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Synthesis and Pharmacology of Halogenated δ -Opioid Selective [DAla²] Deltorphin II Peptide Analogs

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At the time of this publication, Dr. Katlowitz was affiliated with the Memorial Sloan-Kettering Cancer Center, but Dr. Katlowitz is now affiliated with the Children's Hospital of Philadelphia (CHOP).

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Synthesis and Pharmacology of Halogenated δ -Opioid Selective [DAla²] Deltorphan II Peptide Analogs

Abstract

Deltorphins are naturally occurring peptides produced by the skin of the giant monkey frog (*Phyllomedusa bicolor*). They are δ -opioid receptor-selective agonists. Herein, we report the design and synthesis of a peptide, Tyr-d-Ala-(pI)Phe-Glu-Ile-Ile-Gly-NH₂ 3 (GATE3-8), based on the [d-Ala²]deltorphan II template, which is δ -selective in *in vitro* radioligand binding assays over the μ - and κ -opioid receptors. It is a full agonist in [³⁵S]GTP γ S functional assays and analgesic when administered supraspinally to mice. Analgesia of 3 (GATE3-8) is blocked by the selective δ receptor antagonist naltrindole, indicating that the analgesic action of 3 is mediated by the δ -opioid receptor. We have established a radioligand in which ¹²⁵I is incorporated into 3 (GATE3-8). The radioligand has a K_D of 0.1 nM in Chinese hamster ovary (CHO) cells expressing the δ receptor. Additionally, a series of peptides based on 3 (GATE3-8) was synthesized by incorporating various halogens in the para position on the aromatic ring of Phe³. The peptides were characterized for binding affinity at the μ -, δ -, and κ -opioid receptors, which showed a linear correlation between binding affinity and the size of the halogen substituent. These peptides may be interesting tools for probing δ -opioid receptor pharmacology.

Keywords

delta opioid receptor, deltorphan, radioiodination, sandmeyer

Disciplines

Chemicals and Drugs | Neurology | Neuroscience and Neurobiology | Neurosciences

Comments

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Synthesis and pharmacology of halogenated δ -opioid selective [$DAla^2$] Deltorphin II peptide analogs

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ABSTRACT

Deltorphins are naturally occurring peptides produced by the skin of the giant monkey frog (*Phyllomedusa bicolor*). They are δ -opioid receptor selective agonists. We herein report the design and synthesis of a peptide, Tyr-D-Ala-(pI)Phe-Glu-Ile-Ile-Gly-NH₂ **3** (GATE3-8), based on the [$DAla^2$]deltorphin II template, which is δ -selective in *in vitro* radioligand binding assays over the μ and κ opioid receptors. It is a full agonist in [³⁵S]GTP γ S functional assays and analgesic when administered supraspinally to mice. Analgesia of **3** (GATE3-8) is blocked by the

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2
3 selective δ receptor antagonist naltrindole, indicating that the analgesia is mediated by the δ
4 opioid receptor. We have established a radioligand in which ^{125}I is incorporated into **3** (GATE3-
5 8). The radioligand has a K_D of 0.1 nM in Chinese hamster ovary (CHO) cells expressing the δ
6 receptor. Additionally, a series of peptides based on **3** (GATE3-8) were synthesized
7 incorporating various halogens in the para position on the aromatic ring of Phe³. The peptides
8 were characterized for binding affinity at the μ -, δ -, and κ -opioid receptors, which showed a
9 linear correlation between binding affinity and the size of the halogen substituent. These peptides
10 may be interesting tools for probing δ -opioid receptor pharmacology.
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24 Introduction

