded onto a 4-to-20% Tris-glycine polyacrylamide gel (Invitrogen). The gel was run in pre-chilled 1× SDS running buffer in an ice-cold room for 2.5 h at 125 V. Proteins were transferred to nitrocellulose for 2.5 h at 70 V in 1× transfer buffer and detected as described below under *Western Blotting*.

FLAG Coimmunoprecipitation HA Detection and Biochemical Disruption. The N terminus of human iNOS was tagged with either influenza hemagglutinin (HA) peptide (YPYDVPDYA) or FLAG peptide (DYKDDDDK) and linked to iNOS via two glycine residues using insertion mutagenesis following the protocol described previously (Wang and Malcolm, 1999). HEK293 cells were cotransfected with HA- and FLAG-tagged human iNOS for 4 h followed by addition of compound and incubation overnight (no compound was added to the cotransfected cells for the biochemical disruption assay.) Before harvesting cells, 50 µl of media from each well was plated in quadruplicate for NO determination via the DAN assay. Cells were harvested with 1 ml of ice-cold PBS, centrifuged 2 min at 400g and washed once with 1 ml of ice-cold PBS. Cells were lysed with 500 µl of lysis buffer (25 mM Tris, pH 8.0, 10% glycerol, 150 mM NaCl, and 0.5% Triton-X-100) containing protease inhibitors. Extracts were centrifuged at 16,000g for 10 min at 4°C. The concentration of protein was normalized using an advanced protein assay (Cytoskeleton, Inc.). Approximately 200 to 400 µg of protein was immunoprecipitated for 1 h at 4°C using anti-FLAG M2 beads (Sigma) in 1 ml (for biochemical disruption of dimers, compound was added to lysates 1 h before the M2 immunoprecipitation). Immunoprecipitates were washed three times with 1 ml of lysis buffer followed by elution with 50 µl of 2× NuPage loading buffer. DTT was added to each sample at a final concentration of 0.1 M, and samples were loaded onto a 4-to-12% Bis-Tris polyacrylamide gel (Invitrogen). The gels were run in 1× MES running buffer for 1 h at 160 V. Proteins were transferred to nitrocellulose for 2 h at 80 V in 1× transfer buffer and probed as described below (Western blotting).

Western Blotting. Membranes were blocked overnight in Blotto [20 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% (v/v) Tween 20 containing 3% (w/v) nonfat milk at 4°C]. The membranes were incubated in mouse anti-iNOS (1:2500; BD Biosciences, San Jose, CA), mouse anti-Flu 16B12 (1:1000; Covance Research Products, Princeton, NJ), mouse anti-nNOS (1:2500; BD Biosciences), or rabbit anti-calmodulin (1:4000; Abcam Inc., Cambridge, MA) in Blotto for 1 h at room temperature followed by three 5-min washes in 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% (v/v) Tween 20. The blot was then incubated in goat anti-mouse (1:2000; Bio-Rad Laboratories, Her-

cules, CA) or goat anti-rabbit (1:5000; Santa Cruz) analysis of variance horseradish peroxidase-conjugated secondary antibody in Blotto for 1 h at room temperature. After four 5-min washes, the proteins were visualized by chemiluminescence using SuperSignal West Dura (Pierce, Rockford, IL) detection reagent and captured on a CCD-based imaging device (Alpha Innotech, San Leandro, CA).

Mouse LPS Model. Male Balb/C mice were given intraperitoneal injections of LPS solution in saline (10 mg/kg; Sigma). Blood samples were collected under isoflurane anesthesia at predetermined time points for analysis of nitrates (6 h after LPS). KLYP956 was administered by oral gavage immediately before LPS injection. 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; Millipore, Billerica, MA), at 2000g for 10 min at 4°C. Plasma nitrates, a marker of iNOS activity, were measured using a fluorimetric assay kit (Cayman Chemical) after the formation of a fluorophore by the addition of DAN. Fluorescence was measured using the Aquest plate reader (Molecular Devices). All results are expressed as mean \pm S.E.M.

Mouse Formalin Model. Compounds were administered to animals via oral gavage in a vehicle consisting of 9:0.5:0.5:90 of polyethylene glycol 400/Tween 80/polyvinylpyrrolidone K30/Cm-cellulose in water (0.5% w/v) 1 h before formalin challenge. For intraperitoneal dosing, the vehicle composition was 9:0.5:0.5:90 of polyethylene glycol 400/Tween 80/polyvinylpyrrolidone K30/water and the compounds were administered 1 h before formalin challenge. The formalin solution for intraplantar injection was first prepared by diluting a 10% formalin stock solution to a final concentration of 5% with 0.9% saline. A volume of 20 µl was injected into the left hind paw of each animal.

Immediately after injection of formalin, mice were placed in a transparent observation chamber (Kalypsys, Inc.), and a timer was activated. The duration of pain behaviors (hind paw flinches, licking, and biting) displayed by the injured hind paw was counted in 5-min intervals. Phase I was observed from 0 to 5 min after formalin injection. Phase II was observed from 25 to 45 min after formalin injection. Data are presented as time spent in nociceptive behaviors during phases I and II. There was a single observer throughout the study, and observers were not blinded with respect to treatment group. All in vivo experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Results

High-Throughput Screening Identifies a Novel Potent iNOS Inhibitor. In an effort to identify non-imidazole-based inhibitors of iNOS, we conducted a homogeneous cell-based high-throughput screen of 880,000 small molecules for inhibitors of recombinant human iNOS. A series of secondary assays were implemented in a matrix fashion to select for direct, non-substrate-competitive inhibitors of human iNOS with favorable pharmaceutical properties. One compound that emerged from this screening paradigm displayed favorable potency and isoform selectivity, as well as good preliminary absorption distribution metabolism excretion toxicology properties (data not shown). Exploratory chemistry efforts led to the synthesis of KLYP956, a highly potent quinolinone-based human iNOS inhibitor that has no structural similarity to arginine-based inhibitors (such as SEITU) or to *N*-substituted imidazole-based inhibitors (such as BBS-4) (Fig. 1, A and B). Given the novel chemical structure of KLYP956, a series of studies was conducted to characterize its selectivity properties as well as to investigate the mechanism of NOS inhibition.

