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Brief Article

Influence of the Side Chain Next to C-Terminal Benzimidazole in Opioid Pseudopeptides Containing the Dmt-Tic Pharmacophore

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To improve the structure−activity studies of the lead δ opioid agonist H-Dmt-Tic-Asp*-Bid, we synthesized and pharmacologically characterized a series of analogues in which the side chain next to 1*H*-benzimidazole-2-yl (Bid) was substituted by those endowed with different chemical properties. Interesting results were obtained: (1) only Gly, Ala, and Asp resulted in δ agonism, (2) Phe yielded δ antagonism, (3) and all other residues except Glu (devoid of any activity) gave μ agonism.

Introduction

The dipeptide Dmt-Tic[1] represents a versatile opioid pharmacophore able to induce a wide range of activities by interaction with μ and/or δ receptors.[2−5] δ Opioid receptor agonists are known to produce many pharmacological effects in rodents, including analgesia,[6] antidepressant,[7] neuroprotection/neurogenesis,[8] and anti-Parkinson[9] activities. On the basis of the potential utility of δ agonists in different pharmacological fields, here we report a structure−activity study related to the side chain adjacent to Bid in our lead δ agonist H-Dmt-Tic-Asp*-Bid. In preceding studies, we demonstrated that side chain of Asp can be substituted by the methyl side chain of Ala[5] or removed (Gly)[3] with the maintenance of δ agonism. Interestingly, the introduction of a protected or unprotected Lys side chain resulted in μ agonism without δ agonism.[4] To gain a better understanding of the importance of the side chain in the definition of the opioid activity in Dmt-Tic analogues, we synthesized a series of compounds in which the side chain next to the C-terminal Bid is derived from the principal natural amino acids. Further, on the basis of our previous results, the asymmetric carbon carrying the side chain seems to be unimportant for activity.[5,10]

Chemistry

All pseudopeptides (**9**−**18**) were prepared stepwise in solution using conventional synthetic methods as outlined in Scheme 1 (Supporting Information). Carboxylic functions of Boc protected amino acids were transformed into Bid according to the procedure of Nestor et al.[11] Briefly, mixed carbonic anhydride coupling of Boc protected amino acids with *o*-phenylendiamine gave the crude intermediate amides, which were converted without purification to the desired benzimidazole heterocycles (Bid) by cyclization/dehydration in acetic acid at 60 °C for 1 h. Each intermediate, after Boc deprotection with TFA, was condensed with Boc-Tic-OH via WSC/HOBt. Subsequently, Boc deprotection (TFA) and the final condensation (WSC/HOBt) with Boc-Dmt-OH gave the fully protected compounds. Final deprotections of side chains (if present) and N-terminal amine functions (by TFA treatment) gave the crude final compounds (**9**−**18**), which were purified by preparative reverse-phase HPLC.

Results and Discussion

Receptor Affinity Analysis. Receptor binding data for δ- and μ-receptors and δ-selectivity (K_i^{μ}/ K_i^{δ}) are reported in Table 1. All new compounds (**9**−**18**) had subnanomolar affinities for δ-opioid receptors (K_i^{δ} = 0.035−0.457 nM), which are in quite good accordance with the reference compounds containing the Dmt-Tic pharmacophore (**1**−**6**). As expected, the lack of a negative charge decreased δ-selectivity essentially by increasing μ-affinity (K_i^{μ} = 0.077−20.2 nM). In fact, compounds **3** and **18** containing Asp and Glu side chains exhibited δ-selectivity 122 and 36, respectively. All other analogues, except **10**, containing Bid at the C-terminal were essentially nonselective (K_i^{μ}/ K_i^{δ} or K_i^{δ}/ K_i^{μ} = 1.1−14). Compound **10**, in which Tyr substitutes for Dmt, exhibited a behavior in line with results obtained by Schiller et al. concerning the pharmacological characterization of H-Dmt-Tic-Phe-Phe-NH_2 and the corresponding Tyr analogue H-Tyr-Tic-Phe-Phe-NH_2; Tyr increases δ selectivity especially through the lowering of μ affinity.[12]