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27 Opioid analgesics have been used for their pain-relieving properties for centuries. These
28 compounds decrease the sensation of pain by binding opioid receptors found in the central and
29 peripheral nervous system.¹ Morphine, the most widely used opioid analgesic, has served as a
30 structural template for the design and synthesis of novel opioids. Unfortunately, morphine and its
31 clinically used analogs have deleterious side-effects, such as respiratory depression and a high
32 potential for addiction. Therefore, it is desirable to design a drug that is able to retain the pain-
33 relieving properties of morphine without causing dangerous side-effects.
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44 Three classes of opioid receptors have been cloned: μ (MOR), δ (DOR), and κ (KOR).
45 Subtypes of MOR, DOR, and KOR have also been proposed on the basis of a variety of
46 biochemical and pharmacological approaches.² Radiolabeled peptides have been useful in the
47 characterization of opioid receptors. Endogenous enkephalin analog probes with tritium such as
48 [d-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO) and [d-Pen²,d-Pen⁵]enkephalin (DPDPE) are
49 commercially available MOR and DOR-selective ligands, respectively. However, most tritiated
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3 probes have limitations for identifying high affinity binding sites with low expression levels,
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5 given the specific activity of 28.8 Ci/mmol of tritium.³ For example, Zhu et al. could not detect
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7 binding in brains of DOR knockout mice using [³H]DPDPE and [³H]deltorphan-II, despite
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9 findings that both of these compounds retain their analgesic effects when administered
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11 supraspinally to this mouse.⁴
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15 Radioiodinated compounds have numerous other clinically and scientifically relevant uses,
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17 which include tissue ablation, tumor imaging, autoradiography, and binding assays.⁵ High
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19 specific activity, such as that of ¹²⁵I or ¹³¹I, has major advantages when visualizing small receptor
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21 populations. This is illustrated by our recent studies with [¹²⁵I]iodobenzoylnaltrexamide
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23 ([¹²⁵I]IBNtxA). [¹²⁵I]IBNtxA binds with high affinity to the novel opioid target, 6TM/E11.⁶
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27 Since there are currently no iodinated small molecules or peptides commercially available to
28
29 target DOR, our primary goal was to synthesize a DOR-selective, analgesic opioid peptide
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31 bearing an iodine atom. The radioiodinated analog could be used for radioligand binding assays
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33 in rodent brain homogenates, whereas the non-radioactive counterpart could be used as a DOR
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35 analgesic model compound in rodents, thus creating a useful probe for DOR pharmacology.
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39 Secretions on the skin of the amphibian *Phyllomedusa bicolor*, contain opioid peptides such as
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41 deltorphins, which are highly selective for DOR and analgesic.⁷ In an attempt to synthesize a
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43 selective radioiodinated probe for DOR we decided to use the ^DAla²-deltorphan II amino acid
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45 sequence as a template. All peptides synthesized were characterized in radioligand binding
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47 assays in Chinese hamster ovary (CHO) cell lines stably transfected with opioid receptors. The
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49 non-radioactive iodinated deltorphan analog, Tyr-^DAla-(*p*-I)Phe-Glu-Ile-Ile-Gly-NH₂ **3** (GATE3-
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51 8) was further characterized in *in vitro* [³⁵S]GTPγS functional assays and *in vivo* tail flick
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53 analgesia assays. The radioiodinated analog, [¹²⁵I]-**3**, Tyr-^DAla-(*p*-¹²⁵I)Phe-Glu-Ile-Ile-Gly-NH₂
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3 (¹²⁵I-GATE3-8) was made by the introduction of an amino group at the 4' position of Phe³, **5** and
4 followed by a subsequent Sandmeyer reaction.⁸ This radioligand was further characterized in
5 saturation binding assays and competition assays confirming its selectivity for DOR.
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10 11 12 **Results and Discussion**

