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Letter

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NOpiates: Novel Dual Action Neuronal Nitric Oxide Synthase Inhibitors with μ-Opioid Agonist Activity

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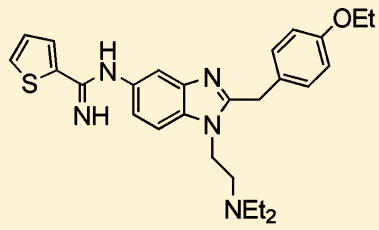
Supporting Information

ABSTRACT: A novel series of benzimidazole designed multiple ligands (DMLs) with activity at the neuronal nitric oxide synthase (nNOS) enzyme and the μ-opioid receptor was developed. Targeting of the structurally dissimilar heme-containing enzyme and the μ-opioid GPCR was predicated on the modulatory role of nitric oxide on μ-opioid receptor function. Structure−activity relationship studies yielded lead compound **24** with excellent nNOS inhibitory activity (IC_{50} = 0.44 μM), selectivity over both endothelial nitric oxide synthase (10-fold) and inducible nitric oxide synthase (125-fold), and potent μ-opioid binding affinity, K_i = 5.4 nM. The functional activity as measured in the cyclic adenosine monosphospate secondary messenger assay resulted in full agonist activity (EC_{50} = 0.34 μM). This work represents a novel approach in the development of new analgesics for the treatment of pain.



Dual action nNOS inhibitor/mu opioid agonist (24)

nNOS: IC50 = 0.44 μM; MOP: Ki = 5.4 nM.

KEYWORDS: *nitric oxide, opioid, dual action, benzimidazole, NOS inhibitor, NOpiate, NOpioid*

Opioid analgesics are one of the most important classes of drugs and represent the cornerstone of pain management. Although opiates have a long history of successful management of pain, there is a continued interest in the development of new opioid analgesics to overcome their numerous liabilities that include respiratory depression, nausea, sedation, constipation, dependency, abuse, and the development of tolerance.[1,2] In the case of chronic and neuropathic pain, opiate use fails to adequately address the features of hyperalgesia and allodynia, yielding suboptimal pain relief in many patients.[3−5] Paradoxically, chronic administration of opiates may be self-limiting as a result of the development of opioid-induced hyperalgesia (OIH),[6] an enhancement of the pain response attributed to adaptative changes in the central nervous system such as upregulation of the cyclic adenosine monosphospate (cAMP) pathway and increased production of nitric oxide (NO).

It has been suggested that compounds acting upon multiple mechanisms of action could better address the shortcomings of current pain therapies by enhancing efficacy and reducing side effects.[3,7,8] The importance of multiple mechanisms acting in concert either additively or synergistically to treat pain has been exemplified by the polypharmacology of opioid combinations such as oxycodone-acetominaphen, morphine-oxycodone, morphine-δ antagonists, and morphine-gabapentin.[9−11] However, the development of combination drugs is complicated by a need to optimally match pharmacokinetic properties and by difficulties in predicting drug interactions, dissolution rates, and stability in the formulated product.[12]

In our approach to address the pressing need for the development of improved analgesics targeting novel pain pathways,[3,13] we adopted the dual action or designed multiple ligand (DML)[14] paradigm with the specific aim of incorporating μ-opioid agonism and nitric oxide synthase (NOS) inhibition into a single new chemical entity (NCE).

NO is synthesized by three highly related isoforms (neuronal or nNOS, endothelial or eNOS, and inducible or iNOS) and regulates neurotransmission, blood pressure, and inflammatory responses, respectively.[15] NO is known to play an important role mediating nociceptive processing in sensitized pain states,[16] and NOS inhibitors are effective in animal models of neuropathic and inflammatory pain.[16−19] In addition, non-selective NOS inhibitors L-nitro arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI) potentiate opioid analgesia in pain models and reduce morphine tolerance. However, this effect is lost in nNOS knockout mice, suggesting that the neuronal form is important in modulating opioid analgesia and tolerance.[20,21]

With this in mind, while recognizing the difficulty inherent in integrating the pharmacophoric elements from two highly dissimilar targets[14,22] (nNOS, a heme-containing enzyme, and the μ-opioid GPCR), we initiated a program to develop a dual-acting selective nNOS inhibitor (i.e., lacking eNOS and iNOS inhibition) with μ-opioid agonist activity. Common structural features between etonitazene,[23] a potent μ-agonist (K_i = 0.2 nM), and previously published nNOS inhibitors were envisioned (Figure 1).[24,25]

Both the central aryl scaffold and the basic amine side chain were apparent in the etonitazene framework. Determination as to whether incorporation of a guanidine isostere [which makes

