Inhibitors of Inducible Nitric Oxide Synthase Dimerization (INOS-DI) are Effective Anti-Nociceptive Agents in Rodent Models

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ABSTRACT

Nitric oxide (NO) generated via iNOS plays an important role in a number of inflammatory diseases and emerging evidence suggests potential for iNOS inhibitors in treating pain states in the inflammation/ neuropathic pain continuum As the enzymatic activity of iNOS requires dimerization of inactive monomers of iNOS, we targeted this mechanism to develop novel and selective iNOS inhibitors. KD7020, a selective iNOS dimerization inhibitor (DI), is a potent inhibitor of human iNOS with an IC50 of ~10 nM and, >1000-fold and ~85-fold selectivity against human e-NOS and human n-NOS, respectively. KD7020 inhibited murine iNOS activity with an IC50 of ~60 nM. Orally dosed KD7020 attenuated endotoxin-induced increases in plasma nitrate levels in the rat, measured 6 hr post-oral dose, with an ID50 ~10 mg/kg. KD7020 exhibited broad spectrum activity both in inflammation and neuropathic pain models. KD7020 (10-50 mg/kg, po or ip) attenuated hyperalgesia in the carrageenan paw inflammation model, post-surgical pain in the Brennan model, pain behaviors in the rat formalin model and allodynia in the Chung model of neuropathic pain. The efficacy of KD7020 is comparable to Gabapentin in the Chung model of neuropathic pain. KD7020 exhibited greater efficacy in neuropathic pain models vs. inflammation models. Interestingly, topically applied KD7020 attenuated intraplantar capsaicin-induced allodynia in the rat and spontaneous pain behaviors (weight-bearing response) both in Bennett model of neuropathic pain and in a mild thermal injury model. KD7020 is equi-efficacious to topically applied lidocaine. Topical application of KD7020 provides high target-tissue concentrations with minimal systemic exposure. KD7020 is not a local anesthetic like lidocaine. A single atom change in chemical structure of KD7020 not only abrogates its iNOS inhibitory activities but also abrogates its anti-nociceptive effects, thus establishing a causal link between iNOS inhibition and efficacy in vivo. These data suggest that ligands such as KD7020 represent novel strategies for pain, especially for topical treatment of neuropathic pain.

INTRODUCTION

Nitric oxide (NO) generated by nitric oxide synthase (NOS), is an important messenger molecule in the signal transduction pathway that amplifies nociceptive transmission both in peripheral and central nervous systems (Zimmermann, 2001; Reidel & Neeck, 2001). There are three isoforms of NOS that include two constitutive forms, endothelial (eNOS) and neuronal (nNOS) and one inducible form, iNOS. Several lines of scientific investigation suggest that NO generated through iNOS plays a critical role in inflammation, central neuronal sensitization, and the generation and maintenance of neuropathic pain states (Levy *et al*, 1999; Wu *et al*, 2001; Pettipher *et al*, 1997; Hallinan *et al*, 2002; LaBuda *et al*, 2005; De Alba *et al*, 2006). As the dimerization of iNOS monomers is a critical step in generating a functionally competent iNOS enzyme complex (Vallance & Leiper, 2002; McMillan et al., 2000), we have focused on inhibitors of this multimeric assembly to generate iNOS inhibitors with significant isoform selectivity. In the current studies, we investigated the effects of an iNOS dimerization inhibitor, KD7020, in a variety of rodent pain models representing both acute and chronic pain states.

MATERIALS & METHODS

LPS-induced plasma nitrate elevation: Bacterial endotoxin (LPS), induces iNOS leading to marked increases in plasma nitrates. A dose of 0.3 mg/kg of LPS in saline (Sigma) was given intravenously. Blood samples were collected 6 hr post LPS for analysis of nitrate and drug levels. Nitrate levels were measured by Nitrite Fluorimetric Assay (Cayman assay kit).

Carrageenan-induced paw edema and thermal hyperalgesia: Intraplantar carrageenan produces an inflammatory hyperalgesia characterized by injection of the elaboration of mediators such as prostaglandins, TNFα and NO. A 1% w/v suspension of carrageenan (Sigma) was injected into right hind paw in rats to induced paw edema and thermal hyperalgesia. Paw edema and thermal hyperalgesia (Hargreaves method) were measured at selected time points as indicated in the figure legends. The paw exudate was collected at the end of assay for the measurement of nitrate levels.