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16 Five analogs of _DAla²-deltorphin II were synthesized using Fmoc-based solid phase peptide
17 synthesis.⁹ The Val⁵-Val⁶ of [_DAla²]Deltorphin II (Table 1) was replaced with Ile⁵- Ile⁶ and *p*X-
18 Phe³ where X at 4' position of Phe varied from F, Cl, Br, I to NH₂ (Table 1 and Figure 2).
19 Valines at positions 5 and 6 were replaced with isoleucine in order to increase hydrophobicity.
20 Isoleucine is more branched than valine and increasing the hydrophobicity has been shown to
21 increase activity at the δ-receptor.¹⁰ Additionally the para hydrogen of phenylalanine was
22 substituted with various halogens. The atomic radius of each halogen increases with molecular
23 weight, resulting in an increase in size of the halogen substituent; F < Cl < Br < I. (Table 2).
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35 In radioligand binding assays versus [¹²⁵I]BNtxA in opioid transfected CHO membranes, all
36 analogs showed selective binding for DOR over MOR, KOR and 6TM/E11 sites. As the size of
37 the halogen substituent increased, the affinity of the peptide for DOR increased raising the
38 possibility of the peptide forming weak halogen bonds in the protein pocket. There was also no
39 significant difference between the affinity of **3** (K_i=0.76 nM) and **4** (K_i= 0.74 nM) compounds
40 (Table 3). **3** was further characterized in [³⁵S]GTPγS functional assays in DOR transfected CHO
41 cells. **3** was a full agonist, similar to the prototypic DOR agonist DPDPE, although it was about
42 18-fold less potent (ED₅₀=57nM) than DPDPE (EC₅₀=3nM) and 10-fold less potent than
43 deltorphin II (Figure 1a). In *in vivo* tail flick analgesia assays **3** was as potent (ED₅₀=0.3 ug) as
44 DPDPE (ED₅₀=0.4 ug) when given supraspinally (Figure 1b). The analgesia was also
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3 antagonized by the DOR selective opioid antagonist naltrindole (NTI), confirming that analgesia
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5 was exhibited through the activation of DOR (Figure 1c).
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8 Direct iodination of opioids (tyrosine in position 1 in this case) presents a problem since
9
10 incorporation of iodine usually leads to a loss of affinity. Thereby the precursor to [¹²⁵I]-**3**, **5**, was
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12 radioiodinated using a modified Sandmeyer reaction (Scheme 1). In saturation binding assays to
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14 CHO-DOR cell membranes, the K_D was 0.1 ± 0.02 nM and the B_{max} was 196.97 ± 12.44 fmol/mg
15
16 (**Figure 2**). We performed competitions assays to validate the site as DOR pharmacologically.
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18 The prototypic DOR agonists DPDPE ($K_i = 4.5 \pm 0.6$ nM) and SNC80 ($K_i = 9.8 \pm 0.7$ nM) bound the
19
20 site with high affinity while DOR antagonists such as naltrindole ($K_i = 0.79 \pm 0.16$ nM) and
21
22 naltriben ($K_i = 0.14 \pm 0.04$ nM) also displayed high affinities consistent with literature values
23
24 (Table 4). We performed competitions assays to validate the site as DOR pharmacologically. The
25
26 prototypic DOR agonists DPDPE ($K_i = 4.5 \pm 0.6$ nM) and SNC80 ($K_i = 9.8 \pm 0.7$ nM) bound the site
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28 with high affinity while DOR antagonists such as naltrindole ($K_i = 0.79 \pm 0.16$ nM) and naltriben
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30 ($K_i = 0.14 \pm 0.04$ nM) also displayed high affinities consistent with literature values (**Table 4**).
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39 **Conclusions**

40 We synthesized halogenated analogs of [_DAla²] Deltorphin II, and all analogs maintained
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42 selectivity for DOR over MOR and KOR. As the size of the halogen substituent increased, the
43
44 affinity of the peptide for the delta opioid receptor increased. Tyr-_DAla-(*p*-I)Phe-Glu-Ile-Ile-Gly-
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46 NH₂ **3**, a [_DAla²] Deltorphin II analog, was found to be highly DOR selective in *in vitro*
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48 radioligand binding assays and was a full agonist in [³⁵S]GTPγS functional assays. **3** was
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50 analgesic in mice when given supraspinally. Analgesia was blocked by the selective DOR
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52 antagonist naltrindole, indicating that the analgesia is mediated by DOR. The peptide **5** was
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3 readily radioiodinated using a modified Sandmeyer reaction and bound DOR with high affinity.
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5 Competition assays confirmed the site is DOR, as prototypical DOR agonists and antagonists
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7 competed [¹²⁵I]-3 (¹²⁵I-GATE 3-8) with affinities consistent with literature values. Together, our
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9 findings suggest the compound may be useful to elucidate the molecular mechanisms of DOR-
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11 mediated analgesia in the brain using radioimaging and *in vivo* behavioral assays.
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15 **Experimental**