KLYP956 Inhibits iNOS and nNOS in Cells but Not in Reconstituted Biochemical Assays. While the inhibitory activity of the high-throughput screening leads against recombinant human iNOS was robust, inhibition of the murine iNOS enzyme was surprisingly poor, with an $IC_{50} > 50 \mu M$ (data not shown). However, the improved potency of KLYP956 against human iNOS was paralleled by improved potency against murine iNOS. In cell-based assays, KLYP956 inhibited both human and murine iNOS activity in a concentration-dependent manner with IC_{50} values of 0.01 and 0.14 μM , respectively (Fig. 2A and Table 1). Next, we investigated the isoform and species selectivity profile of KLYP956. The compound inhibited human nNOS and human eNOS but required significantly higher concentrations than those necessary to inhibit iNOS, with IC_{50} values of 2.6 and 19.0 μM , respectively. Consequently, in humans, the selectivity of KLYP956 for iNOS over nNOS is 260-fold and that over eNOS is 1900-fold (Table 1). Surprisingly, in addition to an approximate 10-fold difference in potency between human and murine iNOS, an inverse potency relationship is seen for human versus murine nNOS. KLYP956 is essentially equipotent on murine iNOS and murine nNOS (Table 1).

The majority of substrate competitive iNOS inhibitors exhibit high potency in biochemical assays, but are considerably less potent in cell-based assays. In contrast, imidazole-based dimerization inhibitors such as BBS-4 display an

opposite profile, being substantially more potent in cell-based assays (Blasko et al., 2002; Davey et al., 2007). As seen in Fig. 2, B and C, BBS-4 and KLYP956 display limited inhibition against human iNOS and nNOS in the biochemical inhibition assay, even at the highest concentration tested (30

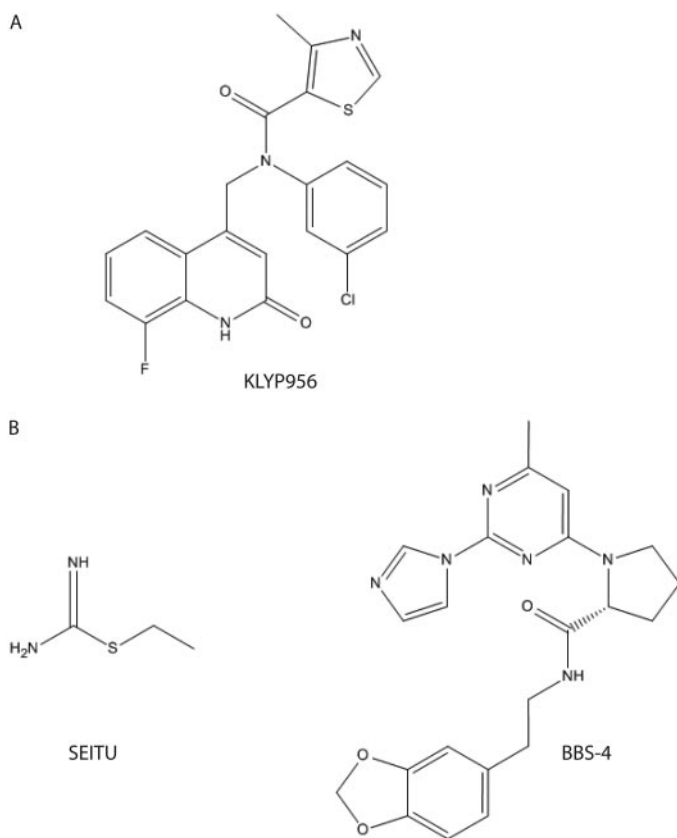


Fig. 1. Chemical Structure of KLYP956 and other known iNOS inhibitors. A, KLYP956 is a novel *N*-(3-chlorophenyl)-*N*-((8-fluoro-2-oxo-1,2-dihydroquinolin-4-yl)methyl)-4-methylthiazole-5-carboxamide inhibitor of iNOS, a precursor of which was identified in a cell-based ultra high-throughput screen. B, SEITU is a nonselective substrate-competitive inhibitor of NOS that is isosteric with L-arginine. BBS-4 is an *N*-substituted imidazole-containing inhibitor of iNOS dimerization.