Post-operative pain in rat (Brennan Model): Under isoflurane anesthesia, 1-cm long incision was made through skin and fascia of plantar aspect of right foot in the rat. After homeostasis with gentle pressure, the skin was sutured and animals allowed to recover. The test of weight distribution was measured from 30 to 120 min post-operation.

Spinal nerve ligation-induced tactile allodynia in the rat (Chung Model): Using an aseptic technique, the left L5 and L6 spinal nerves were exposed and isolated with a surgical hook, distal to their respective dorsal root ganglia. A tight ligation as made on each nerve. The back muscles were sutured closed and the skin laceration was stapled with surgical wound clips. Tactile allodynia was measured 2 weeks post surgery.

Capsaicin-induced pain behavior in rat: Intraplantar injection of capsaicin induces a hyperalgesic pain state accompanied by increased expression of iNOS in the dorsal horn (Wu et al., 2001). Capsaicin (Sigma, 10 μL of 10 mg/mL solution) was injected into right hind paw in rat. Tactile allodynia was measured 30 -60 min post injection.

Induction of Mid Thermal Injury (MTI) in rat (*modified from Nozaki-Taguchi and Yaksh 2002*)**:** The entire planter surface of the right hind paw was placed on a plate at 52°C ± 1°C with a 80 g weight on the dorsal surface of the paw for 25 sec while the animals were under deep anesthesia. This procedure produces hyperalgesic state characterized by a mild burn, redness, but no blistering within 24 hrs. The weight distribution on each hind paw was measured.

Chronic constriction injury (CCI; Bennett Model) in rat: Using anesthesia, the left sciatic nerve at the level of the mid-thigh was exposed and loosely ligated with four ligatures. Each ligation was approximately 1-2 mm apart. The thigh muscles were sutured shut with 3-0 braided black silk and the skin was closed with surgical wound clips. The weight distribution on ipsi- and contra-lateral hind paws were measured at 14 after surgery.

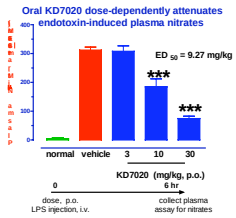
Results

KD7020 is a Potent and selective iNOS Inhibitor

Compound	hiNOS EC_{50} (μM)	heNOS EC_{50} (μM)	hnNOS EC_{50} (μM)
KD7020	0.007	>10	0.6
SEITU (S-ethylisothiourea)	3	6	16

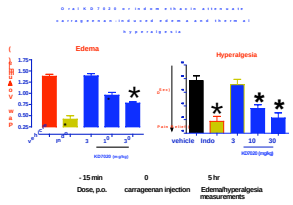
Effect of KD7020 on Pain Behaviors in Rodent Models Following Systemic Administration

Fig. 1



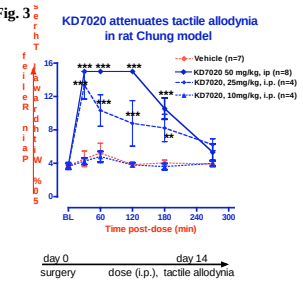
Rats were dosed with KD7020 or vehicle (0.75%DMSO/20%encapsain) at doses indicated. At 1-3 minutes post dosing, a volume of 0.3 mL of 0.3 mg/kg LPS was injected intravenously. Data represent mean ± SE from 5 animals/ group,*** p < 0.001.

Fig. 2



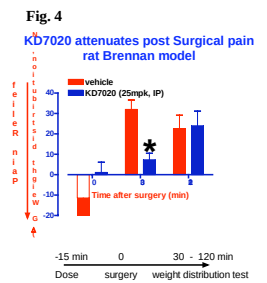
SD rats were dosed with KD7020 (3,10 or 30 mg/kg, p.o.), Indo (10mg/kg, p.o.) or vehicle (1%DMSO/20%encapsain). At 15 minutes post dosing, intraplantar injection of carrageenan (0.1%; 100 mL) was injected into the right hind paw. The measurements of paw volume and thermal hyperalgesia (withdrawal latency) were taken at 5 hr post carrageenan injection. Each data point represents mean ± SE from 5 animals/ group.*, p<0.05 vs Vehicle.

Fig. 3



SD rats underwent Chung Surgery and 14 days after the surgery, baseline (BL) measurements were first taken. Following baseline, rats were dosed (i.p.) with KD7020 in the vehicle of 1%DMSO/20% encapsin at doses of 10, 25, or 50 mg/kg or vehicle only. 50% withdrawal thresholds were recorded at 30, 60, 120, 180 and 270 min post drug administration. Data represent mean ± SE from 4-8 animals/group. ***P<0.001, **p<0.01.

