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Further in vivo studies on attenuating morphine withdrawal: isoform-selective nitric oxide synthase inhibitors differ in efficacy

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Abstract

The *N*-methyl-D-aspartate (NMDA) receptor–nitric oxide (NO) pathway has been linked to opiate withdrawal. Pretreatments with four inhibitors of NO synthase, 7-nitro indazole, 3-bromo-7-nitro indazole, *S*-methyl-L-thiocitrulline and aminoguanidine, which exhibit different isoform selectivity in vitro, were evaluated for their ability to attenuate signs of naloxone-precipitated morphine withdrawal. In separate experiments, effects of NO synthase inhibitors on blood pressure were measured in naïve and morphine-dependent rats. 7-Nitro indazole, 3-bromo-7-nitro indazole and *S*-methyl-L-thiocitrulline, which are specific inhibitors of the constitutive isoforms, produced dose-dependent reductions of several signs of withdrawal. Blood pressure was unaffected by the indazoles, whereas *S*-methyl-L-thiocitrulline produced a strong vasoconstrictor response. Aminoguanidine, which selectively inhibits inducible NO synthase, reduced fewer signs of opioid withdrawal, had a lower relative potency and exhibited no vasopressor activity. These data suggest that constitutive isoforms, but not the inducible isoform of NO synthase, have a primary role in NO-mediated processes that modulate the opioid withdrawal syndrome in the rat. © 1997 Elsevier Science B.V.

Keywords: Morphine withdrawal; Blood pressure; Nitric oxide (NO) synthase inhibitor; 3-Bromo-7-nitro indazole; Aminoguanidine; *S*-Methyl-L-thiocitrulline; 7-Nitro indazole; Nitric oxide (NO)

1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor–nitric oxide (NO) pathway has been pharmacologically linked to the behavioral manifestation of opioid withdrawal. Activation of the NMDA receptor-gated ion channel stimulates Ca^{2+} entry into the cell and the formation of a Ca^{2+}–calmodulin complex. Upon binding to NO synthase, the complex confers the ability to produce NO. Inhibition of this pathway by either competitive or noncompetitive NMDA receptor antagonists (Cappendijk et al., 1993b; Trujillo and Akil, 1991; Rasmussen et al., 1991) or by inhibitors of NO synthase (Adams et al., 1993; Cappendijk et al., 1993a; Kimes et al., 1993; Vaupel et al., 1995b) attenuates signs of opiate withdrawal in rodents.

Previously we demonstrated that four inhibitors of NO synthase produced dose-related reductions in several signs of naloxone-precipitated opioid withdrawal in morphine-dependent rats (Kimes et al., 1993; Vaupel et al., 1995b). The usefulness of NO synthase inhibitors to treat opiate withdrawal may be limited, however, by undesirable cardiovascular effects of these drugs. NO synthase inhibitors that are nonselective with respect to the different isoforms of NO synthase increase blood pressure in awake, morphine-naïve and non-withdrawing, morphine-dependent rats, and also elicit a vasopressor response in humans (Petros et al., 1991). Because elevated blood pressure is a component of the human opiate withdrawal syndrome (Himmelsbach, 1937), vasopressor agents would best be avoided. 7-Nitro indazole, a selective in vitro inhibitor of constitutive NO synthase (Babbedge et al., 1993; Moore et al., 1993a,b), reduces the morphine withdrawal syndrome in rats without affecting blood pressure in naïve (Moore et al., 1993a; Vaupel et al., 1995b) or in morphine-dependent rats (Vaupel et al., 1995b).

This report evaluates three inhibitors of NO synthase in vivo to determine whether they offer advantages based on in vitro isoform specificity over other NO synthase in-

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hibitors previously tested for alleviating signs of opiate withdrawal and for vasoconstrictor activity in the rat. 3-Bromo-7-nitro indazole is selective for neuronal constitutive NO synthase and is four times as potent as 7-nitro indazole as an inhibitor of the enzyme in isolated cell systems (Bland-Ward et al., 1994). *S*-Methyl-L-thiocitrulline, developed as a more potent derivative of thiocitrulline, is 17 times as selective for neuronal constitutive NO synthase when compared with endothelial constitutive NO synthase (Furfine et al., 1994); however, it also effectively inhibits smooth muscle inducible NO synthase (Narayanan and Griffith, 1994). Aminoguanidine inhibits diamine oxidase, aldose reductase and the formation of advanced glycosylation end-products but has been characterized more recently as a relatively selective inhibitor of inducible NO synthase (Wolffenbutterl and Huijberts, 1993). 3-Bromo-7-nitro indazole, *S*-methyl-L-thiocitrulline and aminoguanidine were compared with 7-nitro indazole, which was selected as the standard NO synthase inhibitor based on our previous work (Vaupel et al., 1995a,b). 3-Bromo-7-nitro indazole was as efficacious as 7-nitro indazole in its ability to attenuate morphine withdrawal and was devoid of vasopressor activity. *S*-Methyl-L-thiocitrulline was not as effective as 7-nitro indazole in attenuating withdrawal and produced a marked elevation in blood pressure, whereas aminoguanidine had minimal effects on the withdrawal syndrome and the cardiovascular system.

2. Materials and methods

All animal procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals as endorsed by the National Institutes of Health.

2.1. Animals

Male Fischer-344 rats, 4–6 months of age (Charles River Breeding Laboratories), were used for all experiments. They were housed in a temperature-controlled vivarium under a 12 h on (6 a.m.)/12 h off lighting cycle for 1–2 months, with access to food and water ad libitum. A total of 235 rats were used for the withdrawal experiments and 21 rats for the cardiovascular experiments.