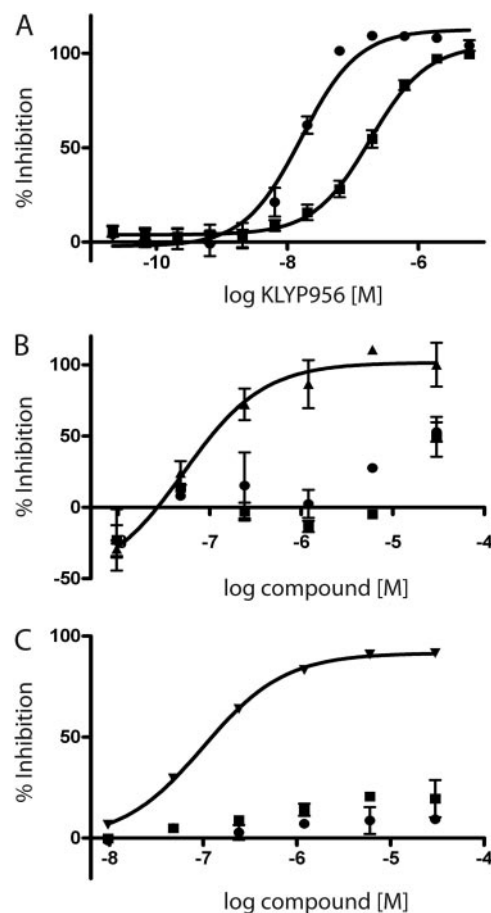


Fig. 2. KLYP956 inhibits iNOS in cells but not in reconstituted biochemical assays. A, cell-based iNOS inhibition assay; recombinant iNOS (human or murine iNOS) was transiently transfected into HEK293 cells, and NO production was measured indirectly by measuring nitrite levels using DAN. Data are shown as dose-response curves of iNOS inhibition \pm S.D. of KLYP956 against human (●) and murine (■) iNOS, respectively. B, biochemical inhibition of human iNOS; compounds (▲, SEITU; ●, BBS-4; ■, KLYP956) were incubated for 20 min with protein extracts derived from HEK293 cells transfected with human iNOS followed by measuring conversion of radioactive arginine to citrulline. Data are shown as dose-response curves of iNOS inhibition, mean \pm S.D. C, biochemical inhibition of human nNOS; compounds (▲, SEITU; ●, BBS-4; ■, KLYP956) were incubated for 20 min with protein extracts derived from HEK293 cells transfected with human nNOS followed by measuring conversion of radioactive arginine to citrulline. Data are shown as dose-response curves of nNOS inhibition, mean \pm S.D.

TABLE 1

KLYP956 isoform and species selectivity profile

Values in second, third, and fourth column represent $IC_{50} \pm$ S.D. for NOS inhibition. Calculations are averages of at least three experiments.

Species	iNOS	nNOS	eNOS	Selectivity	
				i/n	i/e
	μM			<i>-fold</i>	
Human	0.01 \pm 0.01	2.6 \pm 1.0	19 \pm 6.5	260	1900
Murine	0.14 \pm 0.12	0.15 \pm 0.05	12.0 \pm 4.8	Non-selective	86

i/n, iNOS/nNOS; i/e, iNOS/eNOS.

μM). In contrast, the nonselective arginine mimetic SEITU potentially inhibits both NOS isoforms in vitro (Fig. 2, B and C).

To characterize the biochemical mechanism by which KLYP956 inhibits NOS, we then tested the effects of changes in substrate concentration on KLYP956 compared with previously reported NOS inhibitors. Substrate competitive NOS inhibitors display significant shifts in IC_{50} values as a consequence of elevated arginine concentrations. For example, SEITU and 1400W display a 15- and 9-fold potency shift when the arginine concentration in cell culture media is increased from 10 μM to 1 mM, respectively (Table 2). Consistent with the in vitro biochemical inhibition data described above, KLYP956 and BBS-4 display little if any significant change in potency when arginine concentrations in

cell culture media are modulated by 2 orders of magnitude (Table 2).

Likewise, changes in the concentration of the essential cofactor BH_4 can potentially affect the potency of compounds if they are competitive with BH_4 . This possibility can be investigated by supplementing excess sepiapterin, a bipterin precursor, into cell-based inhibition assays (Blasko et al., 2002). To test whether KLYP956 inhibits BH_4 binding, we determined KLYP956-mediated cellular iNOS inhibition with varying concentrations of sepiapterin, ranging from 0 to 10 μM . Under the conditions tested, the potency of neither KLYP956 nor BBS-4 was perturbed by changes in sepiapterin concentrations, indicating that KLYP956 is unlikely to be competitive with BH_4 (Table 3).

KLYP956 Perturbs iNOS_{oxy} Domain Heme Spectrum in a Manner Distinct from That of Imidazole-Based Inhibitors. Direct interaction with the iNOS_{oxy} domain can be detected spectrophotometrically by measuring changes in the heme absorbance spectrum of the purified enzyme (McMillan and Masters, 1993; Sennequier and Stuehr, 1996). Bacterially expressed 6 \times His-tagged human iNOS_{oxy} domain was purified and partially denatured to yield a population of monomeric and loose dimeric enzyme that, in the presence of DTT, displayed a characteristic split Soret absorbance peak at 378 and 460 nm (Fig. 3). The addition of KLYP956 (1–10 μM) results in a concentration-dependent shift in the absorbance spectrum, generating a single Soret maximum at 396 nm (Fig. 3). A similar spectral shift has been described when the enzyme is coincubated with arginine and BH_4 (Sennequier et al., 1999). This spectral shift contrasts sharply with that generated by imidazole-based compounds that produce a single Soret peak centered at 429 nm, indicating a shift in the heme iron to the low spin state (Blasko et al., 2002).

KLYP956 Destabilizes Both Murine and Human iNOS Dimer in Cells. The quaternary structural status of iNOS isolated from activated murine macrophages can be visualized using partially denaturing low-temperature SDS-PAGE followed by immunoblotting with antibodies recognizing the iNOS protein. Under these experimental conditions, a

TABLE 2

KLYP956 is non-arginine-competitive in cells

Values in second and third column represent $\text{IC}_{50} \pm \text{S.D.}$ for iNOS inhibition. Calculations are averages of three experiments.

Compound	Arginine		Shift
	10 μM	1000 μM	
	μM		-fold
KLYP956	0.005 ± 0.001	0.010 ± 0.004	2
BBS-4	0.005 ± 0.002	0.007 ± 0.001	<2
SEITU	2.2 ± 1.0	29.1 ± 8.3	15
1400W	3.9 ± 2.2	34.9 ± 16.3	9

TABLE 3

KLYP956 is non-biopterin-competitive in cells

Values represent $\text{IC}_{50} \pm \text{S.D.}$ for iNOS inhibition. Calculations are averages of three experiments.

