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pK_a and Volume of Residue One Influence δ/μ Opioid Binding: QSAR Analysis of Tyrosine Replacement in a Nonselective Deltorphin Analogue

Deborah L. Heyl,[a,*] Stephen E. Schullery,[a] Kutralanathan Renganathan,[a] Malika N. Jayamaha,[a] David W. Rodgers[a] and John R. Traynor[b]

[a]*Department of Chemistry, Eastern Michigan University, Ypsilanti, MI 48197, USA*
[b]*Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109, USA*

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Abstract—[Gly^4]deltorphin (Tyr-*D*-Ala-Phe-Gly-Val-Val-Gly-NH_2) is a nonselective analogue of the opioid heptapeptides isolated from *Phyllomedusa* amphibian skin. Its nonselective nature allows for simultaneous characterization of the effects of sequence modification on both delta (δ) and mu (μ) receptor binding. The N-terminal regions of opioid peptides are considered to be responsible for receptor recognition, and the tyrosine at position one is relatively intolerant to alteration. In order to further investigate the role of the phenolic hydroxyl group in receptor interaction, a series of peptides was synthesized in which the position-one tyrosine residue was replaced with analogues of varying electronic, steric, and acid/base character, including ring-substituted tyrosines, *para*-substituted phenylalanines, and other nonaromatic and heterocyclic amino acids. The effects of these replacements on δ and μ receptor affinities were measured and then analyzed through quantitative structure–activity relationship (QSAR) calculations. Results support a dual hydrogen bond donor/acceptor role for the Tyr^1 hydroxyl moiety, with *less acidic* hydroxyl groups exhibiting stronger binding to opioid receptors. In addition, steric bulk in the Tyr^1 position independently strengthens μ and possibly δ binding, presumably by either a ligand conformational effect or enhanced van der Waals interactions with a 'loose' receptor site. The pK_a effect is stronger on δ than on μ binding, generating an increase in δ selectivity with increasing residue-one pK_a.

Introduction

The family of opioid peptides known as the deltorphins has been extensively studied, beginning with their isolation from amphibian skin.[1,2] Opioid peptides, as their name suggests, bind to the opioid receptors, which consist of three major classes, delta (δ), mu (μ) and kappa (κ),[3] including δ and μ receptor subtypes.[4,5] The physiological effects connected with a particular ligand/receptor system depend on the selective affinity of the ligand for that receptor type and the ability of the ligand to activate that receptor to transduce a biological response. While each of the opioid receptors is associated with analgesia, side effects such as dependence, respiratory depression, and gastrointestinal effects have been attributed to various receptor types. Selective agonists and antagonists are therefore desirable to reduce adverse side effects. The deltorphins both show a remarkable selectivity for mammalian δ receptors and bind to these receptors to a far greater extent than any known endogenous opioid peptides.[6] This extraordinary behavior has driven the intensive research into these compounds that has taken place over nearly 15 years.

Hundreds of analogues of the various deltorphins have been synthesized[6,7] to determine the precise nature of the binding interactions and the basis for receptor selectivity, with the ultimate goal of designing more effective, less problematic analgesic drugs. Structure–activity relationship (SAR) studies of many of these analogues suggest some important features, including the following.

Residues 5 and 6: δ but not μ binding is favored by narrow side chains. μ binding at residue 6 is favored by

*Corresponding author. Tel.: +734-487-2057; fax: +734-487-1496; e-mail: debbie.heyl-clegg@emich.edu

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side chain bulk but is impeded by crowding at the side chain β carbon.[8] The native Val apparently strikes a favorable balance for δ affinity and selectivity. Hydrophobicity per se, either of the side chains[8] or of the entire dipeptide segment,[9] does not seem to be important.

Residue 4: The acidic functional group (Glu or Asp) is necessary for strong δ binding.[10,11] Discrimination against μ binding occurs due to the side chain's steric bulk.[12]

Residue 3: Proper spatial orientation and/or length of a bulky hydrophobic side chain is required,[13–17] although apparently for similar pockets on δ and μ receptors.[12]

Residue 2: The ability of the deltorphins to demonstrate greatly increased affinities and selectivities over endogenous opioid ligands in mammals is believed to be due in part to the presence of a D-amino acid in the second position of the peptide sequence.[1,2,10] A D-amino acid with similar size and shape to the native D-Ala is necessary.[18–20]

Residue 1: The L-tyrosine is relatively intolerant to modification[21–24] and its spatial orientation is also of importance.[21,25] It is theorized that Tyr^1 is involved in receptor recognition, and the *para*- orientation of the hydroxyl group is generally viewed to be a required element in the binding pharmacophore.[23]

Further work on the nature of the residue-1 interaction is necessary, as there are outstanding questions regarding the role of the steric bulk and aromaticity of the ring and, perhaps more important, the specific role of the phenolic hydroxyl group. To that end, we now report a study of the parent compound Gly^4 deltorphin I/II, a so-called infidelitous deltorphin[6] having comparably tight binding to both the δ and μ receptors and therefore uniquely suited for simultaneous study of both binding regimes. We have synthesized and studied 31 new side chain analogues of the Tyr^1 residue (i.e., X^1Gly^4), including one series of 10 in which substitutions are made to the Tyr ring and a second series of 10 in which the Tyr hydroxyl group is replaced by a variety of aprotic substituents (i.e., *p*-X-Phe^1 analogues). The δ and μ receptor binding affinities of the single-substitution analogues are subjected to correlation and multiple regression QSAR analyses focused on the acidity/basicity role of the Tyr^1 phenolic group. The roles of several other calculated quantum, solubility, and geometric properties are also investigated.

