Effect of anchoring 4-anilidopiperidines to opioid peptides

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ABSTRACT

We report here the design, synthesis, and in vitro characterization of new opioid peptides featuring a 4-anilidopiperidine moiety. Despite the fact that the chemical structures of fentanyl surrogates have been found suboptimal per se for the opioid activity, the corresponding conjugates with opioid peptides displayed potent opioid activity. These studies shed an instructive light on the strategies and potential therapeutic values of anchoring the 4-anilidopiperidine scaffold to different classes of opioid peptides.

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The increasing need for effective pain management prompts the invention of new strategies and pharmacological tools. Among the three opioid receptor types (μ, δ, and κ, the μ-opioid receptor is considered to be essential for efficient pain suppression. However, μ-opioids do not provide adequate treatment of chronic pain since their long-term use results in multiple side effects (e.g.,[1]). Close examination of the opioid system conferred that a μ–δ opioid receptor heterodimer represents the fundamental signaling unit responsible for opioid tolerance and dependence.[2,3] During the last two decades, our laboratory has carried out extensive research on the synthesis of novel opioid analogs. Within the frame of our works on bivalent opioid ligands there seemed a gap between small-molecule and peptide-based opioids. We have sought to bridge this gap by incorporating the 4-ANDP[4] scaffolds into opioid peptides, and also to optimize their physicochemical properties.[5–7] Previously, research efforts to produce bivalent opioid peptides
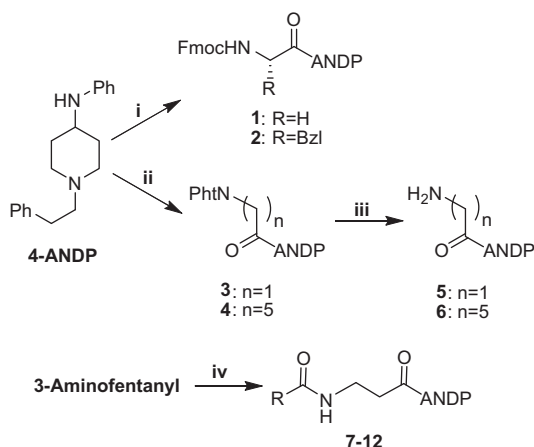
resulted in analogs with exceptional properties. One representative example is biphalin.[8] Despite numerous advantages such as low toxicity, high activity and specificity, opioid peptides are still not being used as pain-relieving agents in general anesthetic practice because of main drawbacks such as poor bioavailability after systemic (subcutaneous or oral) administration, limited ability to cross the blood–brain barrier, and rapid degradation in vivo by peptidases. Although the fact that fentanyl[9–12] is a bioavailable drug does not directly suggest that its addition to a peptide structure will yield a bioavailable hybrid, we hypothesized that its incorporation into opioid peptides may positively impact overall bioavailability of the latter.

A careful analysis of the literature revealed that in the series of fentanyl analogs there was no general consensus regarding the effect of substitution in the propionyl part of the molecule. We thus set out to prepare the desired compound by using Fmoc- and Pht-protected amino acid chlorides (Scheme 1). This allowed us to synthesize 3-aminofentanyl and analogs **1–6**. The synthesis, NMR characterization and binding affinity at the μ and δ opioid receptors of compounds **7–12** have been reported previously by us,[5] but no results of GPI and MVD assays were provided for this series. In this report, we provide additional biological data for compounds **7–12**. The effects of having the amide substituents in the propionyl moiety are summarized in Table 1. It is evident from the results presented in Table 1 that all of these analogs displayed low affinity at the μ and δ opioid receptors and low activity in MVD and GPI assays. For the major part, the chemical nature of these fentanyl

Scheme 1. Attachment of Fmoc and phthaloyl amino acids to 4-anilino-1-phenethyl-piperidine. Reagents and conditions: (i) Fmoc-AA-Cl (AA: Gly, Phe), DCM/10% NaHCO$_3$ in water, 0 °C (83% and 49%, respectively); (ii) Pht-Gly-Cl, TEA or DIPEA, DCM, 0 °C; (iii) N$_2$H$_4$·nH$_2$O, ethanol, reflux; (iv) see Ref. 5, R: Me (**7**), CF$_3$ (**8**), Et (**9**), Ph (**10**), –NHEt (**11**), –CH(Bzl)(NHCOCH$_3$) (**12**).