2.2. Production of physical dependence on morphine and measurement of precipitated withdrawal

Physical dependence was produced by implanting, s.c., one 75-mg morphine pellet (University of North Carolina School of Pharmacy, Drug Products Program, Chapel Hill, NC, USA) while the rats were anesthetized with halothane (4%, <3 min). Withdrawal was precipitated with naloxone (0.5 mg/kg, s.c.) 3 days after pellet implantation and was scored following methods described previously (Kimes et al., 1993; Vaupel et al., 1995b). After allowing each rat at least 15 min to habituate to an open-topped, square, clear Plexiglas observation chamber ($46 \times 46 \times 26$ cm) with lines partitioning the bottom into quadrants, rats were weighed, returned to the chamber and given naloxone. Withdrawal signs were measured over 15 min by one observer who was blind to the treatment conditions. Immediately afterwards, a final body weight was measured. Signs quantified by counting their occurrences were quadrant crossing, rearing, jumping (escape jumps), abnormal posturing, wet dog shakes, teeth chattering, mastication (chewing movements), penile licking/ejaculation and grooming. Exploratory activity was defined as the sum of the quadrant crossings and rearings. Other signs, evaluated as absent (0) or present (1) during each 5-min epoch, included diarrhea, lacrimation, rhinorrhea, salivation, irritability, ptosis and tachypnea. The maximal score for each of the quantal signs was 3 (1 point per 5-min epoch). Secretory activity was defined as the sum of the lacrimation, rhinorrhea and salivation scores (maximal score being 9). The appearance of signs normally not associated with withdrawal, specifically ataxia and impairment of the righting reflex, were determined subjectively based upon observing the gait and attempting to regain an upright posture after spontaneously falling on one side, respectively. Rats were observed for lethality over a period of 3 days after drug administration and withdrawal and then were killed.

Animals were pretreated with an i.p. injection of either a NO synthase inhibitor, saline or peanut oil (vehicle for 7-nitro indazole and 3-bromo-7-nitro indazole) 1 h prior to precipitating withdrawal by a person who did not score withdrawal signs. Rats were returned to their home cages for 45 min until they were placed in the observation chamber for habituation. Each animal was used once for the withdrawal studies. Typically, the responses to four or more doses of one drug, saline and peanut oil (when appropriate) were all evaluated on each test day, with the pretreatment dosages and controls treatments randomized. Experiments were conducted in two sessions, with a different observer for each. Aminoguanidine (3, 10, 30, 100 and 300 mg/kg; $n = 7$, 8, 8, 7 and 9, respectively), 7-nitro indazole (18, 30, 56 and 100 mg/kg; $n = 8$ for all doses), saline ($n = 8$) and peanut oil ($n = 8$) were tested in the first group. 3-Bromo-7-nitro indazole (18, 30, 56 and 100 mg/kg; $n = 8$ for all doses), *S*-methyl-L-thiocitrulline (5.6, 10, 18, 30, 56 and 100 mg/kg; $n = 8$, 9, 8, 8, 8 and 8, respectively), 7-nitro indazole (18, 30, 56 and 100 mg/kg; $n = 8$, 8, 8 and 11, respectively), saline ($n = 16$), and peanut oil ($n = 16$) were evaluated in the second group of experiments, and the sequence for testing the three drugs varied across test days.

2.3. Measurement of blood pressure and heart rate

Halothane-anesthetized rats had their left femoral artery and vein catheterized with PE-50 tubing as previously

described (London et al., 1981). Each animal was partially restrained by a cast that encased the lower body to restrict its movement and was allowed at least 3 h to recover from anesthesia. Blood pressure was recorded using a Transducer Signal Conditioner (Gould, model 13-6615-50) and a chart recorder (Gould, model 42-8440). Patency of the catheters was maintained by administering heparinized saline (110 units/ml) as needed.

The experimental sequence for blood pressure assays consisted of two baseline measurements obtained 15 min apart, an i.p. injection of drug or vehicle, and recordings obtained at 5, 15, 30, 45, 60, 75 and 90 min after the injection. Identical experiments were run in naïve and morphine-dependent rats (see above) using saline, 100 mg/kg 3-bromo-7-nitro indazole, 56 mg/kg *S*-methyl-L-thiocitrulline and 0.1 mg/kg clonidine. These doses represented the highest dose of each NO synthase inhibitor used in the precipitated withdrawal experiments that did not produce signs of behavioral toxicity. Previously, we had evaluated the effects of clonidine on withdrawal, but not on blood pressure (Vaupel et al., 1995b). Each animal was tested with only a single drug. However, three morphine-naïve rats completing the saline vehicle experiments were immediately administered a second injection of clonidine, and measurements were continued for another 90 min. Systolic, diastolic and mean arterial blood pressure and heart rate were measured directly from recordings. Animals were killed with i.v. sodium pentobarbital at the conclusion of the experiment.

2.4. Drugs

7-Nitro indazole (molecular weight (MW) 162.1) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA) and Alexis (San Diego, CA, USA). 3-Bromo-7-nitro indazole (MW 242.0) and *S*-methyl-L-thiocitrulline (MW 241.8) were purchased from Alexis and aminoguanidine hemisulfate (MW 123.1) was obtained from Sigma (St. Louis, MO, USA). *S*-Methyl-L-thiocitrulline was dissolved in saline. 3-Bromo-7-nitro indazole and 7-nitro indazole were injected as a suspension of peanut oil (arachis oil). The suspension was prepared by sonicating the drug in peanut oil for approximately 20 s. Injections for both experiments were administered in a volume of 3 ml/kg.