Compound	Sepiapterin		
	0 μM	1 μM	10 μM
	μM		
KLYP956	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
BBS-4	0.002 ± 0.001	0.003 ± 0.001	0.003 ± 0.001
SEITU	1.8 ± 0.2	1.5 ± 0.8	1.5 ± 0.2

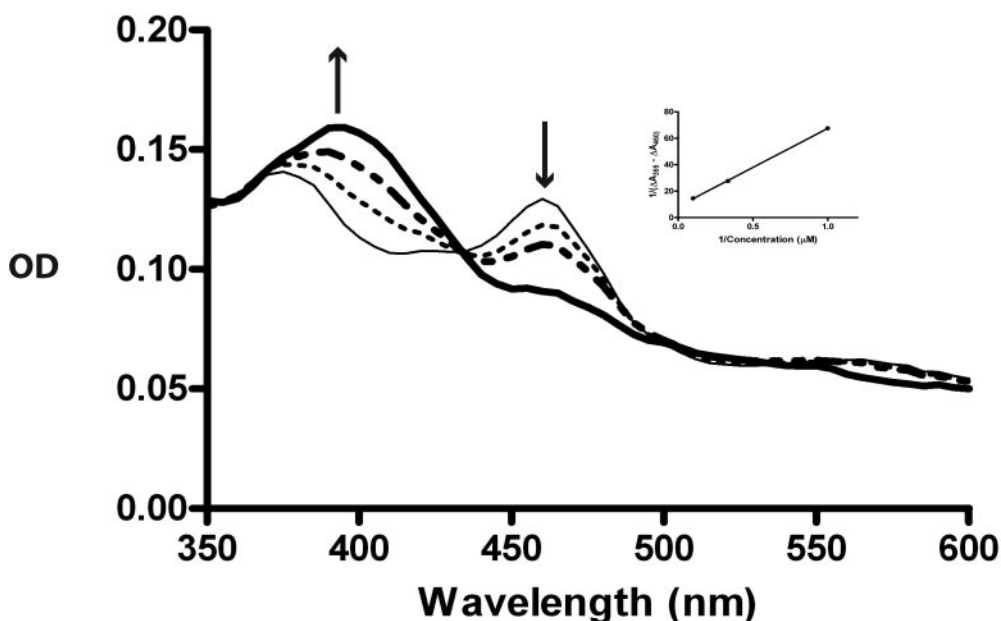


Fig. 3. KLYP956 perturbs purified iNOS_{oxy} domain heme absorbance spectrum in vitro. Urea-dialyzed human iNOS_{oxy} (residues 74–504) displays a characteristic twin Soret absorbance spectrum with peaks at 378 and 460 nm. KLYP956 dose-dependently induces a spin state transition of the heme iron to generate an absorbance maxima of 396 nm. Each trace represents a distinct concentration of KLYP956 as indicated on the graph: 0 (—), 1 (---), 3 (·····), and 10 (— · —) μM . Arrows indicate the increase and decrease in absorbance at 396 and 460 nm, respectively. Inset displays a reciprocal difference plot between changes in absorbance at 396 and 460 nm versus KLYP956 concentration. Line represents best fit using least-squares method ($r^2 = 0.9998$).

subpopulation of untreated or vehicle-treated iNOS remains dimeric. The dimer-to-monomer ratio can be visualized by differences in mobility when separated in the polyacrylamide gel matrix. Incubation of cells with SEITU (50 μ M) efficiently inhibits murine macrophage-derived iNOS activity (Fig. 4B). However, rather than reducing the amount of detectable dimer under LT SDS-PAGE conditions, SEITU increases the ratio of dimer to monomer (Fig. 4A). By contrast, pretreatment with 1 μ M BBS-4 results in complete loss of detectable dimer, consistent with reports indicating that compounds in this series function as inhibitors of dimerization (McMillan et al., 2000; Blasko et al., 2002). RAW264.7 cells were treated with KLYP956 at concentrations ranging from 0.2 nM to 1 μ M. Similar to the results observed for BBS-4, KLYP956 dose dependently reduces levels of dimeric iNOS (Fig. 4A). It is noteworthy that, unlike BBS-4, KLYP956 treatment leads to the formation of a series of one or more higher-order bands that are likely to be composed of inactive multimers or oligomers of iNOS (Fig. 4A). Compounds structurally related to KLYP956 also induce the formation of these multimers (Fig. 4A and data not shown). Reduction in nitrite levels from supernatants collected from RAW264.7 cells treated with KLYP956 paralleled the reduction in dimer levels (Fig. 4B).

To evaluate the dimeric state of iNOS under more native conditions, we generated two epitope-tagged versions of human iNOS containing either a FLAG tag or an HA tag and cotransfected both constructs into HEK293 cells (Fig. 5A). Lysates prepared from cotransfected cells facilitated coimmunoprecipitation of the HA-tagged form using anti-FLAG antibodies (Fig. 5A). Neither HA-tagged nor untagged native human iNOS (UT) coimmunoprecipitate with anti-FLAG antibodies in the absence of coexpressed FLAG-tagged human iNOS (Fig. 5B). This system allowed for the testing of com-

pound effects on human iNOS protein-protein interactions under both in vivo and in vitro conditions without the need for partial denaturation. Similar to the effects seen on murine iNOS in the LT SDS-PAGE assay, SEITU treatment (50 μ M) results in somewhat greater quantities of coimmunoprecipitated HA-tagged human iNOS (Fig. 5B, top). By contrast, BBS-4 has substantially reduced levels of anti-HA immunoreactivity, consistent with a reduction in dimeric human iNOS. Coimmunoprecipitated amounts of HA-tagged human iNOS are also reduced in KLYP956-treated cells, albeit to a lesser extent than that seen for BBS-4 (Fig. 5B, top). In contrast, the relative levels of the iNOS-associated cofactor protein calmodulin were unaffected by any treatment tested (Fig. 5B, third blot). Comparable levels of FLAG-iNOS were immunoprecipitated in all samples (Fig. 5B, second blot). Enzymatic activity from cell culture supernatants treated with SEITU (50 μ M), BBS-4 (0.5 μ M), and KLYP956 (0.5 μ M) were all reduced by >95%, indicating that residual human iNOS-tagged heterodimer/multimers were inactive (Fig. 5C). To ascertain whether any of the compounds tested above could disrupt pre-existing human iNOS dimers, the same cotransfection study was conducted, but with compounds added after protein expression and cell lysis. Under these conditions, vehicle-treatment of lysates results in coimmunoprecipitation of HA-tagged human iNOS, similar to that seen in the vehicle pretreatment experiment (Fig. 5, B and D). However, none of the three compounds tested resulted in detectable changes in the level of coimmunoprecipitated HA-tagged human iNOS, even at concentrations of 50 μ M (Fig. 5D), indicating that none of these compounds can destabilize preformed iNOS dimers.