Table 1
Bioactivities of the 4-anilidopiperidine analogs

No.	hDOR[a] [³H]DPDPE[b] K_i[e] (μM)	rMOR[a] [³H]DAMGO[c] K_i[e] (μM)	IC$_{50}$[d] (μM) MVD (δ)	GPI (μ)
1[f]	1.0	1.0	n.d.	n.d.
2[f]	No response	3.1	n.d.	n.d.
3	13	2.1	26.4%	11%
3[f]	36	3.2	14.2%	5.9 ± 0.89[g]
7	8.4	1.0	7.5%	5.2 ± 0.21[h]
8	12	0.15	4.1%	3.0 ± 0.89[h]
9	0.45	0.45	4.1%	1.4 ± 0.48[h]
10	4.6	7.6	2.9%	3.9 ± 0.22
11	6.6	0.030	26.1%	1.3 ± 0.3
12	7.6	2.7	6.2%	5.6 ± 0.56[h]
Fentanyl	0.25	0.0033	0.0094	0.0034

n.d. not determined.
[a] Competition analyses were carried out using membrane preparations from transfected HN9.10 cells that constitutively expressed the respective receptor types.
[b] K_d = 0.50 ± 0.1 nM.
[c] K_d = 0.85 ± 0.2 nM.
[d] Concentration at 50% inhibition of muscle concentration at electrically stimulated isolated tissues; these values represent the mean of four tissues within 95% confidence limit.
[e] Competition against radiolabeled ligand, data collected from at least 2 independent experiments.
[f] HCl salt.
[g] Naloxone sensitive.
[h] Naloxone insensitive.

surrogates may not be optimal for the opioid activity in terms of ionic and hydrophobic interactions.

Although the opioid activities of the fentanyl surrogates **7–12** have turned out to be suboptimal for our original goal, there were several reasons to further investigate incorporation of these motifs into opioid peptides. A major reason for the continued interest in this direction is the fact that even a subtle structural and conformational change of an opioid ligand can greatly influence its overall activity profile, physicochemical properties, and biological efficacy.[13] Previously, we showed the synthesis and in vitro data for the peptide analog **13**, which was based on a part of fentanyl structure and 'one arm' (the core opioid sequence, H-Tyr-D-Ala-Gly-Phe) of biphalin.[5] In view of the promising bioactivity data of **13**, we then considered replacements for β-alanine for the next

series of analogs (Scheme 2, block A). Although the desired linear isomers could be obtained in a convenient fashion by the standard step-by-step solution phase peptide synthesis, it was expected to be more expedient and efficient to prepare such analogs by applying solid-phase chemistry. Our attempts to employ FMPB-AM resin for the synthesis of novel analogs using the reductive amination technique[14] (Scheme 2, block B) resulted in limited success—although the desired peptide **18** was obtained in the purity exceeding 96%, the yield of the target peptide was rather poor (10%). Our next alternative methods for making novel peptide conjugates by the solid phase methods included the use of N- and C-side chain substitutions. N-side chain modification of a Lys residue was performed on the resin after assembly of the first amino acid by removal of the Fmoc or Alloc group followed by treatment with *p*-nitrochloroformate and then with 3-aminofentanyl (Scheme 2, block C). This series also included analogs in which 3-aminofentanyl is linked to a dynorphin A structure.[15–17] Furthermore, the Tyr and Phe residues in positions 1 and 4 were replaced by a Dmt and Phe(4-Cl), respectively, in **21** to facilitate opioid receptor recognition and enhance metabolic stability. C-side chain modification of the Glu residue was performed using a combination of N^α-Fmoc and N^α-Boc strategies (Scheme 2, block D).