2.5. Statistical analysis

For the naloxone-precipitated withdrawal studies two one-way analyses of variance were performed for each drug. The first determined the presence of a treatment effect for each drug and its corresponding vehicles. These results are presented as the 'Treatment effect' in Table 1. Contingent upon a significant treatment effect, a Dunnett's test was used to determine which dose and peanut oil vehicle (if appropriate) effects differed from the saline control value. A second one-way analysis of variance was used for regression analysis, which partitioned the variance into linear regression and deviations from regression components. Relative potencies were calculated using standard analysis of variance methods for parallel line bioassays (Finney, 1978). A bioassay analysis of variance was con-

Table 1
Effects of nitric oxide synthase inhibitors on signs of opioid withdrawal precipitated by naloxone

	7-NI	3-Br,7-NI	Me-TC	AG
Weight loss	↓	↓	↓	0
Treatment effect	0.0220	0.0173	0.0000	NS
Regression	0.0130	0.0150	0.0001	NS
Diarrhea	0	↓	↓	0
Treatment effect	NS	0.0028	0.0002	NS
Regression	NS	0.0042	0.0001	NS
Wet dog shakes	↓	↓	↓	0
Treatment effect	0.0169	0.0005	0.0384	NS
Regression	0.0303	0.0003	NS	NS
Grooming	↓	↓	0	0
Treatment effect	0.0227	NS	NS	NS
Regression	NS	0.0060	NS	NS
Mastication	↓	↓	↓	↓
Treatment effect	0.0000	0.0000	0.0000	0.0029
Regression	0.0003	0.0003	0.0033	0.0002
Secretory activity	↓	0	0	0
Treatment effect	0.0202	NS	NS	NS
Regression	NS	NS	NS	NS
Penis licks + ejaculations	0	0	↑	0
Treatment effect	NS	NS	0.0221	NS
Regression	NS	NS	NS	NS
Exploratory activity	↑	↑	↑	0
Treatment effect	0.0011	0.0389	0.0041	NS
Regression	0.0195	NS	NS	NS
Abnormal posture	↑	0	↑	0
Treatment effect	0.0012	NS	0.0026	NS
Regression	0.0357	NS	NS	NS
Escape jumps	↑	0	0	0
Treatment effect	0.0006	NS	NS	NS
Regression	0.0184	NS	NS	NS
Ptosis	0	0	↑	↑
Treatment	NS	NS	NS	0.0271
Regression	NS	NS	0.0438	NS

Significance or the lack of significance (NS) of a 'Treatment effect' was based on a one-way ANOVA incorporating all vehicles and doses, except as noted below. A significant 'Regression' represents the a log-linear dose–response curve determined by a second one-way ANOVA, using only the dose effects illustrated in Figs. 1–4. For *S*-methyl-L-thiocitrulline, the 100 mg/kg dose was excluded from all regression analyses because it produced marked behavioral toxicity. $P < 0.05$ was the criterion for statistical significance and the exact probabilities are presented. The net effect of a NO synthase inhibitor pretreatment on a particular withdrawal measure based upon the statistical analyses is indicated by an arrow or a zero. 7-NI, 7-nitro indazole; 3-Br,7-NI, 3-bromo-7-nitro indazole; Me-TC, *S*-methyl-L-thiocitrulline; AG, aminoguanidine.

sidered statistically valid if it exhibited linear regression (significant regression term), had parallel dose–response curves (non-significant parallelism term) and had no difference between the weighted means of the two drug treatments (non-significant preparations term). The criterion for significance was $P \leq 0.05$

Significance of differences in mean arterial pressure and heart rate between baseline (0 min) and 1 h after drug administration were determined on the basis of a one-way analysis of variance followed by a Dunnett's test. The criterion for significance was $P \leq 0.05$.

3. Results

3.1. Pattern of effect on opiate withdrawal signs

Withdrawal signs effectively reduced in a dose-dependent manner by 3-bromo-7-nitro indazole and *S*-methyl-L-thiocitrulline were weight loss, diarrhea and mastication (Table 1, Figs. 1 and 2). 3-Bromo-7-nitro indazole also depressed wet dog shakes and grooming activity. *S*-Methyl-L-thiocitrulline reduced wet dog shakes, but the significance of the treatment effect was biased by a substantial response to the 100 mg/kg dose, which produced behavioral toxicity as described below. Only one sign, mastication, was significantly attenuated by aminoguanidine (Fig. 3). 7-Nitro indazole, the reference standard for this study, decreased weight loss, wet dog shakes, mastication and grooming behavior as expected but did not diminish the presence of diarrhea in a dose-dependent manner as it did in our previous assessment of its activity (Fig. 4) (Vaupel et al., 1995b). The treatment effect for diarrhea determined by one-way analysis of variance (Table 1) was nearly significant ($P < 0.054$), and, as shown in Fig. 4, all four doses of 7-nitro indazole moderately reduced diarrhea. For the 7-nitro indazole-induced decrease in grooming, the 18 mg/kg response was unexplainably low and this produced an inverted U-shaped curve (Fig. 4). When this dose was not included in the regression analysis, the *P* value decreased from non-significant (0.0904) to 0.0089.

The sign most consistently enhanced was exploratory activity, as shown for 7-nitro indazole, 3-bromo-7-nitro indazole and *S*-methyl-L-thiocitrulline (Figs. 1, 2 and Figs. 4); however, the increased activity levels were not uniformly linear with dose (Table 1). 7-Nitro indazole also was the only inhibitor to potentiate escape jumps and abnormal posturing, as both the 56 and 100 mg/kg doses increased escape jumps ($P < 0.01$) and 100 mg/kg increased the incidence of abnormal postures ($P < 0.01$) (data not shown). Both *S*-methyl-L-thiocitrulline and aminoguanidine tended to increase ptosis (Table 1). This effect was dose-related for *S*-methyl-L-thiocitrulline but was less consistent for aminoguanidine. Despite a significant treatment effect for ptosis, no single dose effect of aminoguanidine differed significantly from the control