Results

Table 1 presents the experimental purity, molecular weight, and binding data for each analogue studied. The μ and δ binding affinity data are presented, along with the logarithms of the binding enhancement factors (log E) used in the QSAR analyses. A measure of selectivity is provided as the ratio of μ to δ binding affinities. Names and abbreviations of non-standard amino acids are provided in the Notes to Table 1.

Table 2 shows the QSAR properties calculated for the isolated side chains, and Table 3 shows the correlations of each QSAR variable with both δ and μ binding (log E_δ or log E_μ) for each of the two homologous series studied. The statistical significance levels of all correlations having *p*-values <0.10 are shown in parentheses. The *p*-value is the probability of so-called type-1 or false-positive error, that is, the probability of such a relationship occurring by chance.

An exploratory stepwise multiple regression analysis was performed on the ring-substituted Tyr^1 analogues to search for any variable that might contribute independent explanatory power beyond that provided by pK_a. Similar relationships resulted for log E versus pK_a together with any one of log P (log of the octanol/water partition coefficient, an indicator of hydrophobicity), polarizability, area, or volume. As the latter set of four variables are all size-related and highly correlated, this is interpreted as a general size effect, rather than, for example, a specific solubility effect. Statistical data from the multiple regressions of log E_δ or log E_μ on pK_a and volume are shown in Table 4. The significance level of the size-related variables was weaker for δ than μ binding; the *p*-value of volume in the δ-binding regression does not attain the traditional 0.05 threshold of significance although a weak relationship is visually evident in a partial regression plot.

The quality of the regression relationships of log E_δ versus pK_a and volume, and log E_μ versus pK_a and volume are illustrated by partial regression plots, Figures 1–4. A partial regression plot is interpreted as follows. Figure 1, for example, is a graph of the residuals (deviations) from the least-squares regression of log E_δ on volume plotted versus the residuals from the least-squares regression of pK_a on volume. In effect, it is a graph of the part of log E_δ that is not explained by volume versus the part of pK_a that is not explained by volume. The slope and the residuals of this partial regression plot are the same as the pK_a coefficient and residuals, respectively, from the full multiple regression of log E_δ versus pK_a and volume. Partial regression plots provide a ready visualization of the strength (e.g., degree of scatter), direction, curvature, outliers, and unduly influential points of that part of a relationship between two variables that is independent of the linear effects of other variables in the analysis.[26]

Figure 5 shows the relationship between δ selectivity (K_μ/K_δ) and pK_a of the ring-substituted Tyr^1 analogues. None of the other relationships between opioid binding affinity or selectivity and QSAR variables proved to be significant.

No statistically significant QSAR relationships were found for the aprotic *p*-X-Phe analogues at the bivariate (Table 3) or multivariate levels.

Discussion

Table 1 reveals that the *para* location of the hydroxyl group is important. Repositioning the group to the *meta*

Table 1. Purity, mass and opioid receptor binding affinities of X^1Gly^4 deltorphin I/II analogues

X^1 analogue[a]	Purity[b] (%)	Mol. Wt.	K_δ(nM)[c]	K_μ(nM)[c]	K_μ/K_δ	log E_δ	log E_μ
Tyr	>99.9	711.8	22.7	23.2	1.02	0	0
homoTyr	99.7	725.8	844	880	1.04	−1.570	−1.579
o-Tyr	99.2	711.8	>10,000	>10,000	—	−2.644	−2.635
m-Tyr	98.1	711.8	285	258	0.91	−1.099	−1.046
o-Me-Tyr	98.7	725.8	7.0	6.1	0.87	0.511	0.580
m-NO_2-Tyr	>99.9	756.5	>10,000	1322	<0.13	−2.644	−1.756
m-I-Tyr	99.8	837.5	209	95	0.45	−0.964	−0.612
m-OH-Tyr	99.7	727.8	248	241	0.97	−1.038	−1.017
m-Cl-Tyr	99.9	746.3	62	66.7	1.08	−0.436	−0.459
3,5-Br_2-Tyr	99.5	869.5	1079	161	0.15	−1.677	−0.841
3,5-I_2-Tyr	99.5	963.3	1885	30.8	0.02	−1.919	−0.123
DMT	98.1	739.8	15.6	17.6	1.13	0.163	0.120
TMT	>99.9	753.8	0.42	0.78	1.86	1.733	1.473
2-Pal	>99.9	696.8	>10,000	>10,000	—	−2.644	−2.635
3-Pal	99.7	696.8	355	349	0.98	−1.194	−1.177
4-Pal	>99.9	696.8	70	55.6	0.79	−0.489	−0.380
4-Taz	99.5	702.8	>10,000	>10,000	—	−2.644	−2.635
2-Thi	>99.9	701.6	1754	1003	0.57	−1.888	−1.636
Lys-H^+	>99.9	676.6	>10,000	>10,000	—	−2.644	−2.635
Arg-H^+	>99.9	704.7	>10,000	>10,000	—	−2.644	−2.635
Orn-H^+	>99.9	663.7	>10,000	>10,000	—	−2.644	−2.635
Phe	>99.9	695.8	300	63.7	0.21	−1.121	−0.439
Hfe	>99.9	709.8	>10,000	>10,000	—	−2.644	−2.635
p-F-Phe	99.9	713.4	>10,000	>10,000	—	−2.644	−2.635
p-Cl-Phe	99.8	729.9	1519	276	0.18	−1.826	−1.075
p-Br-Phe	99.7	775.5	922	67.2	0.07	−1.609	−0.462
p-I-Phe	99.8	821.5	773	119	0.15	−1.532	−0.710
p-NH_2-Phe	98.4	710.1	579	600	1.04	−1.407	−1.413
p-OCH_3-Phe	>99.9	725.5	967	224	0.23	−1.629	−0.985
p-CN-Phe	>99.9	720.6	619	201	0.32	−1.436	−0.938
p-NO_2-Phe	99.5	740.6	2957	365	0.12	−2.115	−1.197
p-CF_3-Phe	99.5	763.6	2128	950	0.45	−1.972	−1.612