KLYP956 Destabilizes Human nNOS Dimer. Given that KLYP956 inhibits human nNOS at low micromolar concentrations, we conducted several studies to address the mechanism of inhibition of this isoform. First, we took advantage of the calcium/calmodulin dependence of nNOS to determine the ability of compounds to inhibit the enzyme in either its monomeric or dimerized states. HEK293 cells transiently transfected with human nNOS were treated with compound during the nascent phase of nNOS protein expression (within 4 h after transfection), or immediately before Ca^{2+} -ionophore activation (24 h after transfection), when human nNOS dimers are presumably formed. Upon activation with ionomycin, cellular human nNOS generates significant NO that is detected as nitrites using the 2,3-diaminonaphthalene reagent. Under these conditions, SEITU reduces nitrite levels with an IC_{50} of approximately 4.0 μ M and shows no change in potency or efficacy when incubated at either time point (Fig. 6A). By contrast, KLYP956 reduces nitrite levels effectively when incubated at 4 h (IC_{50} = 3.6 μ M) but shows little nitrite reduction when added immediately before ionophore addition (IC_{50} > 100 μ M) (Fig. 6A).

The dimeric status of nNOS was assessed using LT SDS-PAGE after stably transfected human nNOS-expressing HEK293 cells were incubated with compounds. Given the long half-life reported for nNOS in cells (Kolodziejewski et al., 2004), an incubation time of 36 h was used to ensure that the compounds were present during the synthesis of nascent nNOS protein. Lysates from treated cells were separated by LT SDS-PAGE and probed using an antibody specific for human nNOS. As seen with iNOS, SEITU increases the relative quantity of dimeric enzyme compared with vehicle-

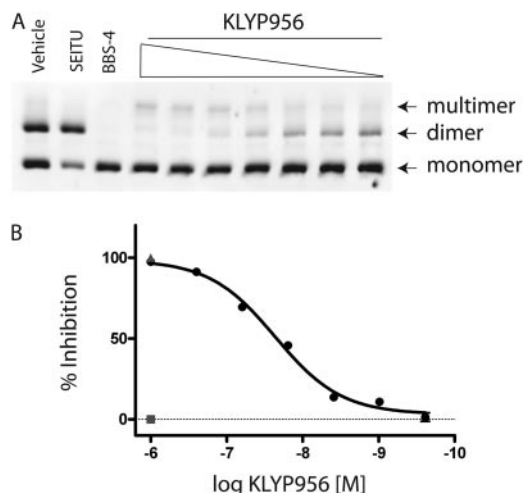


Fig. 4. KLYP956 destabilizes murine iNOS dimer in cells. A, LPS/IFN- γ induced RAW264.7 cells treated with indicated compounds for 18 h were lysed and proteins were separated by low temperature SDS-PAGE and transferred to nitrocellulose and immunoblotted against iNOS. Compounds were tested at the following concentrations: SEITU, 50 μ M; BBS-4, 1 μ M. KLYP956 was tested in dose-response: 1000, 250, 62.5, 16, 4, 1, and 0.2 nM. Proteins cross-reacting with anti-iNOS antibodies corresponding to different forms of iNOS are indicated (monomer, dimer, multimer). B, cell culture supernatant obtained from RAW264.7 cells used in Fig. 4A was tested for nitrite levels using DAN assay. Vehicle (■, DMSO) and SEITU (▲, 50 μ M) are used to define 0 and 100% inhibition, respectively. KLYP956 (●) reduces iNOS activity in a concentration-dependent fashion. Data are shown as percentage inhibition of iNOS activity and are representative of three independent experiments.

treated cells (Fig. 6B). In contrast, KLYP956 (50 μ M) reduces the level of dimeric human nNOS to an extent similar to that of 50 μ M BBS-4 (Fig. 6B), consistent with the observed time-dependence of human nNOS inhibition seen for both compounds in Fig. 6A. In contrast, when compounds were incubated with nNOS-expressing cells for only 1 h, neither KLYP956 nor BBS-4 affected pre-existing dimer (Fig. 6C).

Orally Administered KLYP956 Inhibits LPS-Induced NO Production and Attenuates Formalin-Induced Nocifensive Behavior in Vivo. To address whether KLYP956 could inhibit iNOS activity in vivo, we tested the ability of the compound to reduce the production of plasma nitrates (stable metabolite of NO) in the mouse endotoxemia model. Intraperitoneal administration of lipopolysaccharide (LPS) to rodents induces a systemic inflammatory response coincident with the expression of iNOS and production of NO (Kato et al., 2005). Administration of iNOS inhibitors has been demonstrated to block the production of LPS-induced NO/nitrates (Vos et al., 1997). In agreement with in vitro studies, oral administration of KLYP956 reduced plasma nitrates in a

dose-dependent fashion, with an ED₅₀ of approximately 10 mg/kg (Fig. 7).

Next, we investigated the ability of KLYP956 to reduce pain behaviors in a rodent model of nociceptive pain. The test involves a subcutaneous injection of dilute formalin into the plantar surface of the hind paw that causes a characteristic pattern of behavioral responses, including elevation and licking of the injected paw. Two clearly demarcated phases of pain behavior, referred to as phase I (0–5 min after injection) and phase II (25–45 min after injection) are monitored in the model. Pain behaviors in phase II of the formalin model are thought to involve wind-up of spinal neurons in a process referred to as central sensitization (Dubuisson and Dennis, 1977). Efficacy of KLYP956 in attenuating nociceptive behavior elicited by intraplantar injection of formalin was assessed both after intraperitoneal and oral routes of administration. Intraperitoneal injection of KLYP956 (30 mg/kg) given 1 h before intraplantar injection of formalin attenuated both phases I and II of the pain response with a more robust