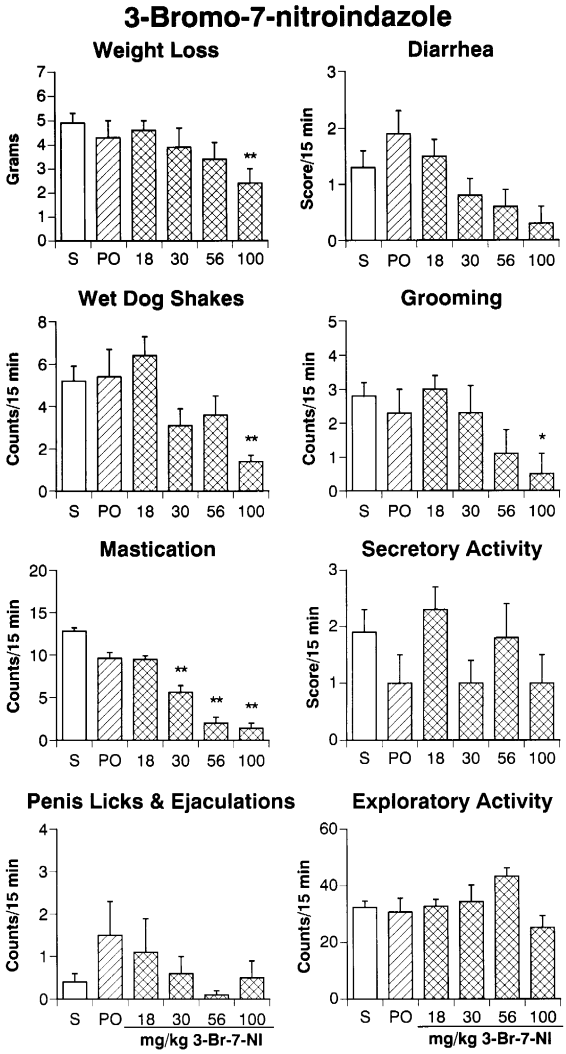

Fig. 1. Mean effects of 3-bromo-7-nitro indazole (3-Br-7-NI) on naloxone-precipitated morphine withdrawal signs. For the saline (S) and peanut oil vehicles (PO), the *n* is 16; the *n* for the 3-bromo-7-nitro indazole dose groups was 8. Vertical lines represent the S.E.M. Significant differences from saline are indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$ (one-way analysis of variance followed by Dunnett's test).

condition, and the dose–response relationship was curvilinear with the largest degree of ptosis observed with the 30 mg/kg dose.

3.2. Relative potency

Relative potency estimates were obtained for weight loss, wet dog shakes, grooming and mastication, and there were no significant differences in potency between 7-nitro indazole, its brominated derivative and *S*-methyl-L-thiocitrulline (Table 2). The largest number of valid parallel line bioassays was obtained for the 7-nitro indazole and 3-bromo-7-nitro indazole comparisons. These findings reflect the similarity of the profiles for 7-nitro indazole and 3-bromo-7-nitro indazole and trends for *S*-methyl-L-thiocitrulline and especially aminoguanidine to be less effica-

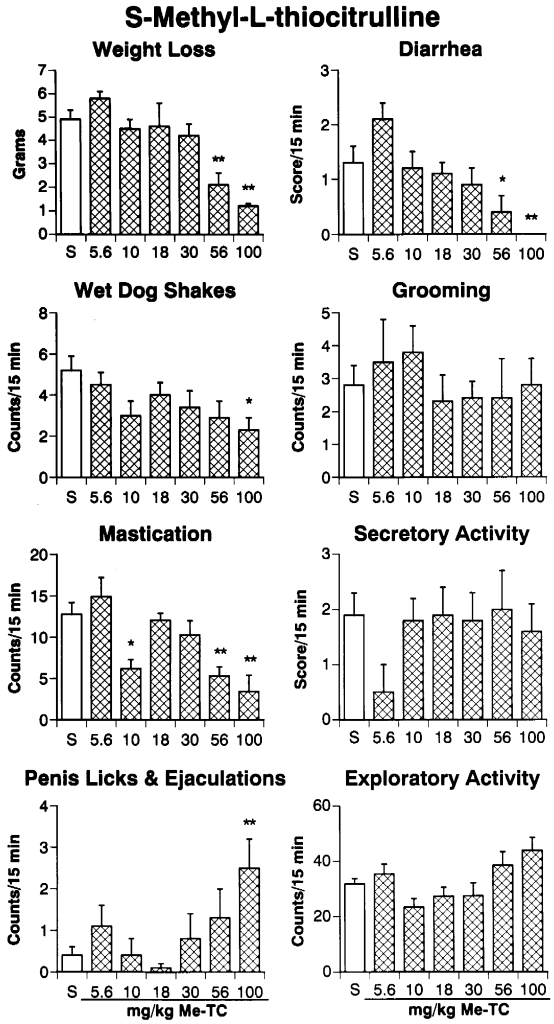

Fig. 2. Mean effects of *S*-methyl-L-thiocitrulline (Me-TC) on naloxone-precipitated morphine withdrawal signs. For the saline (S) vehicle, $n = 16$, and for the *S*-methyl-L-thiocitrulline dose groups, $n = 8$. Vertical lines represent the S.E.M. Significant differences from saline are indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$ (one-way analysis of variance followed by Dunnett's test).