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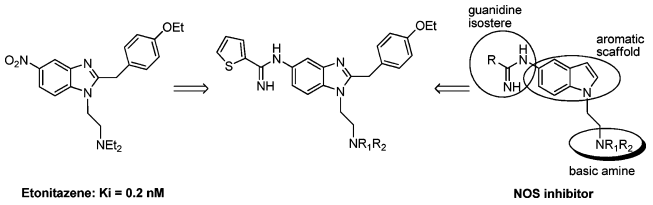


Figure 1. Proposed dual action structure.

an important bidentate interaction with the conserved glutamic acid residue at the arginine (substrate) binding site of the NOS enzyme] would be tolerated without significant loss of μ-opioid binding ability was paramount. Although little has been published on the binding interactions of etonitazene,[26] two literature reports suggested that upon replacement of the nitro group, antinociceptive activity could be retained albeit weaker than etonitazine itself.[27,28] Herein, we report the synthesis of a series of dual action substituted benzimidazoles with selective inhibitory activity toward the human nNOS isoform and activity at the μ-opioid receptor.

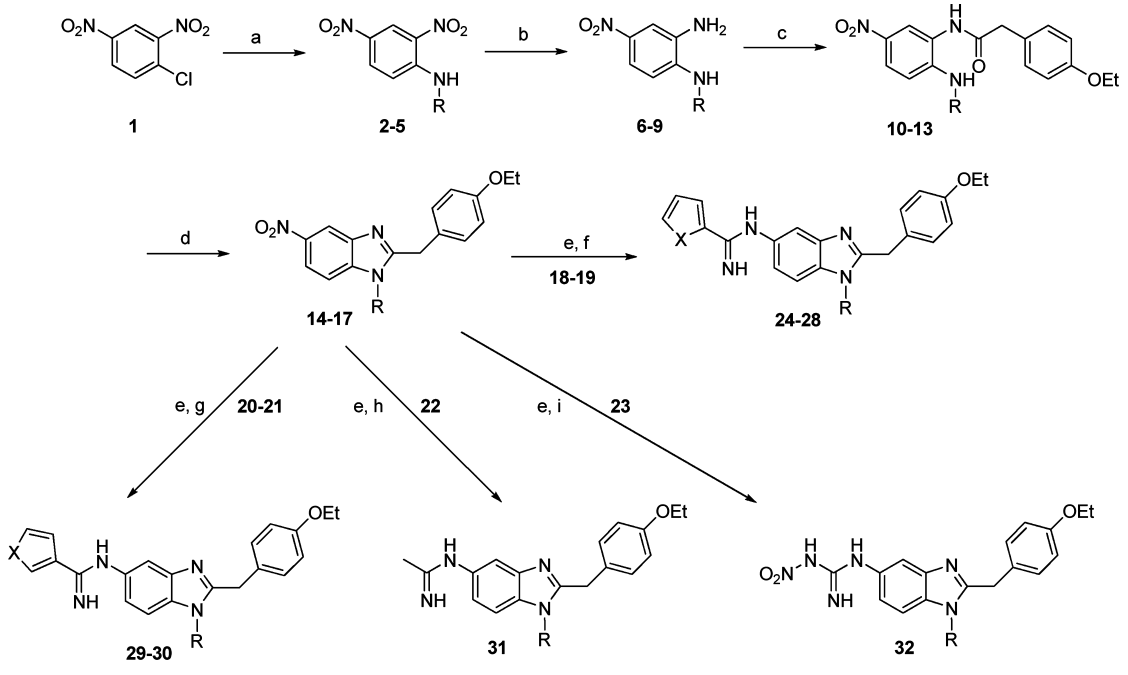
Preparation of the 1,2,5-trisubstituted benzimidazole analogues was achieved as outlined in Scheme 1 utilizing a modified version of the original synthesis of etonitazene.[23]

The tertiary amino side chains were introduced via nucleophilic aromatic substitution of the chloro group of 1-chloro-2,4-dinitrobenzene **1** with various diamines. Recrystallization from EtOH yielded the 2,4-dinitro-substituted anilines **2**–**5**. Preferential reduction (commonly >5:1) of the nitro group *ortho* to the secondary amine was achieved by a Zinin reduction.[29] Subsequent separation from the *para* diamino regioisomer exploiting dry column chromatography techniques yielded the 1,2-diaminobenzenes **6**–**9**. Condensation with 2-(4-ethoxyphenyl)acetic acid in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) followed by PCl_5-induced cyclization[30] yielded the key benzimidazole intermediates **14**–**17**. Reduction of the nitro group to the corresponding amino group under atmospheric hydrogenation conditions and subsequent reaction in situ with one of methyl thiophene-2-carbimidothioate·hydroiodide (HI) (**18**), benzyl furan-2-carbimidothioate·hydrobromide (HBr) (**19**), benzyl thiophene-3-carbimidothioate·HBr (**20**), benzyl furan-3-carbimidothioate·HBr (**21**), naphthalen-2-ylmethyl ethanimidothioate·HBr (**22**), or 1-methyl-3-nitro-1-nitrosoguanidine (**23**) yielded final compounds **24**–**32**.[31] Utilizing the reduction/amidine formation sequence (vide supra), the six-substituted regioisomer of **24** was synthesized from known compound **33**,[25] as shown in Scheme 2. All compounds were converted into their corresponding dihydrochloride salts.