16 **Chemistry:**

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18 **Peptide Synthesis:** Peptide synthesis was performed according to standard Fmoc methodology¹¹
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20 using a Discover SPS microwave system and Accent cleavage system (CEM Corporation,
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22 Matthews, NC). Fmoc-Gly-CLEAR-Amide Resin was purchased from Peptides International
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24 (Louisville, KY). The solvents *N,N*-dimethylformamide (DMF), dichloromethane (DCM),
25
26 acetonitrile, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Fair Lawn,
27
28 NJ). The reagents diisopropylcarbodiimide (DIC), phenol and piperidine were purchased from
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30 Sigma-Aldrich (St. Louis, MO) and Oxyma pure was purchased from Advanced ChemTech
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32 (Louisville, KY). Amino acids Fmoc-D-Ala-OH·H₂O, Fmoc-Glu(OtBu)-OH·H₂O, and Fmoc-Ile-
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34 OH were purchased from Peptides International (Louisville, KY). The amino acids Fmoc-
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36 Tyr(tBu)-OH, and Fmoc-Phe(4-Cl)-OH were purchased from Novabiochem (Billerica, MA). The
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38 amino acid Fmoc-Phe(4-F)-OH was purchased from Advanced ChemTech (Louisville, KY).
39
40 Amino acids Fmoc-(4-Br)-Phe-OH and Fmoc-(4-NHBoc)-Phe-OH were purchased from Chem-
41
42 Impex (Wood Dale, IL). The amino acid Fmoc-(4-I)-Phe-OH was purchased from Anaspec
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44 (Fremont, CA). All reagents were ACS grade or better and used without further purification.
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53 Synthesis was performed on solid support in a Discover SPS microwave synthesizer.
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55 Approximately 250mg (0.1mmol) of Fmoc-Gly-CLEAR-Amide Resin was placed in a 50mL
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3 fritted plastic reaction vessel and the resin was swelled in 50% DMF and 50% DCM for one
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5 hour. The initial deprotection was performed with about 6mL of 20% piperidine in DMF for 30
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7 seconds at 70°C followed by 30 seconds at 75°C. The resin was washed with DMF and a Kaiser
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9 test performed to confirm the presence of a primary amine. A 3-fold excess of the first amino
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11 acid was dissolved in DMF along with a 5-fold excess of Oxyma pure. A 5-fold excess of DIC
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13 was added to the amino acid mixture and then the entire volume was added to the resin. Coupling
14
15 was added to the amino acid mixture and then the entire volume was added to the resin. Coupling
16
17 was performed for five minutes at 75°C. Another Kaiser test was then performed to confirm
18
19 completion of the coupling reaction. The deprotection and coupling cycle was repeated as
20
21 necessary until all amino acids were added to the growing peptide chain. After addition of the
22
23 last amino acid, the deprotection was repeated one last time and the resin was washed with
24
25 DCM. Cleavage of the peptide was performed using the Discover SPS microwave in addition to
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27 the Accent cleavage system. Approximately 6mL of cleavage cocktail (90% TFA/5% water/5%
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29 phenol) was added to the dried resin and cleavage was performed for 30 minutes at 38°C.
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31 Cleaved peptide was collected by filtration and ether precipitation followed by centrifugation for
32
33 30 minutes at 4°C. The peptide was dissolved in water and lyophilized to a powder. HPLC
34
35 analysis revealed crude peptide purity as greater than 95% pure as demonstrated by area under
36
37 the curve. Peptides were analyzed for purity and molecular weight by LC-MS. Purity was greater
38
39 than 95% for all peptides with an M+1 mass spectral analysis within equipment error
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47 **Iodination:** **5** was iodinated using a modified Sandmeyer reaction.^{8, 12} 18-Crown-6 (303mM)
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49 and tetrakis copper(I)tetrafluoroborate (254 nM) were dissolved in 40 uL water and incubated
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51 with 4mCi Na¹²⁵I on ice. A second reaction vial containing 8 mM **5** was incubated with 2 N
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53 sulfuric acid on ice. 50mM sodium nitrite was added and incubated on ice for 5 minutes. The
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3 reaction was quenched with 290 mM sulfamic acid. The reactions were combined and placed on
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5 ice. Following a 3 hour incubation, the reaction was centrifuged and the supernatant was injected
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7 onto a reverse-phase HPLC C18 column (Thermo Scientific, 150x4.6mm, 5 μ m). The gradient
8
9 started at 5% acetonitrile containing 0.1% TFA (1mL/min). During the first 5 min, the gradient
10
11 was increased to 30%, held at 30% for 5 min, increased to 50% during the following 10 min, and
12
13 finally increased to 95% during the final 5 min. The desired product eluted at 15 min and was
14
15 obtained with an unoptimized radiochemical yield of 1.3%. The product was confirmed by
16
17 running **3** as a standard (S1). The yields obtained were lower than expected; we have used the
18
19 Sandmeyer reaction on a different peptide alpha-neoendorphin with yields around ~29%⁸.
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21 Efforts are underway to improve the radiochemical yields so this protocol can be used on a
22
23 variety of peptides.
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29 **Pharmacology:**