[a]Abbreviations: molecular weight obtained by electrospray mass spectrometry (Mol. Wt.); homotyrosine (homoTyr), *ortho*-hydroxylphenylalanine (*o*-Tyr), *meta*-hydroxyphenylalanine (*m*-Tyr), *ortho*-methyltyrosine (*o*-Me-Tyr), *meta*-nitrotyrosine (*m*-NO_2-Tyr), *meta*-iodotyrosine (*m*-I-Tyr), *meta*-hydroxytyrosine (*m*-OH-Tyr), *meta*-chlorotyrosine (*m*-Cl-Tyr), 3,5-dibromotyrosine (3,5-Br_2-Tyr), 3,5-diiodotyrosine (3,5-I_2-Tyr), 2,6-dimethyltyrosine (DMT), 2,3,6-trimethyltyrosine (TMT), 2-pyridylalanine (2-Pal), 3-pyridylalanine (3-Pal), 4-pyridylalanine (4-Pal), 4-thiazolylalanine (4-Taz), 2-thienylalanine (2-Thi), ornithine (Orn), homophenylalanine (Hfe), *para*-fluorophenylalanine (*p*-F-Phe), *para*-chlorophenylalanine (*p*-Cl-Phe), *para*-bromophenylalanine (*p*-Br-Phe), *para*-iodophenylalanine (*p*-I-Phe), *para*-aminophenylalanine (*p*-NH_2-Phe), *para*-methoxyphenylalanine (*p*-OCH_3-Phe), *para*-cyanophenylalanine (*p*-CN-Phe), *para*-nitrophenylalanine (*p*-NO_2-Phe), *para*-trifluoromethylphenylalanine (*p*-CF_3-Phe).
[b]Purity of final product peptide as assessed by RP-HPLC peak integration at 214 or 230 nm (whichever is lower).
[c]Average values from three assays performed in triplicate (>10,000 nM indicates that less than 50% inhibition of the labeled [^{3}H]diprenorphine was reached at a peptide concentration of 10 μM).

Table 2. QSAR variables for side chains of ring-substituted analogues

X^1 analogue	pK_a	Basicity	HOMO	LUMO	Charge	log P	Polarizability (Å^3)	Area (Å^2)	Volume (Å^3)	Width (Å)	Length (Å)
Tyr	10.21	n.a.	−8.88159	0.4260628	−0.253	2.23	12.91	138.07	110.13	4.34	6.41
o-Me-Tyr	10.38	n.a.	−8.80425	0.4329556	−0.253	2.7	14.74	152.7	125.38	5.16	6.31
m-Cl-Tyr	8.79	n.a.	−9.00359	0.1146421	−0.237	2.75	14.83	151.47	123.38	4.98	6.02
m-I-Tyr	8.81	n.a.	−9.08369	0.0356362	−0.240	3.49	17.93	168.66	139.51	5.65	6.21
m-NO_2-Tyr	7.45	n.a.	−9.67559	−0.966262	−0.205	0.25	14.75	162.71	129.1	5.23	6.44
m-OH-Tyr	9.69	n.a.	−8.76349	0.2943208	−0.272	1.95	13.54	145.24	115.9	4.77	6.29
DMT	10.51	n.a.	−8.69834	0.4175412	−0.255	3.16	16.58	174.55	142.32	6.42	6.50
TMT	10.86	n.a.	−8.64437	0.3954772	−0.257	3.63	18.41	190.98	158.25	5.93	6.23
3,5-Br_2-Tyr	7.21	n.a.	−9.24774	−0.328230	−0.229	3.81	18.16	183	153.34	5.60	6.46
3,5-I_2-Tyr	7.38	n.a.	−9.27989	−0.317757	−0.233	4.74	22.96	196.5	168.37	5.83	6.48
Phe	n.a.	0.14	−9.33034	0.520090	0.130	2.51	12.27	128.56	103.63	4.32	5.79
p-F-Phe	n.a.	0.10	−9.26967	0.143766	−0.108	2.65	12.18	133.98	106.1	4.33	6.03
p-Cl-Phe	n.a.	0.07	−9.29898	0.135114	−0.020	3.03	14.2	142.97	115.06	4.31	6.38
p-Br-Phe	n.a.	0.09	−9.35475	0.043342	0.048	3.31	14.9	146.81	120.43	4.32	6.52
p-I-Phe	n.a.	0.12	−9.40761	0.044951	0.139	3.77	17.3	160.07	132.96	4.32	6.63
p-CF_3-Phe	n.a.	n.a.	−10.0277	−0.325754	0.083	3.4	13.83	160.19	128.3	4.35	6.84
p-CN-Phe	n.a.	0.33	−9.71009	−0.394472	−0.135	2.55	14.12	148.67	120.65	4.33	7.28
p-NH_2-Phe	n.a.	0.41	−8.35584	0.614964	0.037	1.73	13.62	142.92	114.21	4.34	6.53
p-NO_2-Phe	n.a.	0.28	−10.3048	−1.04497	−0.153	0.54	14.11	153.72	122.66	4.34	6.80
p-OCH_3-Phe	n.a.	0.29	−8.78618	0.514195	0.173	2.26	14.74	159.45	128.05	4.58	7.91