The target compounds were tested for their affinity and potency in vitro (Table 2). Concerning the functionality of these ligands, no opioid antagonist activity was observed in the MVD or GPI assays. Compared to biphalin, analogs **13–18** represent peptide ligands with higher $c\log P$ values (about two- to fourfold) due to the hydrophobic character of the fentanyl moiety. As shown in Table 2, the binding assays reveal that these compounds possess μ receptor selectivity with binding affinities ranging from 0.09 nM for **14** to 260 nM for **16**. In the functional assays, the selectivity of **17** was enhanced almost threefold for the δ receptor over the μ receptor as compared to biphalin and the previously synthesized **13**. However, in this series, despite a slight increase in the μ receptor selectivity and binding affinity, and the overall lipophilicity, there was a moderate loss of μ bioactivity in the GPI functional assays. In contrast, 'branched' analogs **23** and **24**, in which the 4-ANDP fragment is attached to the side chain of the Glu residue, showed potency comparable to that observed with biphalin. These observations suggested that the branched peptide ligands could be better accommodated in the opioid binding domain as opposed to the case of the linear core analogs.

In the endomorphin series, peptides **26** and **27** were very different in selectivity and potency from the parent opioid peptide, EM-1.[18] Although **26** and **27** gained moderate binding affinity of 64 and 130 nM, respectively, at the δ opioid receptor, both analogs showed a 14- to 28-fold reduction of the affinity for the μ receptor and concomitant reduction in μ/δ potency. Interestingly, the piperidinyl analog **26**, which has a longer spacer between the tetrapeptide and the 4-ANDP moiety, was slightly more potent than the analogous **27**. Because in both cases the anchoring strategy had an adverse affect on affinity and potency, it seems that the 4-ANDP structure disrupts key interactions within the binding site of endomorphins.