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31 **Receptor-Binding Assays.** Competition-binding assays in CHO cells stably expressing MOR,
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33 DOR, or KOR were performed at 25 °C in potassium phosphate buffer (50 mM; pH 7.4), with
34
35 the inclusion of MgSO₄ (5 mM) in the MOR assays. Competition assays were carried out using
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37 [¹²⁵I]BNtxA^{6a} as described. Specific binding was defined as the difference between total binding
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39 and nonspecific binding, determined in the presence of levallorphan (8 μ M). Protein
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41 concentration was determined as described by Lowry et al., using bovine serum albumin as the
42
43 standard. Protein concentrations of MOR-CHO, DOR-CHO, and KOR-CHO membranes were
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45 between 20 and 90 μ g/mL, and incubation times were 90 min. [¹²⁵I]**3** (¹²⁵I-GATE 3-8) assays
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47 (0.25mL; 0.045 mg protein) were performed in the presence of a protease inhibitor containing 2
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49 ug/ml each leupeptin, pepstatin, aprotinin, and bestatin and 0.2mM PMSF.
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3 6TM/E11 competition binding assays^{6b} using [¹²⁵I]BNtxA (1; 0.15 nM) were carried out in
4 whole brain membrane homogenates (0.5 mL; 0.5 mg protein) at 25°C in potassium phosphate
5 buffer (50 mM; pH 7.4) with magnesium sulfate (5 mM) for 90 min in the presence of CTAP,
6 U50488H, and DPDPE, all at 200 nM, to block traditional opioid binding sites.
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11 **Tail Flick Analgesia Assays.** Male CD-1 mice (25–35 g; Charles River Breeding Laboratories,
12 Wilmington, MA) were maintained on a 12 h light/dark cycle with Purina rodent chow and water
13 available ad libitum. Mice were housed in groups of five until testing. All animal experiments
14 were reviewed and approved by the Institutional Animal Care and Use Committee of Memorial
15 Sloan-Kettering Cancer Center. Analgesia was determined using the radiant heat tail flick
16 technique¹³ using a machine (Ugo Basile; model 37360). The intensity was set to achieve a
17 baseline between 2 and 3 s. The latency to withdraw the tail from a focused light stimulus was
18 measured electronically using a photocell. Baseline latencies (2.0–3.0 s) were determined before