Table 3. QSAR correlations for Tyr-ring (protic) and Phe-ring (aprotic) analogues[a]

QSAR variable	Tyr ring analogues		Phe ring analogues	
	log E_δ	log E_μ	log E_δ	log E_μ
pK_a	0.903 (<.0005)	0.706 (.023)	n.a.	n.a.
Basicity	n.a.	n.a.	0.272	−0.050
HOMO	0.880 (.001)	0.730 (0.017)	0.334	−0.005
LUMO	0.859 (.001)	0.712 (0.021)	0.408	0.087
Charge[b]	−0.698 (.025)	−0.553 (0.097)	0.542	0.441
log P	0.242	0.529	0.123	0.145
Polarizability	−0.167	0.229	0.239	0.464
Area	−0.055	0.281	−0.041	0.135
Volume	−0.055	0.301	0.052	0.247
Width	0.111	0.302	0.039	0.018
Length	−0.355	−0.154	0.093	0.134

[a]$n = 10$ for each class of analogues. p-Values for all correlations having statistical significance levels less than 0.10 are shown in parentheses.
[b]The partial charge on oxygen atom of Tyr analogues or total of partial charges on all atoms of the para substituent of Phe analogues.

Table 4. Data for multiple regressions of log E vs. pK_a and volume

Statistic	log E_δ	log E_μ
R	0.940	0.906
Adjusted R^2	85%	77%
SE	0.499	0.430
F[a]	26.68 (.001)	16.09 (0.002)
Regression constant[a]	−11.55 (.001)	−9.428 (.001)
pK_a coefficient[a]	0.917 (<.0005)	0.580 (0.001)
pK_a β-coefficient[a]	0.996 (<.0005)	0.907 (0.001)
Volume coefficient[a]	0.0187 (.082)	0.0283 (0.008)
Volume β-coefficient[a]	0.277 (.082)	0.603 (0.008)

[a]Statistical significance p-values are shown in parentheses.

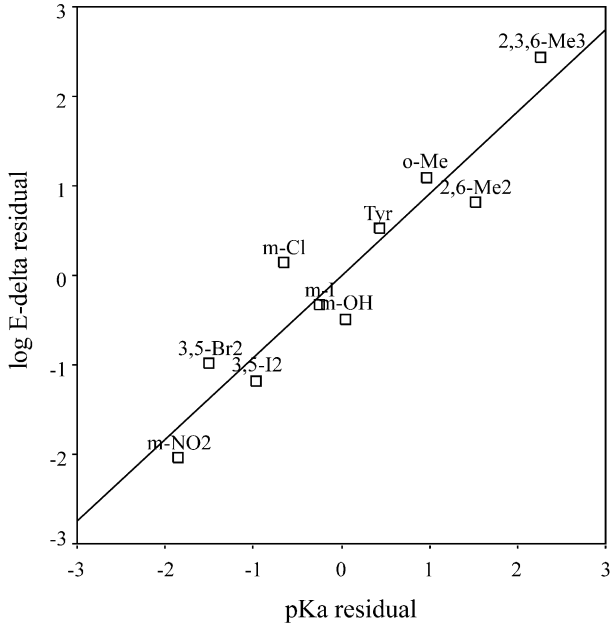

Figure 1. Partial regression plot for pK_a in the regression of δ binding on pK_a and volume of 10 Tyr ring-substituted Tyr[1]Gly[4] analogues. The least-squares best-fit line is shown.

position decreases both δ and μ binding by a factor of ten, and repositioning to the *ortho* position lowers binding beyond detection. Replacing the hydroxyl with hydrogen (Phe[1]) lowers δ binding by a power of 10 and μ binding by a factor of three. Attempts to identify an