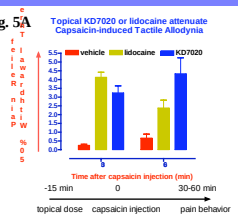
Fig. 4



SD rats were dosed with KD7020 (25 mg/kg, i.p.) 15 min before the surgery. Under a brief isoflurane anesthesia, a sterile incision was made in the right hind paw plantar surface and sutured. Following recovery, the spontaneous pain behavior (differential weight distribution in between normal or surgical paws) were measured 30 and 120 min post-surgery. Data represent mean ± SE from 3 animals/ group, *p<0.05.

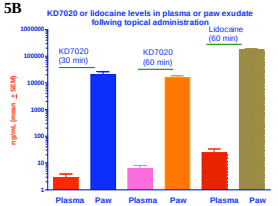
Effect of KD7020 on Pain Behaviors in Rodent Models Following Topical Administration

Fig. 5A



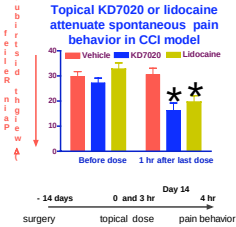
KD7020 or lidocaine was dissolved in a vehicle containing water: ethanol: propylene glycol (WEP; 5mg/mL). The right hind paw was dipped into vehicle or compound for 30 sec. Fifteen minutes after the topical application, capsaicin (0.1 mg) was injected into the right hind paw and tactile allodynia was measured at 30 or 60 min post injection. Data represent mean ± SE and N=7-9 rats.

Fig. 5B



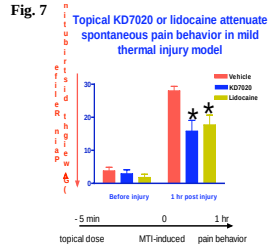
For the measurement of drug level, samples of blood and paw exudate were collected at 0.5 and 1 hr post topical administration and data represent mean ± SE and n=3 rats.

Fig. 6



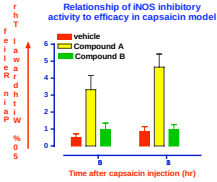
Two weeks after sciatic nerve ligation, rats were tested for weight bearing 24 hrs before the study. Rats received repeated topical applications of KD7020 or lidocaine (10 mg/mL, two applications of 0.5 mL at 3 hr apart in WEP vehicle) on the skin surface covering the surgical area. At 1 hr post second dose, weight bearing distribution was measured. Each data point represent mean ± SE from 6-8 animals/group. *, p< 0.05 vs vehicle (ANOVA).

Fig. 7



MTI produced avoidance behavior as reflected in weight distribution. Rats received topical administration of KD7020 (paw dipped into 5 mg/mL for 30 sec), lidocaine (5 mg/mL), or vehicle (WEP) 15 minutes before the induction of MTI . The measurements of weight distribution at 60-120 min post-injury. Data represent mean ± SEM for 9 animals/ group. *, p< 0.05 vs vehicle (ANOVA).

Fig. 8



	hiNOS EC_{50} (μM)	Drug level in paw exudate (μg/ml)	Drug level in plasma (mg/ml)
Compound A	0.002-0.006	69 ± 8	1.3 ± 1
Compound B	inactive	95 ± 7	18 ± 4

Compound A or Compound B (a structural analog of Compound A that is inactive as an iNOS inhibitor), was dissolved in vehicle (5mg/mL). The right hind paw was dipped into vehicle or compound for 30 sec. Fifteen minutes after topical application, capsaicin (0.1 mg) was injected into the right hind paw and tactile allodynia was measured at 30 or 60 min post injection. Data represent mean ± SE and N=4 rats. For the measurement of drug level, samples of blood and paw exudate were collected at 0.5hr post-injection and data represents the mean ± SE and n=3 rats.

Conclusions:

- KD7020 is a potent and selective inhibitor of iNOS *in vitro*
- KD7020 is an orally active iNOS inhibitor with dose dependent anti-inflammatory and anti-hyperalgesic activity in the carrageenan model
- KD7020 significantly reduced pain behaviors in models of surgical and neuropathic pain
- Topically delivered KD7020 achieves high local concentrations with minimal systemic exposure
- Topical KD7020 attenuates pain behaviors induced by intraplantar capsaicin, mild thermal injury or sciatic nerve injury with an efficacy that is comparable to that of lidocaine
- Correlatin of abrogation of topical analgesia by loss of iNOS inhibitory activity establishes a causal link between iNOS inhibition and efficacy *in vivo*

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