Opioid Receptor Affinities of Tyrosine ω-Phenylalkyl Esters, Simple Enkephalin Pharmacophore-mimics.

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Abstract : In order to substantiate the hypothesis that the μ-, δ-, or κ-opioid receptor Affinity of opioid peptides can be elicited by an amino-terminus tyrosine residue and the 4th phenylalanine aromatic ring lying in the proper spatial disposition, a series of simple peptide-mimic tyrosine esters, in which tyrosine is linked to aromatic ring separated by a variety of methylene spacers, were prepared and evaluated by in vitro radioligand receptor assay. Compounds having the 6 or 7 methylene spacers were more potent in opioid receptor affinity than those having fewer or more methylene spacers. The *N*-monomethyl- and *N*,*N*-dimethyltyrosine congeners were more potent than the corresponding tyrosine esters. The order of the receptor type selectivity of these compounds was $\mu >> \delta > \kappa$.

Key phrases : opioid peptide mimic; tyrosine ω-phenylalkyl ester, opioid receptor, receptor selectivity, molecular modeling simulation (ref 17)

Introduction

In the realm of compounds that act on the central nervous system to combat pain, the opiate alkaloids (for example, morphine) have been the pre-eminent drugs for many years. The discovery and characterization of the naturally occurring opioid receptor agonists, opioid peptides, have renewed interest in this field,[1] increasing the hope that analogs of the enkephalins would lead to drugs having reduced side-effects while maintaining a high analgesic activity. Many opioid peptides were discovered and synthetic opioid peptides were made to focus on the creation of non-narcotic strong analgesics overwhelm to morphine.

In recent years, extensive efforts have been made to clone the receptor proteins. Many cDNAs of chemical mediator receptors have been cloned. The cDNA of the δ-opioid receptor was also cloned by Evans et al.[2a] and by Kieffer et al.[2b], respectively. There has been some success in cloning each of the opioid receptor types. In time, the exact chemical and physical characteristics of

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the receptor proteins will be known, and the direct bonding interactions of selective drugs with their receptors will be studied.

At this time, however, we have no exact conformational information of both the receptor and opioid peptides in the binding sites. We felt that designing nonpeptide compounds which mimic the enkephalins was a means to investigate the interactions between the opioid ligand and the receptors.

Notable points on structure-activity relationships of opioid peptides are:

1) The tyrosine at the first amino acid residue position of all the endogenous opioid peptides is essential for activity. Removal of the phenolic hydroxyl group or the basic nitrogen (amino terminus group) abolishes activity.

2) In addition of the phenol and amine groups of Tyr^1, the next most important moiety in the enkephalin structure is the phenyl group of Phe^4. Removal of this group or changing its distance from Tyr^1 can result in full or substantial loss in activity.

3) The enkephalins have several low-energy conformations, and it is likely that different conformations are bound at different opioid receptor types and subtypes.

Many conformational studies have been published using NMR,[3] fluorescence,[4] theoretical studies,[5-7] and more recent X-ray studies.[8] However, among the many published studies which derived "active conformations" for the enkephalins there has been no real consensus. Most do agree that the tyrosine group is superimposed upon the tyramine part of morphine and that the molecule is bent on itself in a beta-turn. The glycines are generally thought to be positioning groups and set the phenylalanine aromatic system to active conformation.

The conformational comparison between opioid peptides and opiate derivatives has been studied using molecular modeling simulations. The molecular superimposition study of Met-enkephalin and an oripavine indicated the requirements of μ-opioid receptor selectivity of ligands.[9] The molecular dynamics simulation of enkephalin and naltrindole, a naltrexone analog, explained the δ-receptor selectivity of naltrindole.[10]