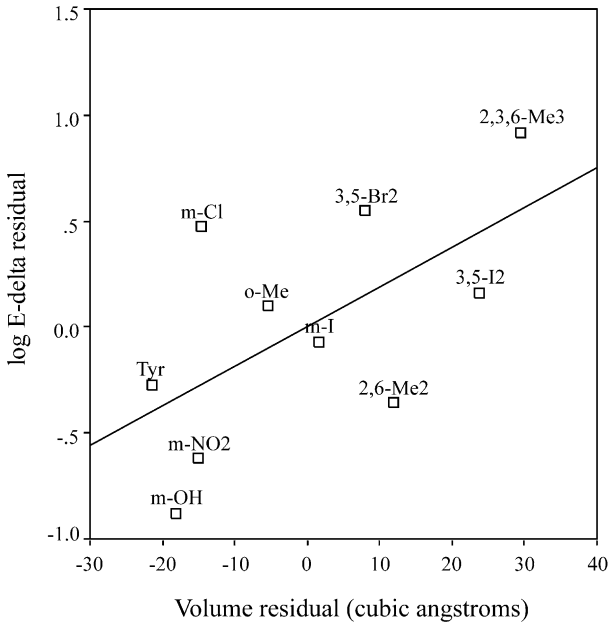

Figure 2. Partial regression plot for volume in the regression of δ binding on pK_a and volume of 10 Tyr ring-substituted Tyr[1]Gly[4] analogues. The least-squares best-fit line is shown.

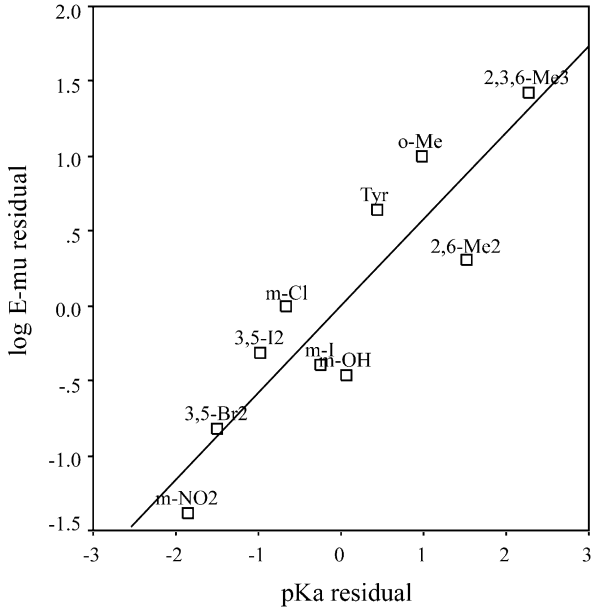

Figure 3. Partial regression plot for pK_a in the regression of μ binding on pK_a and volume of 10 Tyr ring-substituted Tyr[1]Gly[4] analogues. The least-squares best-fit line is shown.

effective hydroxyl replacement failed: all nine of the *para* replacements gave even weaker δ and μ binding than did hydrogen. On average, replacement of the hydroxyl group has a greater effect on δ than on μ binding: mean log E_δ and log E_μ values for the 10 Phe[1] analogues are −1.73 and −1.15, respectively. It may be important that none of the replacements were capable of serving as hydrogen bond donors.

We initially suspected that the Tyr side chain hydroxyl group very likely serves as a hydrogen bond donor during receptor binding, as it commonly does in other

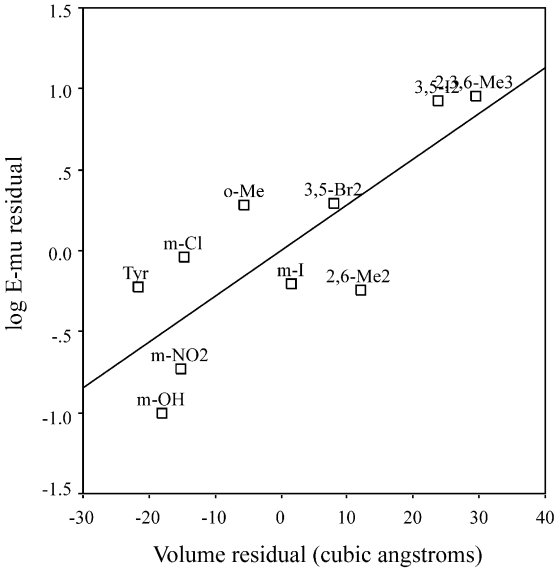

Figure 4. Partial regression plot for volume in the regression of μ binding on pK_a and volume of 10 Tyr ring-substituted Tyr[1]Gly[4] analogues. The least-squares best-fit line is shown.

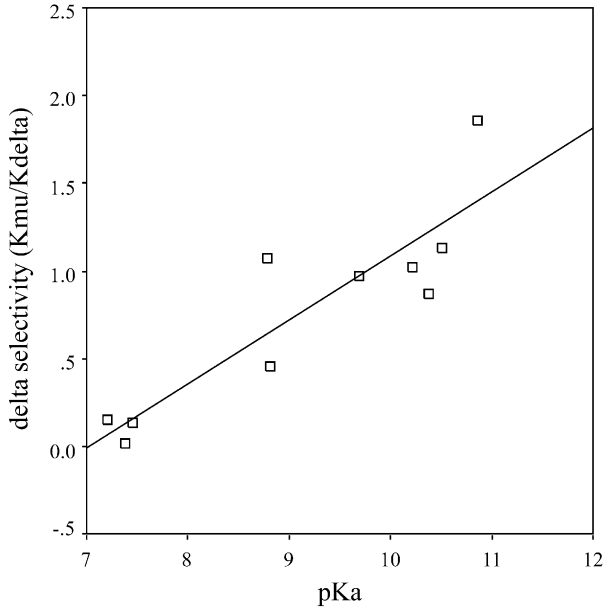

Figure 5. Plot of δ selectivity versus pK_a of 10 Tyr ring-substituted Tyr[1]Gly[4] analogues. The least-squares best-fit line is shown.