Introduction of the 4-ANDP scaffold into the deltorphin peptide sequence[19,20] (ligands **28** and **29**) resulted in significant changes in binding affinities at the μ and δ opioid receptors. The incorporation of the 4-ANDP through the side chain of the Glu acid residue at a distance from the opioid message sequence afforded compound **28** with a subnanomolar binding affinity (K_i = 0.28 nM) for δ opioid receptors. As shown in the functional assays, **28** is potent at both δ and μ receptors with IC$_{50}$ = 6.7 ± 3.9 and 49 ± 16 nM, respectively. In sharp contrast, the deltorphin analog **29** with the 4-ANDP closely adjacent to the opioid message region showed a reversed selectivity compared to deltorphin II and **28**. In the binding assay, ligand **29** displayed a 16-fold higher selectivity for μ over the δ
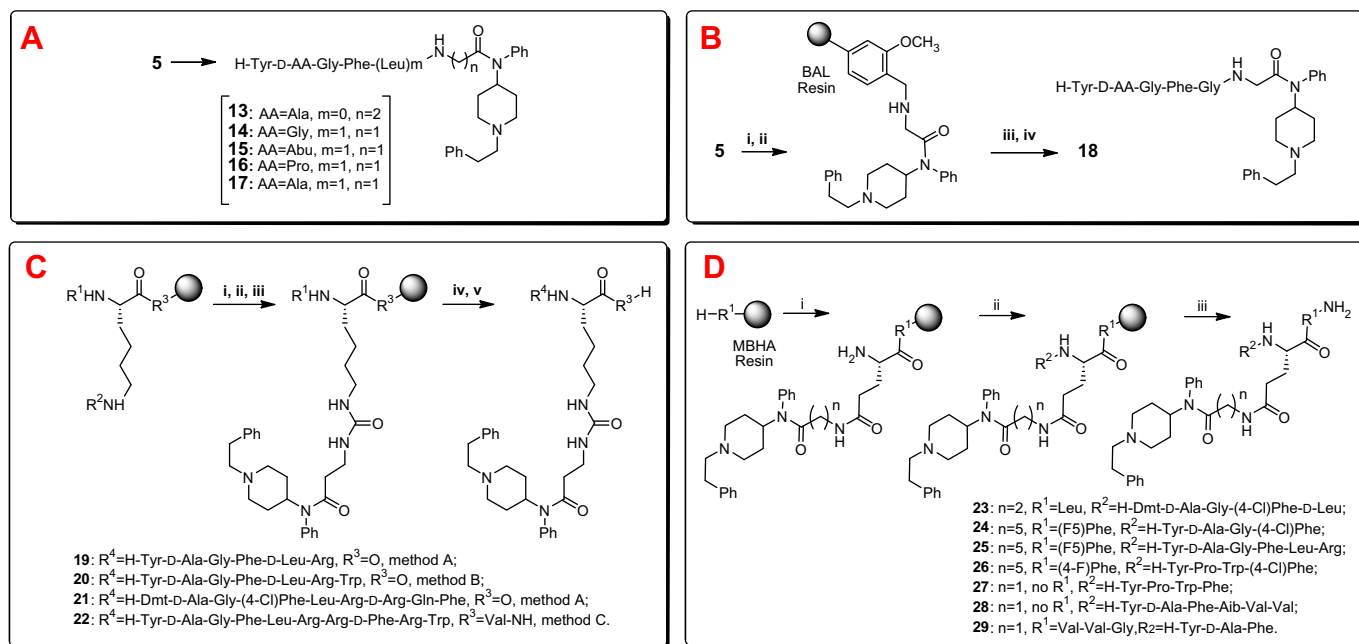
Scheme 2. Our strategies to introduce 4-anilidopiperidine functionality to opioid ligands. Reagents and conditions: (A) stepwise solution phase peptide synthesis, analogs **13–17**; (B) reductive alkylation on solid support using (i) 4-(4-formyl-3-methoxy-phenoxy)butyryl AM (BAL) resin, NaBH(OAc)$_3$, DMF/DCM (1/3), (ii) Fmoc-AA-OH, HOBt, HBTU, DIPEA in DMF, (iii) 50% Piperidine in DMF, and (iv) TFA/i-PrSiH$_3$/H$_2$O (90/5/5); (C) N-side chain modification by *method A*: Merrifield resin (R^3=O, R^1 = Boc, R^2 = Fmoc), (i) 50% Piperidine in DMF, (ii) p-nitrochloroformate, DIPEA, DCM, 1 h, (iii) 2–3 equiv of 3-aminofentanyl, microwave in a closed vessel for 1 h at 80 °C, (iv) 50% TFA in DCM, (v) SPPS followed by TFMSA cleavage; *method B*: 2-chlorotrityl resin (R^3=O, R^1 = Fmoc, R^2 = Alloc), (i) Pd(PPh$_3$)$_4$, PhSiH$_3$ in DCM, (ii) p-nitrochloroformate, DIPEA, DCM, 1 h, (iii) 2–3 equiv of 3-aminofentanyl, microwave in a closed vessel for 1 h at 80 °C, (iv) 50% piperidine in DMF, (v) SPPS followed by TFA cleavage; *method C*: MBHA resin (R^3 = Val-NH, R^1 = Fmoc, R^2 = Boc), (i) 50% TFA in DCM, (ii) p-nitrochloroformate, DIPEA, DCM, 1 h, (iii) 2–3 equiv of 3-aminofentanyl, microwave in a closed vessel for 1 h at 80 °C, (iv) 50% piperidine in DMF, (v) SPPS followed by TFMSA cleavage; (D) C-side chain modification using (i) Fmoc-Glu(Boc)-OH, HOBt, DIC, DMF → 50% TFA in DCM → 5% DIEA in DCM → amine (**1**, **5**, 3-aminofentanyl), HOBt, DIC, microwave in DMF for 5 min at 80 °C → 25% piperidine in DMF, (ii) solid phase peptide synthesis, and (iii) TFMSA cleavage.