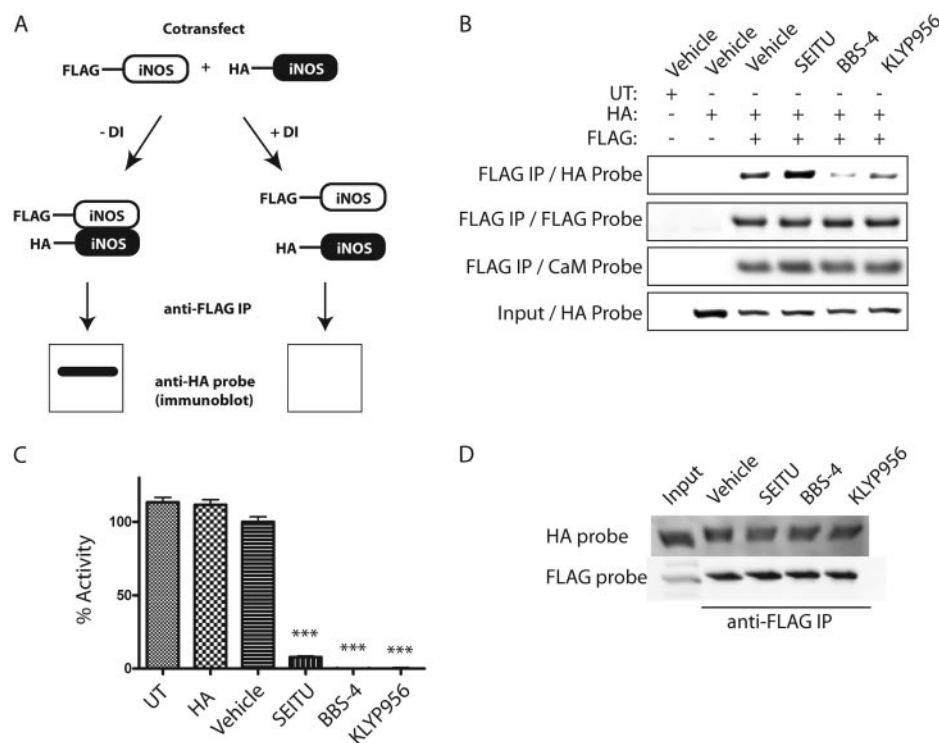


Fig. 5. KLYP956 destabilizes human iNOS dimer in cells. **A**, schematic representing coimmunoprecipitation/Western blotting experimental procedure used to investigate quaternary structure of recombinant human iNOS. In the absence of DI, iNOS dimerization is permitted, allowing for the formation of FLAG-HA heterodimers (as well as FLAG-FLAG and HA-HA homodimers). Anti-FLAG immunoprecipitation (IP) facilitates isolation of complexes containing FLAG-tagged proteins. Anti-HA antibodies detect immunoprecipitated proteins containing the HA sequence. However, in the presence of DI, dimerization of iNOS is blocked resulting in the absence of coimmunoprecipitated HA-tagged iNOS. **B**, HA-tagged (HA) and FLAG-tagged (FLAG) human iNOS expression constructs were cotransfected into HEK293 cells treated with compounds at the following concentrations: SEITU, 50 μ M; BBS-4, 0.5 μ M; KLYP956, 0.5 μ M. Cells were lysed after 18 h of coincubation with compound. FLAG-tagged proteins from lysates were immunoprecipitated with anti-FLAG antibodies and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA antibody (top), anti-FLAG antibody (second blot), or anti-calmodulin (CaM) antibody (third blot). Lysate input was examined to ensure equivalent expression of HA-tagged iNOS (bottom). Singly transfected untagged native human iNOS (UT) and HA-tagged human iNOS demonstrates specificity of anti-HA Western blotting and anti-FLAG immunoprecipitating antibodies, respectively. **C**, iNOS enzymatic activity from compound-treated cells: cell culture supernatant from compound-treated cells in Fig. 5B were tested for nitrite levels (DAN assay). Comparable levels of iNOS activity are present in UT, HA, or cotransfected FLAG and HA samples. All three inhibitors reduce enzyme activity by >95%. Data are shown as mean \pm S.E.M., ***, $p < 0.0001$, t test. **D**, KLYP956 does not disrupt pre-existing iNOS dimers in vitro. HA-tagged and FLAG-tagged human iNOS expression constructs were cotransfected into HEK293 cells as described above, except in the absence of compounds, and lysates were prepared. Compounds were then incubated with lysates for 1 h before immunoprecipitation. Lysates were incubated with anti-FLAG antibodies for an additional hour, and immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose and probed with anti-HA antibody (top blot) or anti-FLAG antibody (bottom blot). Input is equivalent to 1/6 of the material used for immunoprecipitated samples.

effect on phase II (86% reduction, $p < 0.01$ versus vehicle), indicating that this compound affects central sensitization (Fig. 8A). Oral administration of KLYP956 (30 and 100 mg/kg) given 1 h in advance of formalin injection led to dose-related decrements in both phase I and phase II nocifensive behaviors (Fig. 8B). The attenuation of formalin-induced nocifensive behavior by KLYP956 was similar to the previously described iNOS inhibitor AR-C102222 and was not sensitive to the opiate antagonist, naloxone (Fig. 8A and data not shown).

Discussion

A plethora of iNOS inhibitors have been described, the majority representing classic substrate competitive inhibitors that share structural similarity to the amino acid arginine (Vallance and Leiper, 2002). Given the potential issues

associated with this class of molecules and the paucity of viable clinical candidates, efforts to identify alternative mechanisms of enzyme inhibition have been undertaken by several groups (Paige and Jaffrey, 2007). The identification of imidazole-containing anti-fungal agents that interfere with iNOS dimerization has prompted the discovery of several classes of related small molecule dimerization inhibitors (DI) (Sennequier et al., 1999). Much like the antifungal inhibitors, the inhibitory activity of these compounds is completely dependent upon an imidazole group, which coordinates the heme iron within the catalytic domain of the enzyme (McMillan et al., 2000). Although these agents may overcome some of the pitfalls associated with arginine mimetics, we hypothesized that alternatives to an imidazole-dependent mechanism of dimer destabilization may prove beneficial in minimizing potential liabilities such as interaction with other heme-containing enzymes, including cytochrome P450s (Albengres et al., 1998; Zhang et al., 2002).