19 experimental treatments for all animals. Post-treatment tail flick latencies were determined as
20 indicated for each experiment, and a maximal latency of 10 s for tail flick was used to minimize
21 tissue damage. Naltrindole was given subcutaneously and cumulative dose–response
22 experiments carried out with two independent assays with each group (n = 10). **3** (GATE3-8)
23 was delivered intracerebroventricularly (i.c.v.) as previously described.¹⁴ Briefly, the mice were
24 anesthetized by isoflurane. A small incision was made, and **3** (2 ul/mouse) was injected using a
25 10 uL Hamilton syringe fitted to a 27 gauge needle. Injections were made into the right lateral
26 ventricle at the following coordinates: 2 mm caudal to bregma, 2 mm lateral to sagittal suture,
27 and 2 mm in depth. Mice were tested for analgesia 15 minutes post injection. The combined
28 results are presented as the ED₅₀ with 95% confidence limits (n = 20). Analgesia was defined
29 quantally as a doubling, or greater, of the baseline latency. Similar results were obtained
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3 analyzing the data in a graded response manner. Analgesic ED₅₀s and confidence limits were
4 determined using nonlinear regression analysis Graph Pad Prism (Graphpad Software, La Jolla,
5 CA).
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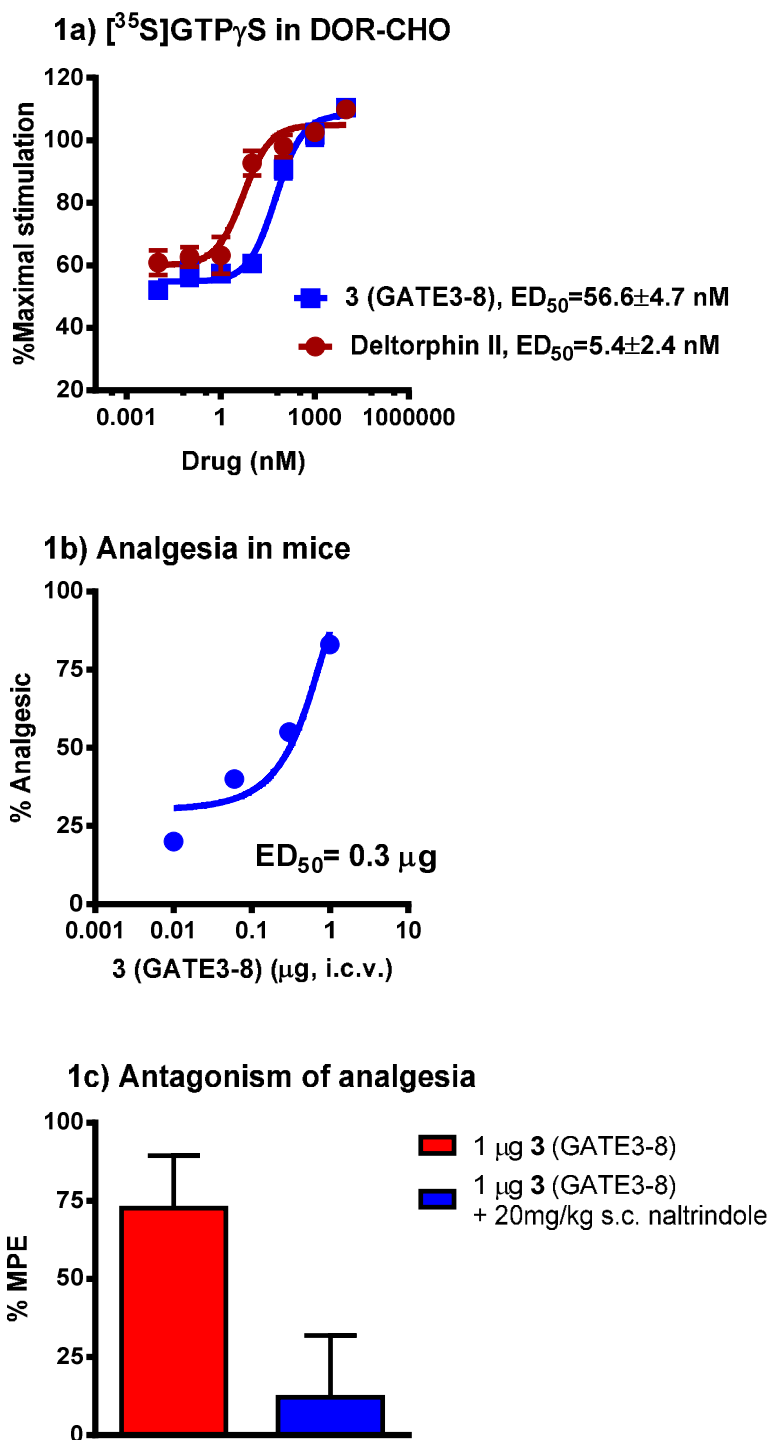
10 **[³⁵S]GTPS-Binding Assay:** [³⁵S]GTPS binding was performed on membranes (60 ug; 1 ml)
11 prepared from transfected cells in the presence and absence of the indicated opioid for 60 min at
12 30°C in the assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.2 mM EGTA, and 10 mM
13 NaCl) containing 0.05nM [³⁵S]GTPS; 2 μg/ml each leupeptin, pepstatin, aprotinin, and bestatin;
14 and 30μMGDP, as previously reported.¹⁵ After the incubation, the reaction was filtered through
15 glass-fiber filters (Whatman Schleicher & Schuell, Keene, NH) and washed three times with 3
16 ml of ice-cold 50 mM Tris-HCl, pH 7.4, on a semiautomatic cell harvester. Filters were
17 transferred into vials with 5 ml of Lquiscent (National Diagnostics, Atlanta, GA), and the
18 radioactivity in vials was determined by scintillation spectroscopy in a Tri-Carb 2900TR counter
19 (PerkinElmer Life and Analytical Sciences). Basal binding was determined in the presence of
20 GDP and the absence of drug.
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Scheme 1. Radioiodination of **5** to ^{125}I -3 (^{125}I -GATE3-8)Tyr-DAla-(pNH₂)Phe-Glu-Ile-Ile-Gly-NH₂**5**