opioid receptors. However, **29** was 18- and 39-fold less potent at δ-receptors and μ-receptors, respectively, than the related compound **28** in the functional assays. In line with the previous observations, these results indicate that in addition to endowing molecules with higher lipophilicity, incorporation of the 4-ANDP structure confers μ opioid receptor selectivity. However, in the case of deltorphins, a synergistic or potentiating antinociceptive effect of anchoring the 4-ANDP structure is questionable.

In the dynorphin A series, potent opioid agonist activities for both receptors were observed in several ligands with binding affinities in the nanomolar and subnanomolar ranges. In this category, the introduction of 4-ANDP structure increased the lipophilicity of the parent peptide. The binding affinity of **21** at the μ receptor was subnanomolar ($K_i = 0.15$ nM), and nanomolar at the δ receptor ($K_i = 1.8$ nM), which represents almost a 13-fold improvement in selectivity for μ-opioid receptors as compared to Dyn A-(1-13). In the functional assays, a reversed trend was observed: analog **21** was about twofold more potent than the parent peptide in the MVD (IC$_{50}$ = 3.2 ± 1.3 nM) assay, but threefold less potent in the GPI (IC$_{50}$ = 3.8 ± 0.8 nM) assay. In the [^{35}S]GTP-γ-S assays this compound had a EC$_{50}$ value of 0.34 at the μ, and 0.2 nM at the δ opioid receptors. How much of an improvement both in affinity and bioactivity was introduced by the 4-ANDP attachment alone in this case, however, is a question that remains to be answered. In terms of useful opioid design, this result is more likely to be attributed to the introduction of the Dmt residue, as observed in many cases. A similar trend in affinity was observed for analogs **19** and **22**. Analog **22** presents a case where a correlation in selectivity is maintained between binding affinities and bioactivities at the μ and δ opioid receptors. Similarly to **19**, ligand **22** showed a reasonable ninefold selectivity for the μ receptor ($K_i = 0.54$ nM) over the δ receptor ($K_i = 4.6$ nM) in binding assays, albeit

somewhat lower than that observed for **19** (31-fold selectivity for the μ receptor). Analog **22** was almost 13-fold more potent than the corresponding **19** in the GPI functional assay, although it also showed a moderate threefold loss of potency in the MVD assay. Ligands **20** and **25** were endowed with subnanomolar affinities of 0.26 and 0.63 nM, respectively, and selectivity (to twofold in both cases) for δ opioid receptors. Noticeably, most of the dynorphin A ligands displayed subnanomolar affinities at the μ opioid receptor. Moreover, compounds from this series also maintained good opioid potency with the 4-ANDP modification. This is in agreement with the earlier model for the dynorphin A binding pocket,[21] and is also consistent with previous results, which showed that introduction of lipophilic residues into dynorphin A structure resulted in more potent analogs.[22] It might be well to point out that although the high molecular weights and PSA values of the dynorphin A analogs are prohibitive of their CNS exposure, the presence of multiple positively charged residues in their structures suggests the possibility of their transport across biological membranes via the adsorptive-mediated endocytosis by binding to negatively charged sites on the surface of brain capillary endothelial cells.[23]

In conclusion, binding assays showed that incorporation of the 4-anilidopiperidine moiety strongly impacts both affinity and functional activity of the opioid peptides. We demonstrated that anchoring the 4-ANDP structure to short ekephalin-related or dynorphin A peptide structures represents a useful tactical approach for further enhancement of opioid potency. Attachment of the 4-ANDP moiety to the short ekephalin-related analogs appears to be a logical choice, considering the relatively small size, more lipophilic nature and high potency at both μ and δ opioid receptors. In case where the fentanyl moiety was conjugated to the dynorphin A, a superior opioid activity profile was reached to dynorphin A itself. The present findings suggest, however, that