Functional Bioactivity. Compounds **9**−**18** were tested in the electrically stimulated MVD and GPI pharmacological assays for intrinsic functional bioactivity (Table 1). As is quite usually observed with opioids containing the Dmt-Tic pharmacophore, a close correlation between binding and functional bioactivity data is often lacking.[10] A similar lack of correlation was also observed by Hruby et al. in a series of 4-anilidopiperidine analogues.[13] On the other hand,

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Table 1. Receptor Binding Affinities and Functional Bioactivities of Compounds **1**−**18**

	H-Dmt-Tic-Xaa*-Bid						
		receptor affinity[b] (nM)		selectivity	functional bioactivity		
	Xaa*	K_i^δ	K_i^μ	K_i^μ/K_i^δ	MVD pA_2[d]	MVD IC_{50}[c] (nM)	GPI IC_{50}[c] (nM)
				Reference Compounds			
1	Gly*[e]	0.035	0.50	14		0.13	26.92
2	Ala*[f]	0.063	0.411	6.5		0.26	71.7
3	Asp*[f]	0.443	53.9	122		0.12	1724
4	Lys*[g]	0.49	0.16	3.1[L]	na		39.7
5	Lys(Z)*[g]	0.64	0.37	1.7[L]		9049	375
6	Lys(Ac)*[g]	0.18	0.13	1.4[L]	na		53.9
7	[Dmt^1]DALDA[h]	2100	0.143	14685[L]		23.1	1.41
8	E-2[i]	6085	1.33	4575[L]		344	6.9
				Compound			
9	Phe*	0.049 ± 0.008 (3)	0.25 ± 0.024 (5)	5.1	8.79		273 ± 48
10	[Tyr^1]Phe*	0.457 ± 0.074 (4)	20.2 ± 1.7 (5)	44	8.60		398 ± 19.3
11	Leu*	0.082 ± 0.007 (4)	0.12 ± 0.04 (4)	1.5	na		23.4 ± 1.5
12	Arg(NO_2)*	0.065 ± 0.005 (4)	0.095 ± 0.008 (5)	1.5	na		106 ± 36
13	Arg*	0.138 ± 0.047 (3)	0.13 ± 0.02 (5)	1.1[L]	na		10.2 ± 2.6
14	Asn*	0.051 ± 0.009 (3)	0.23 ± 0.02 (3)	4.5	na		8.5 ± 0.13
15	Trp*	0.135 ± 0.051 (3)	0.30 ± 0.03 (3)	2.2	na		35.4 ± 6.5
16	Ser(Ac)*	0.035 ± 0.004 (5)	0.077 ± 0.007 (5)	2.2	na		7.84 ± 0.32
17	Ser*	0.179 ± 0.003 (3)	0.13 ± 0.016 (5)	1.4[L]	na		34.9 ± 9.2
18	Glu*	0.04 ± 0.006 (3)	1.45 ± 0.39 (3)	36	na		494 ± 19.3

[b] The K_i values (nM) were determined according to Cheng and Prusoff.[31] The mean ± SE with *n* repetitions in parentheses is based on independent duplicate binding assays with five to eight peptide doses using several different synaptosomal preparations. [c] Agonist activity was expressed as IC_{50} obtained from dose−response curves. These values represent the mean ± SE for at least four tissue samples. DPDPE and PL017 were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparation, respectively. [d] The pA_2 values of opioid antagonists against the δ and μ agonists (deltorphin II and endomorphin-2, respectively) were determined by the method of Kosterlitz and Watt.[32] [e] Data taken from Lazarus et al.[3] [f] Data taken from Lazarus et al.[5] [g] Data taken from Balboni et al.[4] [h] Data taken from Schiller et al.[14] [i] Data taken from Zadina et al.[18] [L] μ-receptor selectivity K_i^δ/K_i^μ. na = no antagonism.