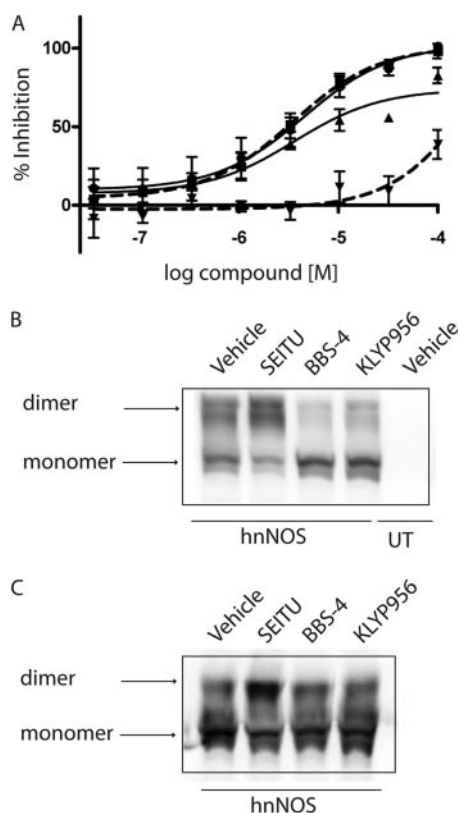


Fig. 6. KLYP956 destabilizes human nNOS dimer. A, transiently transfected HEK293 cells expressing recombinant human nNOS were treated with compound either 4 or 24 h after transfection. Cells were activated with calcium ionophore for 18 h and nitrites (nNOS activity) were measured using DAN assay. Data are represented in dose-response as percentage enzyme inhibition mean \pm S.D. for compounds as indicated: SEITU, 4 h (\bullet) and 24 h (\blacksquare); KLYP956, 4 h (\blacktriangle) and 24 h (\blacktriangledown). B, immunoblot of human nNOS LT-SDS-PAGE with compound pretreatment. HEK293 cells stably transfected with human nNOS were treated with compound indicated (50 μ M) for 36 h and lysates were prepared. Proteins from lysates were separated by low-temperature SDS-PAGE and transferred to nitrocellulose and probed with an anti-nNOS antibody. Dimeric (dimer) and monomeric (monomer) nNOS are indicated with arrows. Untransfected wild-type HEK293 cells (UT) do not express proteins that cross-react with anti-nNOS antibodies under these conditions. C, immunoblot of human nNOS LT-SDS-PAGE with preformed dimer. HEK293 cells stably transfected with human nNOS were treated with compound indicated (50 μ M) for 1 h and lysates were prepared. Proteins from lysates were separated by low temperature SDS-PAGE and transferred to nitrocellulose and probed with an anti-nNOS antibody as described above.

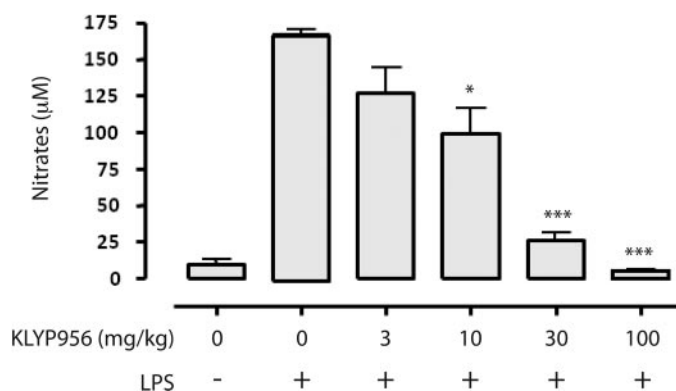


Fig. 7. KLYP956 inhibits LPS-induced nitrite production in vivo (oral administration). Plasma collected from compound-treated Balb/C mice 6 h after LPS injection was measured for nitrite/nitrate concentrations (indirect measure of iNOS activity). KLYP956 was administered orally at the doses indicated (milligram per kilogram) immediately before LPS challenge. Calculated ED_{50} = 10 mg/kg. Data are represented as mean \pm S.E.M.; *, $p < 0.05$; ***, $p < 0.001$ compared with LPS alone, Dunnett's test.

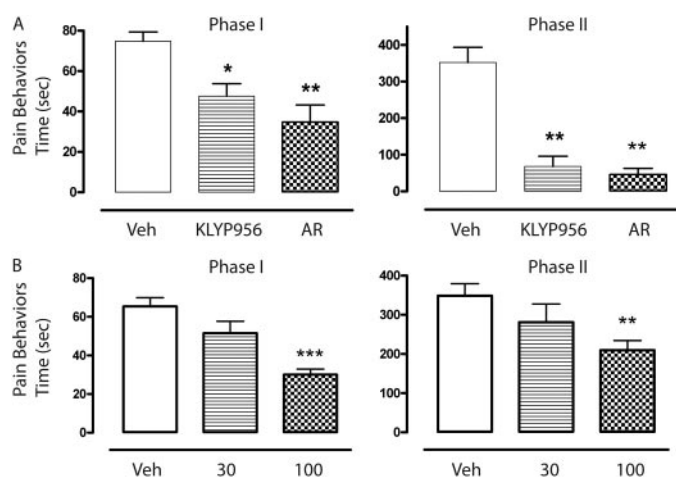


Fig. 8. KLYP956 inhibits nocifensive behaviors in the mouse formalin model. KLYP956 (30 mg/kg) or AR-C102222 (AR; 30 mg/kg) (LaBuda et al., 2006) were delivered via intraperitoneal injection (A) or via oral gavage for KLYP956 at 30 and 100 mg/kg (B) 1 h before formalin injection into the hind paw of male Balb/C mice. Duration of pain behaviors (flinching, biting, licking) were quantified for both phase I (0–5 min) and phase II (25–40 min), and results are shown as mean \pm S.E.M. [*], $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle (Veh)].