Table 2
Bioactivities of the designed peptide analogs

No.	hDOR[a] [³H]DPDPE[b] K_i (nM)[f]	rMOR[a] [³H]DAMGO[c] K_i (nM)[f]	[³⁵S]GTP-γ-S binding				IC₅₀[e] (nM)	
			hDOR[d]		rMOR[d]			
			EC₅₀[g] (nM)	E_{max}[h] (%)	EC₅₀ (nM)[g]	E_{max} (%)[h]	MVD (δ)	GPI/LMMP (μ)
13[i]	1.1	0.90	60	140	60	140	33 ± 6	110 ± 50
13[j]	0.99	0.97	60	140	69	140	35 ± 6	42 ± 16
14	12	0.09	37	49	45	43	180 ± 20	950 ± 130
15	2.8	0.43	5.8	22	35	77	22 ± 5	97 ± 18
16	630	260	n.r.		n.r.		7500 ± 700	580 ± 150
17	27	13	5.9	64	0.59	13	9.5 ± 2.9	86 ± 16
18[k]	4.0	0.73	46	120	9.1	50	360 ± 170	150 ± 40
19	11	0.35	2.2	84	2.8	31	8.6 ± 1.7	98 ± 24
20	0.26	0.50	0.22	19	1.5	59	16 ± 4	120 ± 20
21	1.8	0.15	0.34	10	0.20	32	3.2 ± 1.3	3.8 ± 0.8
22	4.6	0.54	8.3	7	1.4	31	27 ± 8.1	7.7 ± 2.1
23	0.35	0.17	170	11	380	45	40 ± 5	12 ± 2
24	0.51	0.28	1.1	17	2.2	68	8.8 ± 3.0	67 ± 15
25	0.63	1.5	2.8	27	12	29	12 ± 2	130 ± 60
26	84	64	n.r.		56.	21	510 ± 130	480 ± 910
27	590	130	n.r.		n.r.		1000 ± 200	2100 ± 30
28	0.28	9000	1.7	59	n.r.[l]		6.7 ± 3.9	49 ± 16
29	24	1.5	90	33	220	21	120 ± 20	1900 ± 40
Biphalin	2.6 ± 0.4	1.4 ± 0.2	2.5 ± 0.5	27 ± 4	6.0 ± 0.2	25 ± 5	27 ± 2	8.8 ± 0.3
Endomorphin-1	5100 ± 660	4.6 ± 0.2					26 ± 3	11 ± 1
Deltorphin II	1.3	500 ± 40					0.37 ± 0.04	>3000
Dyn A-(1-13)-OH	3.8 ± 0.4	4.0 ± 0.4					6.3 ± 0.9	1.2 ± 0.1

n.r. no response.
[a] Competition analyses were carried out using membrane preparations from transfected HN9.10 cells that constitutively expressed the respective receptor types.
[b] K_d = 0.50 ± 0.1 nM.
[c] K_d = 0.85 ± 0.2 nM.
[d] Expressed from CHO cell.
[e] Concentration at 50% inhibition of muscle concentration at electrically stimulated isolated tissues; these values represent the mean of four tissues within 95% confidence limit.
[f] Competition against radiolabeled ligand, data collected from at least 2 independent experiments in duplicate.
[g] Anti-logarithmic value of the respective EC₅₀.
[h–g] Net total bound/basal binding × 100 ± SEM.
[i–h] Compound **13** is a hydrochloride salt of **13**.
[j–i] Effect is not reversed by naloxone.
[k–g] Tested in the free base form.
[l–k] Tested as a trifluoroacetate salt.

due to very weak opioid activity offered by the 4-ANDP conjugates, other similar sized, lipophilic adducts might be equally effective and easier to incorporate for the same purpose. Taken together, the results of these studies shed an instructive light on the development of mixed classes of opioid agonists as analgesic drug candidates.

Acknowledgment

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.065.

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