The inhibitory activities of the target compounds against human NOS isoforms,[32] their binding affinity to the human μ opioid receptor,[33] and a functional measurement of agonist-like activity (the ability to inhibit forskolin mediated cAMP production)[33] were assessed (Table 1).

Compound **24** was identified as the most potent nNOS inhibitor [IC_{50} = 0.44 μM, more potent than the clinically active nonselective NOS inhibitor (L-NMMA)], while

Scheme 1. Synthesis of Trisubstituted Benzimidazole Final Compounds 24–32[a]



2, 6, 10, 14, 31, 32: R = $CH_2CH_2NEt_2$; **24,29**: R = $CH_2CH_2NEt_2$, X = S; **28,30**: R = $CH_2CH_2NEt_2$, X = O
3, 7, 11, 15: R = $CH_2CH_2NMe_2$; **25:** R = $CH_2CH_2NMe_2$, X = S
4, 8, 12, 16: R = 2-(1-methylpyrrolidin-2-yl)ethanamine; **26**: R = 2-(1-methylpyrrolidin-2-yl)ethanamine, X = S
5, 9, 13, 17: R = 1-methylpiperidin-4-ylamine; **27:** R = 1-methylpiperidin-4-ylamine, X = S

[a]Reagents and conditions: (a) Various diamines (H_2NR), EtOH, reflux. (b) Aqueous $(NH_4)_2S$, EtOH, H_2O, 70 °C. (c) 2-(4-Ethoxyphenyl)acetic acid, EEDQ, CH_2Cl_2 or THF, 35–60 °C. (d) PCl_5, $CHCl_3$, reflux. (e) Pd–C/H_2, EtOH, room temperature. (f) Methyl thiophene-2-carbimidothioate·HI (**18**) or benzyl furan-2-carbimidothioate·HBr (**19**), EtOH, room temperature. (g) Benzyl thiophene-3-carbimidothioate·HBr (**20**) or benzyl furan-3-carbimidothioate·HBr (**21**), EtOH, room temperature. (h) Naphthalen-2-ylmethyl ethanimidothioate·HBr (**22**), EtOH, room temperature. (i) 1-Methyl-3-nitro-1-nitrosoguanidine (**23**), EtOH, reflux.

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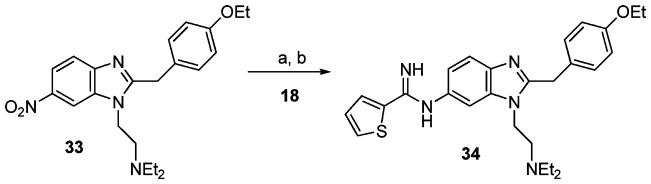
Scheme 2. 6-Regioisomer of Compound 24[a]

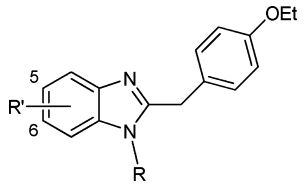


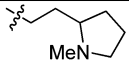
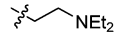
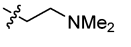
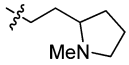
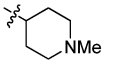
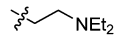
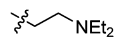
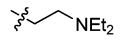
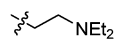
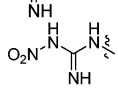
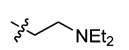
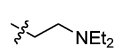
[a]Reagents and conditions: (a) Pd−C/H_2, EtOH, room temperature. (b) Methyl thiophene-2-carbimidothioate·HI (**18**), EtOH, room temperature.

demonstrating selectivity over eNOS (10-fold preference for nNOS); iNOS (125-fold) and importantly showed potent binding affinity (K_i = 5.4 nM, comparable to morphine) at the μ-opioid receptor in a competitive radioligand binding assay.