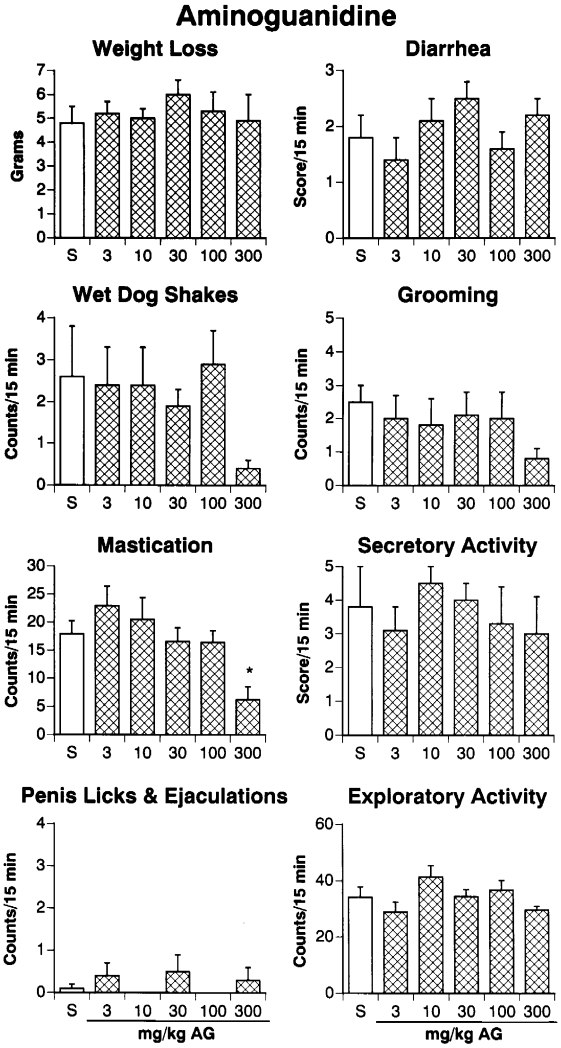

Fig. 3. Mean effects of aminoguanidine (AG) on naloxone-precipitated morphine withdrawal signs. For the saline (S) vehicle, $n = 8$, and for the aminoguanidine dose groups, $n = 8$. Vertical lines represent the S.E.M. Significant differences from saline are indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$ (one-way analysis of variance followed by Dunnett's test).

Table 2
Relative potency estimates calculated from parallel line bioassays

	Weight loss	Wet dog shakes	Grooming	Mastication
7-Nitro indazole	1.0	1.0	1.0	1.0
3-Bromo-7-nitro indazole	1.1 (0.5–2.6)	1.1 (0.6–2.1)	1.0 (0.5–1.8)	1.0 (0.6–1.7)
S-Methyl-L-thiocitrulline	1.6 (0.7–2.9)	2.9 (0.4–36.5)	Prep difference	Prep difference
Aminoguanidine	Not parallel	0.2 (0.1–0.4)	0.2 (0.04–0.4)	0.09 (0.05–0.16)

7-Nitro indazole was selected as the standard drug, and its relative potency was set equal to 1.0. Relative potency was defined as the mg of the standard drug equal to 1 mg of the test drug. For the test drugs, the 95% confidence limits of the relative potency estimates are shown in parentheses for a valid parallel line bioassay. Confidence limits that do not include 1.0 indicate a significant difference in potency. For weight loss, wet dog shakes and mastication 18, 30, 56 and 100 mg/kg 3-bromo-7-nitro indazole and 5.6, 10, 18, 30 and 56 mg/kg *S*-methyl-L-thiocitrulline were compared to 18, 30, 56 and 100 mg/kg 7-nitroindazole. For grooming activity the 18 mg/kg dose of 7-nitro indazole was not used. The comparisons of aminoguanidine and 7-nitro indazole used the 7-nitro indazole data published by Vaupel et al. (1995b) because scoring was done by the same observer. 100 and 300 mg/kg aminoguanidine were compared to 18, 30, 56 and 100 mg/kg 7-nitro indazole for weight loss and wet dog shakes, 5.6, 8, 30 and 56 mg/kg 7-nitro indazole for mastication, and 30 and 56 mg/kg 7-nitro indazole for grooming. The 100 mg/kg dose of 7-nitro indazole was not used for mastication and grooming because a maximum effect was obtained at 56 mg/kg. Cells without values indicate bioassays that were not valid because either the dose–response curves were not parallel ('Not parallel') or the mean effect of the two drug preparations (i.e., treatments) differed significantly ('Prep difference').

Table 3
Effect of nitric oxide synthase inhibitors on rat mean arterial pressure and heart rate

	Mean arterial pressure (change in mm Hg)		Heart rate (change in beats/min)	
	Morphine-naïve	Morphine-dependent	Morphine-naïve	Morphine-dependent
Saline	15 ± 8	8 ± 5	-2 ± 13	-30 ± 26
Peanut oil [a]	1 ± 6	0 ± 1	-10 ± 20	10 ± 6
3-Bromo-7-nitro indazole (100 mg/kg)	2 ± 6	13 ± 14	3 ± 32	-33 ± 58
7-Nitro indazole (100 mg/kg) [a]	3 ± 10	-10 ± 5	-67 ± 32	-160 ± 15 [c]
S-Methyl-L-thiocitrulline(56 mg/kg)	48 ± 5 [b]	56 ± 10 [b]	-120 ± 24 [b]	-60 ± 15
Aminoguanidine (300 mg/kg)	-2 ± 5	-7 ± 7	5 ± 13	-25 ± 32
Clonidine (0.1 mg/kg)	-12 ± 8 [b]	-7 ± 5	-53 ± 22	-83 ± 23

Differences in cardiovascular effects measured 1 h after i.p. administration. The doses represent the highest used in the naloxone-precipitated morphine withdrawal study that did not elicit signs of behavioral toxicity. Results show mean ± S.E.M., $n = 3$. [a] Values taken from Vaupel et al. (1995b). [b] $P < 0.05$ and [c] $P < 0.01$ indicate significant differences from saline (Dunnett's test).