intra- and inter-protein interactions. This is consistent with the finding of Toth et al.[27] that the phenolic hydroxyl of constrained enkephalins must be protonated for δ binding to occur, although a hydrogen bonding role was not proposed. We prepared three analogues specifically to test the hydrogen bonding hypothesis: Lys[1], Arg[1], and Orn[1]. These would all be protonated under assay conditions, and we assumed that at least one from among them should be suitably positioned to serve as hydrogen bond donor. However, none exhibited detectable δ or μ binding. Of course, it also is possible that this might be due to the absence of an aromatic ring. Although the present data set can not confidently assess the necessity for a ring (only three non-ring analogues), the data do suggest that a phenyl ring *per se* may not be crucial. Three of the five heterocyclic side chain analogues (3-Pal, 4-Pal, 2-Thi) had detectable δ and μ binding comparable to or better than some analogues containing the phenol group, and several analogues containing the benzene ring had undetectable binding (e.g., Hfe, *p*-F-Phe). The lack of affinity of the Hfe analogue may be attributable to a different spatial positioning of the aromatic ring as a consequence of the increased side chain length.

The hydrogen bond donor hypothesis was further tested by correlation/regression analyses of the binding dependence on pK_a of the Tyr ring-substituted series of analogues. Hydrogen bonding should be favored by stronger acids (i.e., lower pK_a), unless the acid is so strong as to ionize to the solvent water. As seen in Table 2, only three of the 10 Tyr ring-substituted analogues should be significantly ionized under assay conditions. However, the data (Table 3) show a strong *positive* correlation between pK_a and δ binding and a similar but weaker relationship with μ binding. Thus, on-average, *less-acidic* hydroxyl groups bind more strongly. We suggest that this seeming contradiction may be explained by the hydroxyl group serving as both hydrogen bond donor *and* acceptor. In fact, a recent ligand–receptor interaction model published by Mosberg and Fowler[28] proposes just such dual hydrogen bonding between the Tyr[1] hydroxyl group and a His and conserved Tyr residue in both the δ and the μ opioid receptors. Their conclusion was based on molecular modeling of docking by two conformationally constrained tetrapeptide ligands with the X-ray structure of the opioid receptor-like 'activated' rhodopsin transmembrane helix bundle. Our suggested model for these findings would be as follows: Following hydrogen donation (albeit incomplete[29]) by the ligand's Tyr[1] to the receptor His, the resultant 'hydroxylate' conjugate base is available to form a second hydrogen bond by accepting a hydrogen from the receptor Tyr. However, whereas all of the Tyr[1]-ring analogues are sufficiently protic to form the first bond, formation of the second bond depends on the basicity of the resulting complex, which increases with pK_a of the Tyr[1] analogue.

The non-phenolic analogues can, at most, play a hydrogen bond acceptor role. The results in that regard are mixed. The effect of the position of the putative hydrogen-acceptor atom in the rings of heterocyclic analogues is consistent with such a role: Receptor binding increases the closer the pyridylalanine ring nitrogen is to the normal *para* location of the Tyr hydroxyl oxygen, that is, in the order 4-Pal > 3-Pal > 2-Pal. Similarly, the sulfur of 2-Thi and the nitrogen of 4-Taz, both of which are weak binders, are in more of an '*ortho*' position of their respective rings. Also, the *p*-X-Phe[1] analogues—which can accept but not donate hydrogen bonds—exhibit reduced but detectable receptor binding; in contrast, the protonated-N analogues—which can donate but not accept hydrogen bonds—exhibit no detectable receptor binding. However, receptor binding by the *p*-X-Phe[1] analogues does not show the correlations with basicity, partial charge, or HOMO energy that might

be expected if the analogues were serving as hydrogen bond acceptors. This failure might be due to insufficient range of the variables or unfavorable geometry.