The quinolinone KLYP956 arose from an ultra-high-throughput screening and medicinal chemistry effort that was designed to identify iNOS inhibitors with novel mechanisms of action, such as dimer destabilization. The sensitivity and low cost of the homogeneous primary assay used in the screen enabled profiling of a larger compound collection than has heretofore been reported for other iNOS screening campaigns. A previous report proposed that activated murine macrophage RAW264.7 cells could be used in a primary screen to identify inhibitors of human iNOS, based on the considerable amino acid and structural conservation between the active sites of these enzymes (Naureckiene et al., 2007). However, the precursor to KLYP956 identified in the screening campaign was virtually inactive against murine iNOS (K.T.S. and C.A.H., unpublished observations). KLYP956 itself exhibits a 14-fold preference for human iNOS versus murine iNOS. Therefore our studies clearly demonstrate the existence of species-selective iNOS inhibitors. Perhaps even more surprising are the apparent differences in KLYP956 potency against iNOS and nNOS in the two species. There exists a 260-fold difference in potency of KLYP956 in human iNOS and nNOS. In contrast, KLYP956 inhibits murine iNOS and nNOS with nearly identical potencies. Recent structural studies may provide insight into the molecular basis of these effects. Examination of the binding mode of a series of quinazoline and aminopyridine inhibitors (Garcin et al., 2008) revealed that flexibility in a triad of sequence divergent second- and third-shell residues can regulate conformational switching in the absolutely conserved residues lining the active site of iNOS, particularly that of a highly conserved glutamine residue (Gln257/263 in murine/human iNOS). Molecular modeling of the binding mode of KLYP956 suggests that it could form nonpolar contacts with Gln257/263. If this were to be the case, KLYP956 inhibitory potency might be determined by residues significantly distal to the active site. These sequence differences, combined with evidence that eNOS forms a more stable dimer than the other two isoforms (Panda et al., 2002), may also explain the considerable selectivity of KLYP956 against both human and murine eNOS. Further studies will be required to determine the features that underlie the isoform and unexpected species selectivity of KLYP956.

Mechanistic analysis from cell-based and biochemical inhibition studies suggest that, unlike substrate competitive inhibitors such as SEITU, KLYP956 cannot inhibit NOS activity of stable intact dimers. Data suggest that KLYP956 may bind iNOS and nNOS in a monomeric or loose dimeric state and interfere with the formation of a stable, enzymatically active dimer. The identification of a quinolinone-based NOS dimerization inhibitor provides evidence that direct imidazole-based coordination of the heme iron is not required for iNOS and nNOS dimer destabilization. Several lines of evidence suggest that chemical differences between BBS-4 and KLYP956 lead to differences in the inhibitory mechanism of the two compounds. First, heme spectral studies indicate an interaction for both compounds in the vicinity of the active site. However, binding of imidazole is mediated through direct coordination with the heme iron, leading to a single Soret band with an absorbance maximum at 429 nm (Sennequier and Stuehr, 1996). This contrasts sharply with the spectral shift induced by KLYP956, where a single broad maximum at 396 nm is generated (Fig. 3). The shift to 396

nm is more akin to that induced by the addition of arginine and tetrahydrobiopterin to iNOS monomers, although in this latter case, dimerization is actually augmented (Sennequier et al., 1999). The binding mode may be similar to that of previously described coumarin-based substrate-competitive inhibitors (Jackson et al., 2005). A second difference in mechanism of dimer destabilization can be inferred from LT SDS-PAGE data from RAW264.7 cells treated with compounds. In these studies, treatment with KLYP956 and related molecules induces higher-order species that are detected with iNOS-specific antibodies (Fig. 4A and data not shown). The formation of inactive multimeric iNOS may help explain the less pronounced reduction in HA-tagged human iNOS protein that is detected in KLYP956-treated versus BBS-4-treated coimmunoprecipitation experiments (Fig. 5B). Calmodulin is an essential protein cofactor known to form a tight interaction with iNOS even at low levels of intracellular calcium (Vallance and Leiper, 2002). It is noteworthy that the association of iNOS with calmodulin seems to be unaffected by KLYP956, based on coimmunoprecipitation studies, suggesting that disruption of this protein-protein interaction does not contribute to the compound's mechanism of action (Fig. 5B). In summary, the overall similarity between the NOS inhibitory profiles of BBS-4 and KLYP956 suggest that the latter acts via destabilization of iNOS and nNOS dimers, although details of the modes of action may differ between the two compounds. A peptide-based compound lacking an imidazole has been described to have iNOS dimer destabilizing properties, providing further evidence that an imidazole is not essential for this mechanism of inhibition (Chida et al., 2005).

In addition, several endogenous proteins have been described that modulate the quaternary structure of iNOS and nNOS (Kone et al., 2003). The cumulative data suggest that both NOS isoforms may have evolved intrinsic capacities to modulate activity by regulating the extent and stability of homodimerization. This is further supported by earlier site-directed mutagenesis studies demonstrating that single amino acid changes in the dimerization interface of iNOS abrogate dimer formation (Ghosh et al., 1999). Given the multifaceted role of arginine in the body, mechanisms may have evolved to regulate this enzyme class independently of substrate concentration. Our work, along with that of several other groups, indicates that this regulation of NOS quaternary structure can be exploited to generate novel therapeutics. Toward this end, we have identified KLYP956, which inhibits iNOS and nNOS dimerization and enzymatic activity both in biochemical assays and in cells. Even more importantly, administration of KLYP956 in animals results in the inhibition of iNOS activity and attenuation of nociceptive behaviors, indicating that the mechanism of action is functional *in vivo*. In conclusion, the potency, selectivity, and mechanism of inhibition of KLYP956 provide the potential that such an agent could become a viable pharmaceutical alternative to existing NOS inhibitors.

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