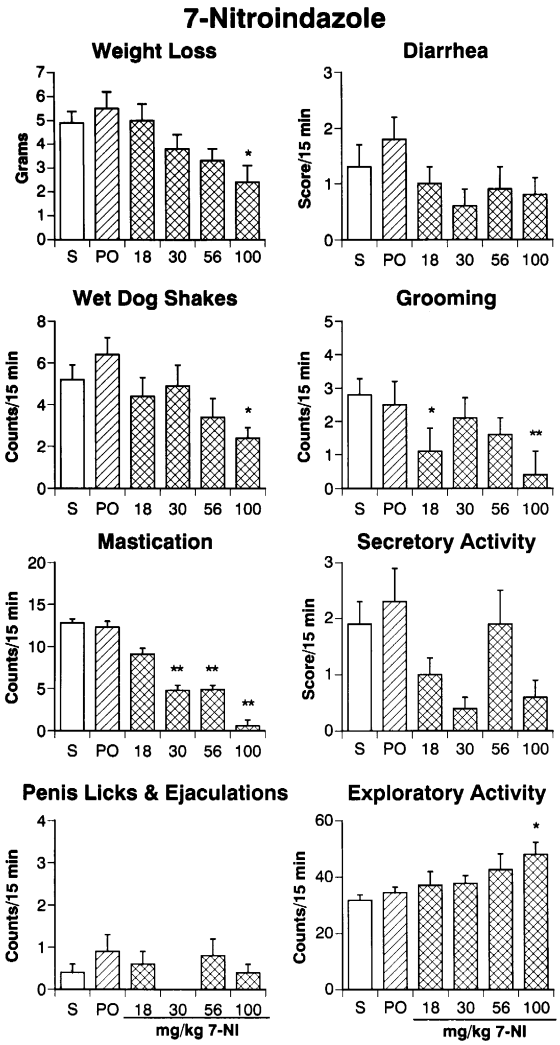

Fig. 4. Mean effects of 7-nitro indazole (7-NI) on naloxone-precipitated morphine withdrawal signs. For the saline (S) and peanut oil vehicles (PO), the *n* is 16; the *n* for the 7-nitro indazole dose groups ranged from 8 to 11. Vertical lines represent the S.E.M. Significant differences from saline are indicated as * $P < 0.05$ or ** $P < 0.01$ (one-way analysis of variance followed by Dunnett's test).

cious than 7-nitro indazole. Two bioassay comparisons of 7-nitro indazole and *S*-methyl-L-thiocitrulline demonstrated linear regression and parallel dose–response curves, but there was a difference in experimental preparations. For these measures, grooming and mastication, *S*-methyl-L-thiocitrulline produced a smaller mean effect than 7-nitro indazole. Aminoguanidine clearly stood out as being the least potent NO synthase inhibitor tested.

3.3. Cardiovascular effects

3-Bromo-7-nitro indazole and aminoguanidine did not affect blood pressure or heart rate, whereas *S*-methyl-L-thiocitrulline produced a marked elevation in mean arterial pressure in both naïve and morphine-dependent animals (Table 3). Clonidine produced a relatively small reduction in mean arterial pressure only in naïve rats. Heart rate was affected only by *S*-methyl-L-thiocitrulline, and that was in the naïve but not in the morphine-dependent condition.

3.4. Toxicity

None of the NO synthase inhibitors produced deaths within the 3-day post-test observation period, and *S*-methyl-L-thiocitrulline was the only compound associated with a pronounced behavioral toxicity. The initial dosages evaluated for *S*-methyl-L-thiocitrulline ranged from 5.6 to 56 mg/kg. A 100 mg/kg dose was added because the data suggested that small to moderate effects on certain measures were produced by 56 mg/kg. Severe ataxia and a sluggish righting reflex clearly were apparent following the 100 mg/kg dose of *S*-methyl-L-thiocitrulline even before naloxone was administered. For this reason, this dose was excluded from the regression and parallel line analyses, although the data are illustrated in Fig. 2.

4. Discussion

The ability of 7-nitro indazole to reduce specific signs of morphine withdrawal generally replicated our previous

results (Vaupel et al., 1995b), and nearly equivalent effects were produced by its 3-bromo analogue. As before, 7-nitro indazole reduced weight loss, wet dog shakes, grooming and mastication, increased exploratory activity and escape jumps, and did not affect ptosis. In the current study, the reduction in diarrhea did not attain statistical significance. Other behaviors were less consistently affected by 7-nitro indazole in both studies and included secretory activity, penis licks/ejaculations, and abnormal posturing. 3-Bromo-7-nitro indazole closely resembled 7-nitro indazole with regard to effects on weight loss, wet dog shakes, grooming, mastication, penis licks/ejaculations, exploratory activity and ptosis. In contrast to 7-nitro indazole, 3-bromo-7-nitro indazole did not affect escape jumps, abnormal posturing or secretory activity although it did reduce diarrhea. Considered together, the actions of the two nitro indazoles on opiate withdrawal yielded similar behavioral profiles; however, 3-bromo-7-nitro indazole did not enhance as many signs as 7-nitro indazole. Whether this represents an advantage over 7-nitro indazole in the rat model of opiate withdrawal is unclear.

Some of the NO synthase inhibitors enhanced (worsened) exploratory activity and escape jumps, which are signs of opiate withdrawal. It is worth noting that clonidine, also, enhances certain signs of morphine withdrawal in the rat although this α_2 agonist is used effectively to alleviate autonomic signs of opiate withdrawal in humans (Jaffe, 1990). In the rat, clonidine enhanced fewer signs, including exploratory activity, escape jumps and penis licks + ejaculations, than it attenuated (Vaupel et al., 1995b). The relevance of the enhancement of these signs of abstinence to predict clinical usefulness of the NO synthase inhibitors in treating opiate dependence is not clear.