Multiple regression analysis shows that the size of the Tyr ring-substituted side chains provides additional and independent explanation of variance in μ-binding and possibly in δ-binding as well (Table 4). Larger groups bind more tightly, suggesting that the Tyr[1] side chain does not bind in a sterically confined binding pocket. Rather than obstruct the binding interaction, bulky ring substituents strengthen binding, perhaps by supporting a favorable ligand conformation or by providing non-specific stabilizing van der Waals interactions in an otherwise 'loose' binding site. The binding enhancement for the 2,3,6-Me_3-Tyr (TMT^1) analogue, which is over 50-fold at δ receptors and almost 30-fold at μ receptors, is thus explained by the methyl groups providing both favorable bulk and an inductive increase in pK_a. Similar positive effects on binding and/or efficacy have been noted when ring-methylated Tyr analogues (particularly 2, 6-Me_2Tyr, DMT) were substituted at the N-terminus of deltorphins and other opioid peptides.[21,27,30–34] In fact, the DMT-Tic pharmacophore is well known to give rise to potent δ-selective antagonism.[32] However, it is surprising that the o-Me-Tyr^1 analogue actually displayed 2 to 3-fold higher opioid binding affinity than the DMT^1 analogue; based on pK_a and steric bulk criteria, the expected order of affinity would be $Tyr^1 < $o-Me-$Tyr^1 < DMT^1 < TMT^1$, as has been reported for corresponding dermorphin tetrapeptide analogues.[33] In addition, it should be noted that while DMT^1 analogues in other opioid series have given rise to much greater μ and δ receptor binding enhancements, by one to two orders of magnitude,[31,33] only a slight improvement in binding (1.5-fold) was observed in the present case. In contrast to the methyl substituent effect, any binding enhancement by the nitro substituent's bulk is apparently more than offset by that group's negative effect on pK_a, causing it to bind poorly. It is important to note that the dependence of binding on size would not be evident from consideration of bivariate correlations alone (Table 3); the size effect is only apparent after the pK_a effect is partialled out. The relative sizes of the standardized β coefficients, which are in comparable standard-deviation units, provide a measure of the importance of the pK_a and volume variables relative to each other. Although the pK_a effect is larger than the volume effect for both δ and μ binding, the gap is smaller for μ binding.

Finally, we consider how the relative impacts of these factors on δ versus μ binding is reflected in binding selectivity. Figure 5 shows that δ selectivity increases with pK_a, consistent with both the steeper slope of δ-binding versus μ-binding pK_a partial regression plots (Figs. 1 and 3) and the larger pK_a regression coefficient for δ binding (Table 4). This may reflect slight conformational/ligand alignment differences between the two receptor types. However, multiple regression analysis failed to detect any additional significant variable. Specifically, the effects of volume on δ and μ binding do not produce a net effect on selectivity.

Conclusion

The data support a dual hydrogen bond donor/acceptor role for the Tyr^1 hydroxyl moiety, consistent with molecular model predictions reported for related model systems.[28] Analogues with *less acidic* hydroxyl groups exhibit stronger binding to opioid receptors, presumably by strengthening the less-favored hydrogen bond acceptor role of Tyr^1. Additional steric bulk in the Tyr^1 position strengthens μ binding, perhaps by either a ligand conformational effect or enhanced van der Waals interactions with a 'loose' receptor site. The weaker steric effect for δ binding, although visually evident, is of borderline statistical significance. The stronger pK_a effect on δ than on μ binding results in an increase in δ selectivity with pK_a. While modification of any of the deltorphin residues usually results in a reduction in opioid receptor binding affinity relative to the parent deltorphin, three of the position-1 analogues in the present study displayed improved binding at both δ and μ receptors, along with increased δ selectivity: *o*-$MeTyr^1$, DMT^1, and TMT^1. The binding enhancements seen with all three of these analogues are understandable in terms of the methyl groups' steric bulk and inductive effect on pK_a.

Experimental

Peptide synthesis

The following novel protected amino acids were purchased from Synthetech, Inc: *p*-Br-Phe, *p*-Cl-Phe, *p*-CN-Phe, *p*-F-Phe, *p*-I-Phe, *p*-NO_2-Phe, *p*-CF_3-Phe, 2-pyridylalanine (2-Pal), 3-pyridylalanine (3-Pal), 4-pyridylalanine (4-Pal), 4-thiazolylalanine (Taz), and 2-thienylalanine (Thi). The Boc-protected methyl-substituted tyrosine analogues *o*-Me-Tyr, 2,6-DMT and 2,3,6-TMT were provided by a colleague at Monsanto. The remaining protected amino acids, coupling agents and resins were purchased from Bachem. Solvents and deprotecting agents were obtained from Fisher Scientific and Aldrich Chemical Co. Radioligands were purchased from New England Nuclear, Multiple Peptide Systems and Amersham. The peptides were prepared on a St. John's Associates manual shaker using standard solid phase techniques for N-α-*t*-butyloxycarbonyl (Boc) protected amino acids on *p*-methylbenzhydrylamine (MBHA) resin (0.6–1.1 mmole/g). The side chain of Tyr was protected as the 2,6-dichlorocarbobenzyloxy derivative. The deprotection solution for the N-terminal amine was 30% trifluoroacetic acid (TFA) in dichloromethane (DCM). Dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were used as coupling agents. The protocol for peptide synthesis in each cycle was as described previously.[12] Simultaneous deprotection and cleavage from the resin were accomplished by treatment with 90% anhydrous HF (Immunodynamics apparatus) and 10% anisole scavenger (10 mL of HF and 1 mL of anisole per g of resin) at 0 °C for 1 h. After evaporation of the HF, the peptide resin was washed with diethyl ether and the peptide was extracted with 70% acetoni-

trile/30% water (with 0.1% TFA), concentrated under reduced pressure, diluted with water, and lyophilized. Crude peptides were purified to homogeneity by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Waters instrument with a Vydac C18 column (2.2×25.0 cm, 10 mL/min). A linear gradient of water (0.1% TFA) to 50% acetonitrile (0.1% TFA)/water (0.1% TFA) was employed, followed by lyophilization.