Compounds **24**, **25**, **28**, **29**, and **30** were selective (5−23-fold) for the nNOS over the eNOS isoform. To obtain compounds devoid of the cardiovascular liabilities associated with eNOS inhibition,[34] selective nNOS inhibition is required. In this series of compounds, the acyclic basic amine side chains showed improved nNOS/eNOS selectivity in comparison to the cyclic amino side chain **27**. Thiophene amidines **24** and **29** were more potent for the nNOS and eNOS isoforms when compared to the corresponding furanyl amidines **28** and **30**, respectively. Suprisingly, compounds **31** and **32** show weak inhibitory activity at NOS despite the presence of the acetamidine (**31**) and nitroguanidine (**32**) moieties, two functional motifs that have been utilized successfully in previous NOS inhibitors.[35] However, **32** displayed excellent activity in the μ-opioid functional assay (52 nM), suggesting an important interaction of the nitro group of etonitazene and potentially **32** that facilitates potent functional activity. In

Table 1. Inhibition of Human NOS Enzymes and MOP Binding and Functional Data[a]



Compound ID	R'	R	nNOS IC_{50} (μM)	eNOS IC_{50} (μM)	iNOS IC_{50} (μM)	Binding h-μ(MOP) Ki (nM)	Functional h-μ(MOP) EC_{50} (nM)
16	5-			113 (38.8-331)	317 (21.5-4650)	NT[b]	0.43 (0.37-0.66)	NT
24	5-			0.44 (0.30-0.66)	4.74 (2.57-8.73)	55.2 (35.5-90.8)	5.41 (4.40-6.64)	340 (218-515)
25	5-			0.74 (0.45-1.22)	17 (10.0-29.2)	NT	110 (95.4-156)	1500 (1054-2157)
26	5-			1.77 (1.20-2.60)	4.04 (2.14-7.61)	NT	170 (122-238)	2200 (982-4854)
27	5-			12.8 (8.79-18.8)	8.00 (5.99-10.7)	NT	2900 (1972-4045)	NT
28	5-			5.05 (2.62-9.75)	28.7 (17.2-47.8)	15.0 (1.22-18.6)	27.0 (21.1-33.6)	400 (316-520)
29	5-			2.76 (1.81-4.23)	29.5 (18.4-47.5)	118 (75.6-184)	12.0 (9.46-14.1)	NT
30	5-			14.4 (10.0-20.8)	117 (71.8-190)	NT	42.0 (35.5-50.3)	590 (391-876)
31	5-			>100[c]	>100	NT	48.0 (33.2-66.3)	940 (329-2740)
32	5-			35.7 (25.2-50.7)	37.1 (14.7-93.8)	NT	7.5 (5.34-10.3)	52.0 (35.0-77.9)
34	6-			37.2 (15.2-90.6)	47.1 (17.1-129)	NT	78.0 (71.1-91.1)	170 (103-279)
L-NMMA				0.95 (0.63-1.4)	0.65 (0.45-0.94)	1.8 (0.47-6.7)		
Tramadol								1300 (845-2043)
Des-Methyl tramadol								120 (92.6-120)
Tapentadol							160[d]	670[d]
Morphine							2.2	3.7

[a]Values reported in parentheses are 95% confidence intervals. [b]NT, not tested. [c]>100, not active at the maximum test concentration of 100 μM. [d]Data from ref 38.

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contrast to the 5-substituted analogue **24** and other 1,6-substituted bicyclic scaffolds,[36] the six-substituted regioisomer **34** shows much weaker nNOS inhibition (85-fold).

Select compounds showed nanomolar level potency in the opioid binding assay but with reduced functional activity. However, these compounds displayed full agonist properties at the μ-opioid receptor. Because of the potential synergies of the dual mechanisms, the functional activity may not need to be as potent as morphine. For example, both Tramadol (and its more active desmethyl metabolite; see Table 1) and Tapentadol (30-fold weaker than morphine in a [^{35}S]GTPγS functional assay) are clinically utilized centrally acting analgesics despite showing modest functional activity at the μ-opioid receptor, likely due to the synergy of nonopioid mechanisms (primarily monoamine reuptake inhibition).[37,38]

In conclusion, we have designed and synthesized a series of novel dual action nNOS inhibitors with μ-opioid agonist activity and selectivity for nNOS over eNOS. This is the first report of a DML combining μ-opioid activity and selective nNOS inhibitory activity. It is notable that this represents one of the few cases of the successful design for two structurally distinct macromolecular targets (GPCR and oxygenase enzyme) as the majority of reported DMLs target similar subclasses.[14,22] The lead compound **24** inhibited nNOS more potently than L-NMMA and displayed a level of potency similar to morphine in a μ-opioid binding assay. Thus, having achieved proof of concept of dual targeting of these dissimilar pain targets, future efforts will be focused on evaluating the potential synergistic effects of combined nNOS/μ-opioid mechanisms in animal models of acute and chronic pain.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, analytical characterization and purity assessment of final products, and biological assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

cAMP, cyclic adenosine monosphospate; DML, designed multiple ligand; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; eNOS, endothelial nitric oxide synthase; HBr, hydrobromide; HI, hydroiodide; iNOS, inducible nitric oxide synthase; L-NAME, L-nitro arginine methyl ester; NCE, new chemical entity; 7-NI, 7-nitroindazole; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; OIH, opioid-induced hyperalgesia

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