The attenuating effects of *S*-methyl-L-thiocitrulline pretreatment on opiate withdrawal were not dramatically different from the profiles of most other NO synthase inhibitors (Vaupel et al., 1995b). Most notable among the differences observed for *S*-methyl-L-thiocitrulline were absences of reduced grooming behavior and of a robust, dose-dependent decrease in wet dog shakes. However, there were disadvantages associated with *S*-methyl-L-thiocitrulline. The qualitative trend for an increased incidence of penis licks and ejaculations has been observed with NO synthase inhibitors that markedly elevate blood pressure (Vaupel et al., 1995b). Indeed, our results confirmed that *S*-methyl-L-thiocitrulline is active as a vasopressor agent (Narayanan et al., 1994). Additionally, the abnormal posturing observed at the 100 mg/kg dose of *S*-methyl-L-thiocitrulline undoubtedly reflects components of behavioral toxicity, as discussed below.

By far the least effective inhibitor of NO synthase in attenuating opiate withdrawal in either the present or previous (Vaupel et al., 1995b) study was aminoguanidine. Several reports have established that in intact cells, cell-free extracts and isolated tissue preparations, aminoguanidine selectively inhibits inducible NO synthase relative to the constitutive isoforms (Corbett et al., 1992; Misko et al., 1993; Joly et al., 1994). Other less specific inhibitors, such as N^G-nitro-L-arginine and N^G-monomethyl-L-arginine, are as potent as aminoguanidine in inhibiting inducible NO synthase; however, aminoguanidine is 10 to 100 times less potent in inhibiting constitutive NO synthase. Based upon the aminoguanidine dose–response data, the threshold dose for reducing withdrawal signs exceeded 100 mg/kg, albeit only for three measures. This observation suggested that at doses up to 100 mg/kg, aminoguanidine primarily affected inducible NO synthase, but that at 300 mg/kg, effects of aminoguanidine on opiate withdrawal reflected inhibition of neuronal and endothelial constitutive NO synthase.

The emergence of any behavioral toxicity was a concern because of the potential to observe changes in withdrawal signs that reflected nonspecific effects. The highest dose of both the 3-brominated and unsubstituted 7-nitro indazoles produced some ataxia, but it was not severe enough to prevent an increase in exploratory behavior. None of these animals fell over on their sides, as was observed for *S*-methyl-L-thiocitrulline. *S*-Methyl-L-thiocitrulline, at 100 mg/kg, produced severe ataxia and, more importantly, depressed the righting reflex observed when the animals fell over on their sides. Still, exploratory activity was enhanced. These data suggest that at least two motoric pathways are being affected differently, resulting in visible depression and stimulation. Nevertheless, data obtained with this behaviorally impairing dose were omitted from assessing the efficacy and relative potency of *S*-methyl-L-thiocitrulline. We had previously observed a relatively high incidence of fatalities over a 3-day period resulting from the administration of N^G-nitro-L-arginine or N^G-nitro-L-arginine methyl ester with morphine-dependent rats undergoing withdrawal (Vaupel et al., 1995b). However, no deaths occurred in the present study over the identical time period.

Our expectation was that 3-bromo-7-nitro indazole and *S*-methyl-L-thiocitrulline would be more potent than 7-nitro indazole. This prediction was based on IC_{50} values for inhibiting rat cerebellar constitutive NO synthase, for which 3-bromo-7-nitro indazole (0.17 ± 0.01 μM) was four times as potent as 7-nitro indazole (0.71 ± 0.01 μM) (Bland-Ward et al., 1994) and on our assumption that neuronal constitutive NO synthase had a dominant role in modulating opiate withdrawal relative to the endothelial or inducible isoforms. In terms of enzyme specificity, it is noteworthy that Bland-Ward et al. (1994) also reported that 7-nitro indazole and 3-bromo-7-nitro indazole were equipotent in inhibiting bovine endothelial constitutive NO synthase but that the 3-bromo derivative was 20 times as potent in inhibiting rat lung inducible NO synthase. *S*-Methyl-L-thiocitrulline is a more potent inhibitor of neuronal than endothelial constitutive NO synthase or inducible NO synthase (Furfine et al., 1994; Narayanan and

Griffith, 1994). Under the present conditions, however, no differences in relative potency between 7-nitro indazole, 3-bromo-7-nitro indazole and *S*-methyl-L-thiocitrulline were demonstrated in vivo. The relatively low potency and low efficacy of aminoguanidine in vivo in attenuating withdrawal and the isoform selectivity of aminoguanidine determined in vitro coupled with the equivalent in vivo potency of the two nitro indazole compounds in this study, despite the much greater potency of 3-bromo-7-nitro indazole relative to 7-nitro indazole in vitro for inhibiting inducible NO synthase, further support the concept that endothelial and neuronal constitutive NO synthase and not inducible NO synthase have a significant modulatory effect in the opiate withdrawal syndrome.

The goal of developing selective inhibitors of NO synthase to treat opiate withdrawal is to gain a therapeutic advantage over currently available pharmaceuticals. In the rat model of morphine withdrawal, the nitro indazoles afford more benefits than other NO synthase inhibitors. Heroin-dependent patients undergoing withdrawal develop an increase in blood pressure (Himmelsbach, 1937), and any substantial enhancement of the vasopressor response by a therapeutic agent could reduce its clinical utility. The α_2 agonist clonidine, similar to inhibitors of NO synthase, attenuates morphine withdrawal in rats (Kimes et al., 1990; Vaupel et al., 1995b) but does not elevate blood pressure. In patients, clonidine is used to alleviate signs of opiate withdrawal but has the disadvantage of producing orthostatic hypotension (Jaffe, 1990). The absence of vasopressor activity, previously seen with 7-nitro indazole in rodents (Moore et al., 1993a,b; Vaupel et al., 1995b), was demonstrated for 3-bromo-7-nitro indazole. While aminoguanidine is without effect on blood pressure, it lacks efficacy in suppressing opiate withdrawal in the rat. Together, the absence of vasoconstrictor activity and good efficacy in attenuating the morphine withdrawal syndrome suggest that 7-nitro indazole and 3-bromo-7-nitro indazole offer the best therapeutic potential among the NO synthase inhibitors evaluated for attenuating the opioid withdrawal syndrome.