Schiller et al. observed the opposite behavior for the lead μ agonist [Dmt^1]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2) (**7**), which had very high selectivity in the receptor affinity (K_i^δ/K_i^μ = 14685) but only minor difference in functional bioactivity (GPI IC_{50} = 1.41 nM; MVD IC_{50} = 23.1 nM).[14,15] Starting from the first identified δ selective antagonist containing the Tyr-Tic pharmacophore H-Tyr-Tic-Phe-Phe-OH (TIPP),[16] and our initial findings on the correlation between the introduction of 1*H*-benzimidazol-2-yl (Bid) at the C-terminal of peptides containing the Dmt-Tic pharmacophore and the induction of δ agonism,[3,5] we synthesized compounds **9** and **10** containing Phe* next to Bid. As seen in Table 1, these derivatives exhibited δ antagonist activity (pA_2 = 8.79 and 8.60, respectively) despite the introduction of Bid at their C-terminal. Furthermore, the difference in receptor affinity and selectivity derived from the substitution of Tyr with Dmt, as noted above, is not confirmed by functional bioactivity; in fact, whereas **9** and **10** had comparable pA_2 values, H-Tyr-Tic-Phe-Phe-OH (pA_2 = 8.32) and H-Tyr-Tic-Phe-Phe-NH_2 (pA_2 = 7.74) appear less active than the corresponding analogue containing Dmt: H-Dmt-Tic-Phe-Phe-OH (pA_2 = 9.70)[17] and H-Dmt-Tic-Phe-Phe-NH_2 (pA_2 = 9.68).[12] Compounds (**1**,[3] **2**,[5] **4**,[4] **6**,[4] **11**−**17**) characterized by different chemical functions in their side chains, are all endowed with μ agonist activity with IC_{50} values ranging from 7.84 to 106 nM, whereas reference compounds (**1**−**3**) showed potent δ agonism and compounds (**9**, **10**) were δ antagonists, confirming the activity reported by Schiller et al.[16] The other reference substances (**4**−**6**) and new compounds (**11**−**18**) completely lacked any activity toward δ receptors. Quite unexpectedly, compound **18** containing the negatively charged side chain of Glu in place of Asp (**3**, our lead δ agonist),[5] did not exhibit δ agonism, but only a low degree of μ agonism. It seems that bulky side chains are not allowed for δ agonism and they are also detrimental for μ agonism (**4** and **6** vs **5**, **12** vs **13**). Finally, compound (**14**) H-Dmt-Tic-Asn*-Bid appears to be an interesting μ agonist, which is endowed with activity comparable to the endogenous opioid endomorphin-2 (**8**)[18] and only slightly less active than the lead μ agonist [Dmt^1]DALDA.[14] Its potential importance is related to the spontaneous deamidation reaction of Asn residues that can occur under physiological conditions;[19] in this case, the μ agonist (**14**) has the potential to be transformed into a potent and selective δ agonist H-Dmt-Tic-Asp*-Bid (**3**). As suggested by Kieffer et al., this behavior could be important in the regulation of δ-opioid receptor trafficking via μ-opioid receptor stimulation, which may be able to increase the potency of δ agonists with some potentially important clinical implications.[20,21]

Conclusions

On the basis of the importance which δ opioid agonists assume in different pharmacological fields,[22] we extended the correlation between the presence of the C-terminal 1*H*-benzimidazol-2yl chemical function and the induction of agonist activity in pseudopeptides containing the Dmt-Tic pharmacophore of general formula H-Dmt-Tic-(L)or(D)Xaa-OH/-NH_2. Previously, we demonstrated that these tripeptides are all endowed of δ antagonist activity (pA_2=7.60−10.07), except H-Dmt-Tic-Glu-NH_2, which resulted a δ agonist (IC_{50} = 2.5 nM).[23] The majority of these peptides were transformed at their C-terminal to the corresponding Bid analogues to yield a wide range of activities: only Gly (**1**), Ala (**2**), and Asp (**3**) gave δ agonism; Phe (**9**, **10**) yielded δ