Peptide analysis

Peptide purity was assessed by analytical RP-HPLC. Peaks were monitored at 214, 230, 254 and 280 nm. All compounds were at least 98% pure as analyzed by peak integration. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Bruker spectrometer at 250 MHz. Samples (approximately 1 mg) were dissolved in DMSO. Diagnostic resonances and peak patterns confirmed the presence of all indicated residues. Electrospray mass spectroscopy confirmed the appropriate molecular weights.

Opioid receptor binding assays

C_6 rat glioma cells stably transfected with rat μ [C_6(μ)] or δ [C_6(δ)] opioid receptors were used.[35] Cells were grown to confluence under 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum in the presence of 1 mg/mL Geneticin.

Membrane preparation

Cells were rinsed twice with ice-cold phosphate-buffered saline (0.9% NaCl, 0.61 mM Na_2HPO_4, 0.38 mM KH_2PO_4, pH 7.4), detached from dishes by incubation with lifting buffer (5.6 mM glucose, 5 mM KCl, 5 mM HEPES, 137 mM NaCl, 1 mM EGTA, pH 7.4) and collected by centrifugation (500×*g*). The cells were resuspended in ice-cold Tris–HCl (pH 7.4, 50 mM) and homogenized using a tissue tearer, then centrifuged for 20 min at 20,000×*g*. The resulting membrane pellets were resuspended in 50 mM Tris–HCl buffer (pH 7.4), centrifuged again, and finally resuspended in Tris buffer at a protein concentration of 0.5 mg/mL. Samples were stored at −80 °C. All procedures were performed at 4 °C.

Ligand binding assays

C_6 cell membranes (25–50 μg) were incubated for 3 h at 4 °C with 0.2 nM [^{3}H]diprenorphine in 50 mM Tris–HCl buffer (pH 7.4) in the presence of eight concentrations of test peptide. The reactions were terminated by the addition of 3 mL ice-cold Tris–HCl buffer. The contents of the tubes were then rapidly vacuum-filtered through glass fiber filters (Schleicher & Schuell no. 32, Keene, NH) and the tubes and filters rinsed with 3 mL ice-cold Tris–HCl an additional three times. Radioactivity retained by the filters was determined by liquid scintillation counting. Non-specific binding was defined with 10 μM naloxone. Non-linear regression analysis of the data was performed using GraphPad Prism (GraphPad, San Diego, CA) using a one site binding analysis. K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation.[36] Each K_i value reported is the mean of three determinations, each performed in triplicate.

QSAR analysis

For all QSAR analyses, binding affinity was analyzed in terms of the logarithm of binding enhancement factors, log E_δ and log E_μ. The enhancement factors E_δ and E_μ are defined as the ratios of the native K_δ or K_μ to the analogue K_δ or K_μ, respectively. This normalization allows direct comparison of data from different laboratories, from which K values of the same compound can differ by two orders of magnitude.[7] Use of the logarithm function provides a variable that is more likely to depend linearly on energy or entropy, and that conveniently gathers and distributes data spanning orders of magnitude. So defined, positive values of log E correspond to binding stronger than that of the parent deltorphin.

The QSAR data were calculated as properties of the isolated side chains cleaved at the α-carbon of Tyr^1 and capped with a hydrogen atom. Two software packages were used to calculate QSAR data: ACD/Labs pK_a Calculator v. 3.0 (Advanced Chemistry Development, Inc., Toronto) and HyperChem v. 5 (HyperCube, Inc.). Effective solute hydrogen bond basicities were assigned to the aprotic *p*-Phe analogues using Abraham's $\Sigma\beta_2^H$ scales.[37] Values for pK_a were calculated for all protic analogues. As additional possible hydrogen bonding variables, HOMO, LUMO, and atomic charge data[38] were calculated, all using HyperChem's AM1 semi-empirical quantum procedure. For the Tyr^1 ring-substitution analogues, the partial charge on the Tyr oxygen atom was used; for the Phe^1 *para*-substitution analogues, the total of partial charges on all atoms of the para substituent was used. Calculations of log P and polarizability employed the methods of Viswanadhan et al.[39] and Miller,[40] respectively. Those calculations were not dependent on structure optimization. Calculation of van der Waals surface area and volume employed the grid method of Bodor et al.[41] based on the atomic radii of Gavezotti,[42] and is weakly dependent on the three-dimensional structure. For this purpose, the structures of the isolated side chains were optimized using HyperChem's MM+ implementation of Allinger's MM2 molecular mechanics force field.[43] The equivalent-box dimensions of width and length (excluding smallest rectangular dimension) were determined using HyperChem's periodic box facility to fit the structure to the smallest possible rectangular box.[12]

QSAR analysis was performed at two levels: first, observation of singular effects of unique modifications that are not part of a homologous series and, second, correlation and multiple regression analysis of homologous series of analogues. Two homologous series were studied: 10 analogues modified by addition substitutions to the Tyr^1 ring and 10 analogues in which the Tyr hydroxyl group is replaced by a variety of substituents (i.e., *p*-X-Phe^1 analogues). Analysis of the homologous

series involved both bivariate correlations (Pearson's product moment r) and stepwise multiple regression analysis of log E_δ and log E_μ versus the entire set of QSAR variables. The pK_a and basicity data were analyzed only with the protic (Tyr) and aprotic (p-X-Phe) analogues, respectively. Data analysis used the SPSS® v. 8.0 statistics package.

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