Using estimates of selectivity based on enzyme assays, it is difficult to reconcile that both 3-bromo-7-nitro indazole and 7-nitro indazole do not affect blood pressure and *S*-methyl-L-thiocitrulline does because the vasopressor effects of the three drugs are not readily correlated with their relative abilities to inhibit constitutive neuronal and constitutive endothelial NO synthase in vitro. Consider the following endothelial NO synthase : cerebellar NO synthase ratios determined from published IC_{50} values (μM) (note: higher ratios indicate a greater selectivity to inhibit neuronal NO synthase): 7-nitro indazole – $0.78/0.71 = 1.1$ (Bland-Ward et al., 1994); 3-bromo-7-nitro indazole – $0.86/0.17 = 5.1$ (Bland-Ward et al., 1994); and *S*-methyl-L-thiocitrulline – $5.4/0.31 = 17$ or $5.4/0.047 = 114$ (Furfine et al., 1994). On this basis, it is surprising that *S*-methyl-L-thiocitrulline, which shows the greatest selectivity for neuronal constitutive NO synthase, elicits a pronounced increase in blood pressure and the less selective nitro indazoles are inactive. Attempts to relate chemical structure, vasopressor activity, and IC_{50} values for endothelial NO synthase do not readily explain these differences (Narayanan et al., 1994; Garvey et al., 1994; Nakane et al., 1995; Wolff and Gribin, 1994; Moore et al., 1993b; Vaupel et al., 1995b). Consequently, other factors (e.g., permeability barriers, pharmacokinetics) or more complex, unidentified mechanisms (e.g., isoforms of endothelial NO synthase or dependence of a cardiovascular response upon both neuronal and endothelial NO synthase) may contribute to the vasoconstrictor reactions to NO synthase inhibitors.

Overall, changes in heart rate exhibited more variability than the vasopressor responses, which precluded the significance of relatively substantial decreases in rate. However, bradycardia measured after 56 mg/kg *S*-methyl-L-thiocitrulline in morphine-naïve rats likely represents a baroreceptor-mediated response to the increase in mean arterial pressure. 7-Nitro indazole, which was without effect on blood pressure, reduced heart rate, but only in morphine-dependent rats was this significant. A stable blood pressure in the presence of reduced cardiac output would be achieved by a peripheral vascular vasoconstriction. Whether this is due to a direct effect of endothelial NO synthase inhibition is unknown. In comparison, clonidine-induced bradycardia, which was not significant in this study, elicits this effect by decreasing sympathetic and increasing parasympathetic inputs to the heart (Langer et al., 1980).

The locus coeruleus is a neuroanatomical substrate that has an integral role in mediating the expression of opiate withdrawal, particularly autonomic signs (Taylor et al., 1988). This brain region, which has a dense concentration of noradrenergic neurons, exhibits increased cellular firing rates during withdrawal that are depressed by clonidine administration (Aghajanian, 1978). Two recent, preliminary reports suggest that inhibiting NO production reduces the withdrawal-dependent noradrenergic hyperactivity occurring within the locus coeruleus. The NO synthase inhibitor L-*N*-nitro arginine *p*-nitroanilide decreased the elevated quantity of dihydroxyphenylacetic acid (a metabolic marker of noradrenergic activity) released during precipitated morphine withdrawal, as determined by microdialysis, and attenuated wet dog shakes and mastication (Javelle et al., 1996). Additionally, both increased locus coeruleus cell firing rates and the emergence of wet dog shakes, evoked by precipitated morphine withdrawal, were markedly reduced by systemically administered NO synthase inhibitors N^G-nitro-L-arginine methyl ester (Pineda and Ugedo, 1996) and 7-nitro indazole (Pineda, personal communication). Together, these data suggest similar effects at the cellular level within this site for NO synthase inhibitors and clonidine, even though the mechanisms of action of the drugs are clearly distinct. Precisely how α_2

noradrenergic mechanisms and NO-generating enzymes are integrated within locus coeruleus and the general role of NO synthase in opiate dependence and withdrawal are questions that remain to be addressed.

The present data confirm that NO synthase inhibitors attenuate signs of morphine withdrawal and imply that nitric oxide is a modulator in the opiate abstinence syndrome. Because aminoguanidine has been both the weakest of seven inhibitors tested in terms of potency and efficacy and also the most selective inhibitor of inducible NO synthase tested to date, we suggest that it is the isoforms of constitutive NO synthase rather than inducible NO synthase that participate in the physiological changes in NO-associated morphine dependence and withdrawal. Furthermore, the equal in vivo potency of 7-nitro indazole and 3-bromo-7-nitro indazole in this study, despite the greater potency of 3-bromo-7-nitro indazole relative to 7-nitro indazole as an inhibitor of inducible NO synthase in vitro, supports this concept. Our data do not distinguish between the relative importance of neuronal and endothelial constitutive NO synthase in opiate withdrawal. Yet, among three inhibitors that exhibit a degree of selectivity for constitutive NO synthase, the two nitro indazole derivatives, although they lacked the potency of less selective inhibitors, were the most efficacious in reducing signs of opiate withdrawal without elevating mean arterial pressure. It is possible that NO synthase inhibitors also may reduce a motivational component of opiate withdrawal (Funada et al., 1996; Higgins and Sellers, 1994; Schulteis et al., 1994), but this hypothesis remains to be tested.

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