a),b)

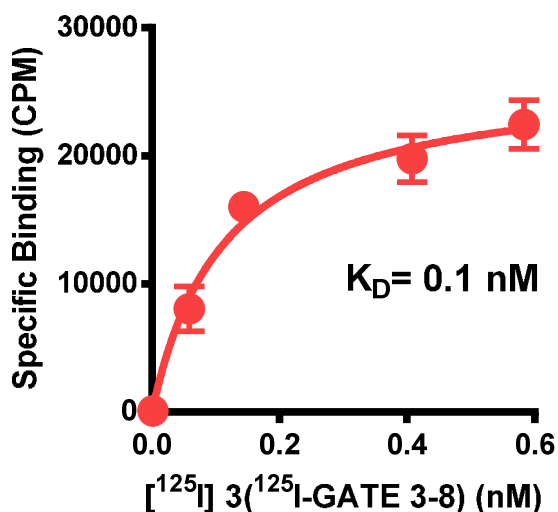
Tyr-DAla-(p¹²⁵I)Phe-Glu-Ile-Ile-Gly-NH₂ ^{125}I -3 (^{125}I -GATE3-8)

- a) NaNO₂, 2N H₂SO₄, H₃NSO₃, 0°C, 5 min b) 18-Crown-6, H₂O, Na¹²⁵I, Cu(I)(CH₃CN)₄BF₄, 3h

Figure 1: Pharmacology of (Tyr-DAla-(pI)Phe-Glu-Ile-Ile-Gly-NH₂) 3 (GATE3-8)

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3 a) **GTP γ S stimulation:** Efficacy data were obtained using agonist induced stimulation of
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5 [35S]GTP γ S binding assay. Efficacy is represented as EC₅₀ (nM) and percent maximal
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7 stimulation relative to standard agonist DPDPE (DOR) at 100nM. All values are
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9 expressed as the mean \pm SEM of three separate assays performed in triplicate.
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12 b) **Analgesia:** Groups of mice (n=10) received **3** (i.c.v.) at the indicated doses and were
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14 tested 15 min later at peak effect to generate the analgesic dose-response curve. ED₅₀
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16 values (and 95% confidence limits) were 0.3 μ g (0.1, 0.47) in CD1 mice by using the
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18 radiant heat tail-flick assay.
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22 c) **Sensitivity of 3 to opioid antagonists:** Groups of mice (n=10) received a fixed dose of
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24 **3**(GATE 3-8, 1 ug, i.c.v.) alone or NTI (20 mg/kg, s.c.) given 15 min before **3** . Tail flick
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26 analgesia was measured 15 min after **3** . Similar results were observed in two
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28 independent replications. Analgesia of **3** was antagonized by NTI (ANOVA followed by
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30 Bonferroni multiple comparison test (p < 0.05)).
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Figure 2. Saturation curve of [¹²⁵I]-3 (¹²⁵I-GATE3-8) in DOR-CHO membranes



[¹²⁵I]-3 saturation studies were carried out on DOR-CHO membranes. Results are from a representative experiment, and only specific binding is reported. Ratios of total to nonspecific binding were 2.5:1. Experiments were replicated at least three times. K_D and B_{max} were determined by nonlinear regression analysis, and the means \pm SEM of the replicates were determined. The K_D value was best fit with a single site, $K_D = 0.11 \pm 0.02$ nM, $B_{max} = 196.97 \pm 12.44$ fmol/mg.

Table 1. Sequences of peptides synthesized

Peptide Name	Amino Acid Sequence
[^D Ala ²]Deltorphin II	Tyr- ^D Ala-Phe-Glu-Val-Val-Gly-NH ₂
1	Tyr- ^D Ala-(pF)Phe-Glu-Ile-Ile-Gly-NH ₂
2	Tyr- ^D Ala-(pCl)Phe-Glu-Ile-Ile-Gly-NH ₂
3	Tyr- ^D Ala-(pI)Phe-Glu-Ile-Ile-Gly-NH ₂
4	Tyr- ^D Ala-(pBr)Phe-Glu-Ile-Ile-Gly-NH ₂
5	Tyr- ^D Ala-(pNH ₂)Phe-Glu-Ile-Ile-Gly-NH ₂

Table 2. HPLC data of Peptides Synthesized; Tyr-DAla-(pX)Phe-Glu-Ile-Ile-Gly-NH₂.

Peptide	(pX)Phe	HPLC k' (Solvent 1)	HPLC k' (Solvent 2)	M+1 (Calculated)	Mass Spectral Analysis (M+1)	Purity
1	(pF)Phe	5.20	13.64	828.94	829.93	>98%
2	(pCl)Phe	5.72	13.86	845.39	844.94	>95%
3	(pI)Phe	6.65	14.73	936.85	936.36	>95%
4	(pBr)Phe	5.87	14.24	889.85	890.10	>98%
5	(pNH ₂)Phe	3.35	7.99	825.96	825.68	>98%

HPLC k' = [peptide retention time - solvent retention time] / solvent retention time in Solvent 1 (10-90% acetonitrile in 0.01% TFA/water over 15 minutes) or Solvent 2 (10-90% methanol in 0.01% TFA/water over 15 minutes) with a flow rate of 1.5 mL/min. HPLC analysis was performed using a Perkin Elmer HPLC System with a Phenomenex Kinetex C18 column (2.6 μ m, 4.6x100mm) at a wavelength of 214nm. Mass spectral analysis took place at CEM Corporation (Matthews, NC) using a ThermoScientific LCQ Advantage System with an Atlantis dC18 analytical column (2.1x150mm). Peptide purity was determined by comparing the area under the main peptide peak as compared to other peaks present.