antagonism, Glu (**18**) and Lys(Z) (**5**) were inactive, and all other aminoacids (**11**−**17**) exhibited μ agonist activity ,which in some cases (**13**, **14**, **16**) was comparable to the endogenous μ agonist endomorphin-2 (**8**). Surprisingly, all the new analogues (**11**−**18**) were devoid of any δ activity. Interestingly, the μ agonist H-Dmt-Tic-Asn*-Bid (IC_{50} = 8.5 nM) (**14**) in which Bid was formed from the α carboxylic function of the C-terminal Asn could represent a new tool in the opioid field. Asn and Gln side chains are known to undergo spontaneous nonenzymatic deamidation to form Asp and Glu residues under physiological conditions, with mechanisms including direct hydrolysis and/or succinimide-mediated deamidation, depending on pH of the environment.[19] Whether specific deamidases exist to further accelerate this process or not is presently unknown.[24] However, on the basis of this assumption, the μ agonist H-Dmt-Tic-Asn*-Bid could potentially be deamidated to form the potent and selective lead δ agonist H-Dmt-Tic-Asp*-Bid with some important pharmacological implications. For example, mixtures of selective μ and δ agonists have been found to produce additive or synergistic antinociceptive effects in both rodents and nonhuman primates without enhancing or attenuating most of each other's nonantinociceptive effects.[25] As described by Schiller, bi- or multifunctional drugs may have improved potency due to synergistic effects or may produce fewer side effects than compounds acting at a single target.[15] Bifunctional μ agonists/δ agonists and their usefulness were described by Ananthan[26] and more recently by Zhang et al.[27] Considering that opioid bivalent ligands reported herein were designed for the simultaneous interaction with both receptors, H-Dmt-Tic-Asn*-Bid and some other published opioids such as H-Dmt-Tic-Asp-Bid(N^1-Me),[10] RWJ-394674,[28] and *N*-desmethylclozapine[29] could represent a new class of "timed" bifunctional ligands able to interact at different times with different receptors. These may be suitable for example in trafficking of opioid receptors.[30]

Experimental Section

Chemistry. 2TFA·H-Dmt-Tic-Phe*-Bid (9). Boc-Dmt-Tic-Phe*-Bid (0.14 g, 0.2 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.16 g (96%); R_f (A) 0.43; HPLC K' 5.1; mp 149−151 °C; $[\alpha]^{20}_D$ +29.7; *m*/z 589 (M+H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.92−3.17 (m, 6H), 3.95−4.51 (m, 3H), 4.92−5.26 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 13H). Anal. $C_{40}H_{39}F_6N_5O_7$: C; H; N.

2TFA·H-Tyr-Tic-Phe*-Bid (10). Boc-Tyr-Tic-Phe*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.1 g (95%); R_f (A) 0.40; HPLC K' 4.82; mp 145−147 °C; $[\alpha]^{20}_D$ +13.8; *m*/z 561 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.92−3.22 (m, 6H), 3.95−4.51 (m, 3H), 4.90−5.23 (m, 2H), 6.68−7.70 (m, 17H). Anal. $C_{38}H_{35}F_6N_5O_7$: C; H; N.

2TFA·H-Dmt-Tic-Leu*-Bid (11). Boc-Dmt-Tic-Leu*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.11 g (95%); R_f (A) 0.40; HPLC K' 4.8; mp 155−157 °C; $[\alpha]^{20}_D$ +14.1; *m*/z 555 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.01−1.83 (m, 9H), 2.35 (s, 6H), 2.92−3.17 (m, 4H), 3.95−4.51 (m, 3H), 4.87−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{37}H_{41}F_6N_5O_7$: C; H; N.

2TFA·H-Dmt-Tic-Arg(NO2)*-Bid (12). Boc-Dmt-Tic-Arg-(NO_2)*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.1 g (97%); R_f(A) 0.38; HPLC K' 4.62; mp 155−157 °C; $[\alpha]^{20}_D$ +12.3; *m*/z 643 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.55−2.65 (m, 12H), 2.92−3.17 (m, 4H), 3.95−4.51 (m, 3H), 4.87−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{37}H_{41}F_6N_9O_9$: C; H; N.

3TFA·H-Dmt-Tic-Arg*-Bid (13). Boc-Dmt-Tic-Arg*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.07 g (97%); R_f(A) 0.32; HPLC K' 4.43; mp 169−171 °C; $[\alpha]^{20}_D$ +15.7; *m*/z 598 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.55−2.65 (m, 12H), 2.92−3.17 (m, 4H), 3.95−4.51 (m, 3H), 4.87−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{39}H_{43}F_9N_8O_9$: C; H; N.

2TFA·H-Dmt-Tic-Asn*-Bid (14). Boc-Dmt-Tic-Asn*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.1 g (97%); R_f(A) 0.35; HPLC K' 4.3; mp 155−157 °C; $[\alpha]^{20}_D$ +19.2; *m*/z 556 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.61−3.17 (m, 6H), 3.95−4.51 (m, 3H), 4.92−5.10 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{35}H_{36}F_6N_6O_8$: C; H; N.

2TFA·H-Dmt-Tic-Trp*-Bid (15). Boc-Dmt-Tic-Trp*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.06 g (94%); R_f(A) 0.42; HPLC K' 5.06; mp 178−180 °C; $[\alpha]^{20}_D$ +30.5; *m*/z 627 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.86−3.17 (m, 6H), 3.95−4.51 (m, 3H), 4.92−5.26 (m, 2H), 6.29 (s, 2H), 6.80−7.70 (m, 13H). Anal. $C_{42}H_{40}F_6N_6O_7$: C; H; N.

2TFA·H-Dmt-Tic-Ser(Ac)*-Bid (16). Boc-Dmt-Tic-Ser-(Ac)*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.07 g (96%); R_f(A) 0.37; HPLC K' 4.25; mp 143−145 °C; $[\alpha]^{20}_D$ +20.7; *m*/z 570 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.01 (s, 3H), 2.35 (s, 6H), 2.92−3.17 (m, 4H), 3.93−3.97 (m, 1H), 4.41−4.76 (m, 4H), 4.92−5.47 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{36}H_{37}F_6N_5O_9$: C; H; N.

2TFA·H-Dmt-Tic-Ser*-Bid (17). Boc-Dmt-Tic-Ser*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.06 g (95%); *Rf*(A) 0.31; HPLC K' 3.92; mp 150−152 °C; $[\alpha]^{20}_D$ +22.6; *m*/z 529 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.92−3.17 (m, 4H), 3.95−4.20 (m, 3H), 4.41−4.51 (m, 2H), 4.92−4.96 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{34}H_{35}F_6N_5O_8$: C; H; N.

2TFA·H-Dmt-Tic-Glu*-Bid (18). Boc-Dmt-Tic-Glu*-Bid was treated with TFA as reported for 2TFA·H-Dmt-Tic-Phe*-Bid: yield 0.16 g (97%); R_f(A) 0.69; HPLC K' 5.11; mp 173−175 °C; $[\alpha]^{20}_D$ +21.5; *m*/z 571 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.23−2.25 (m, 4H), 2.35 (s, 6H), 2.92−3.17 (m, 4H), 3.93−3.97 (m, 1H), 4.41−4.51 (m, 2H), 4.87−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{36}H_{37}F_6N_5O_9$: C; H; N.

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Supporting Information Available: Abbreviations, chemistry general methods, synthesis of intermediates, and pharmacology. This material is available free of charge via the Internet at http://pubs.acs.org.

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