Table 3. Competition assays against ¹²⁵IBNtxA, Tyr-DAla-(pX)Phe-Glu-Ile-Ile-Gly-NH₂.

Peptide	(pX)Phe	MOR (nM)	DOR (nM)	KOR (nM)	6TM/E11 (nM)
(DAla-2)DeltorphinII		>1000	0.34±0.1	>1000	>1000
1	(pF)Phe	>1000	6±2.47	>1000	>1000
2	(pCl)Phe	>1000	2.44±0.08	>1000	>1000
3	(pI)Phe	>1000	0.76±0.45	>1000	>1000
4	(pBr)Phe	>1000	0.74±0.36	>1000	>1000
5	(pNH ₂)Phe	>1000	26±4.16	>1000	>1000

Competition studies were performed with the indicated compounds against [¹²⁵I]BNtxA (0.1 nM) in membranes from CHO cells stably expressing the indicated cloned opioid receptor or in mouse brain membranes for 6TM/E11 sites with blockers to prevent binding to traditional mu,

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kappa₁ and delta receptors as described in the methods section. K_i values were calculated from the IC₅₀ values and represent the means ± SEM of at least three independent replications. K_D's used for calculating K_i's were 0.11, 0.03, 0.24 and 0.16 nM for MOR-1, KOR-1, DOR-1 and 6TM/E11 respectively.

Table 4. Competition assays against [¹²⁵I]-**3** (¹²⁵I-GATE3-8) in DOR-CHO.

Compound	DOR-CHO K _i (nM)
DPDPE	4.5±0.6
SNC80	9.8±0.7
Naltrindole	0.79±0.16
Naltriben	0.14±0.04

Competition studies were performed with the indicated compounds against [¹²⁵I]**3** (0.2 nM) in membranes from CHO cells stably expressing DOR. K_i values were calculated from the IC₅₀ values¹⁶ and represent the means ± SEM of at least three independent replications. The K_D used for calculating K_i's was 0.1nM.

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Author Contributions

+ RP and GFM contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Supporting Information

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ABBREVIATIONS

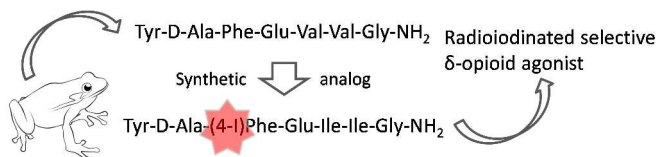
6TM/E11, six transmembrane exon 11; MOR, mu opioid receptor; KOR, kappa₁ opioid receptor; DOR, delta opioid receptor; IBNtxA, 3'-iodobenzoylnaltrexamide; s.c., subcutaneous; CHO, Chinese hamster ovary cells; DPDPE, [D-Pen²,D-Pen⁵]Enkephalin; K₂CO₃, potassium carbonate; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; TFA, trifluoroacetic acid; Tyr, tyrosine; _DAla, D-Alanine; Phe, phenylalanine; (pF)Phe, para-fluoro-phenylalanine; (pCl)Phe, para-chloro-phenylalanine; (pBr)Phe, para-bromo-phenylalanine; (pI)Phe, para-iodo-phenylalanine; (pNH₂)Phe, para-amino-phenylalanine; Glu, glutamic acid; Ile, isoleucine; Gly, glycine; HPLC, high pressure liquid chromatography; diisopropylcarbodiimide (DIC)

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TOC Graphic:

Synthesis and pharmacology of halogenated δ -opioid selective

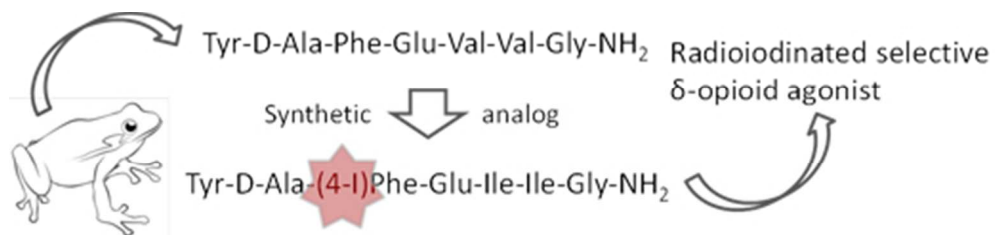
[DAla²] Deltorphan II peptide analogs

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