Chemistry

ω-Phenylalkanols with 2 to 4 methylene carbons are commercially available. ω-Phenylalkanols with more than 5 methylene carbons were prepared by the procedure as shown in Chart 1. Reaction of phenylmagnesium bromide and cycloalkanone gave a mixture of 1-phenylcycloalkene and 1-phenylcycloalkanol. The mixture was treated with chromium trioxide in acetic acid gave an ω-benzoylalkanoic acid,[11] which was converted into an ω-phenylalkanol by Huang-Minlon reduction[12] followed by diborane reduction.[13] Tyrosine ω-phenylalkyl esters were prepared with tyrosine and a corresponding ω-phenylalkanol in methanesulfonic acid (Chart 2).[14]

ATIn derivatives are tyrosine phenylalkyl esters with 2 to 10 methylene carbons. The simple methylene-carbons chain of the esters was anticipated only to have a hydrophobic interaction

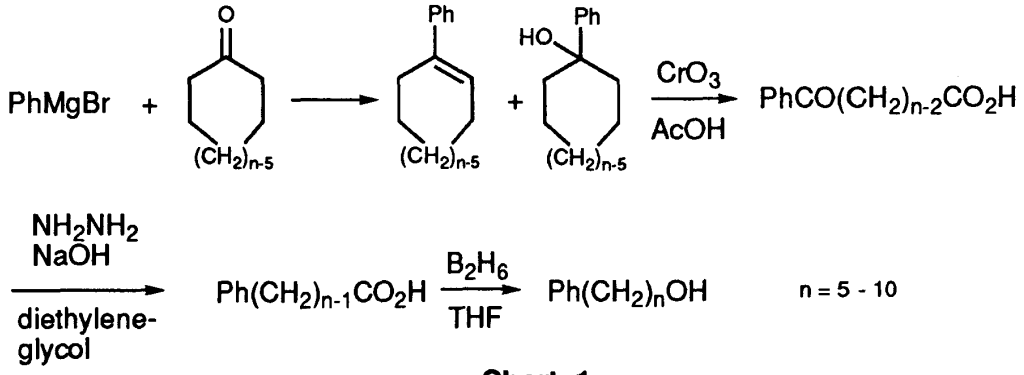


Chart 1

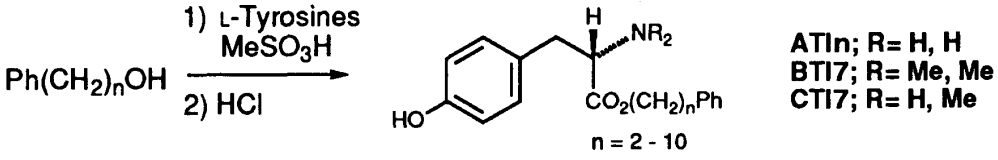


Chart 2

Table 1. L-Tyrosine Phenylalkyl Esters.

Compd	Yield(%)	Mp(°C)*	Elemental Analysis*					
			Calcd			Found		
ATIn			C	H	N	C	H	N
ATI2	90	192-3	63.26	6.33	4.24	63.45	6.26	4.35
ATI3	87	191-2	64.33	6.66	3.97	64.38	6.60	4.17
ATI4	83	146-7	65.23	6.90	3.99	65.23	6.91	4.00
ATI5	33	178-9	66.16	7.32	3.84	66.02	7.20	3.85
ATI6	90	137-8	66.48	7.54	3.51	66.74	7.47	3.71
ATI7	95	171-3	67.20	7.74	3.30	67.42	7.72	3.57
ATI8	86	148-9	67.77	7.99	3.37	68.05	7.95	3.45
ATI9	69	159-161	68.64	8.16	3.34	68.55	8.16	3.35
ATI10	95	142-3	69.18	8.36	3.23	69.38	8.34	3.24
ATI13N	94	143-5	66.72	9.74	3.38	66.47	9.89	3.34
BTI7**	54	109-110	68.64	8.16	3.34	68.36	8.20	3.25
CTI7***	61	98-100	68.05	7.95	3.45	67.78	8.03	3.44

* Data of HCl salts are described.
** N,N-Dimethylated ATI7.
*** N-Monomethylated ATI7.

during binding with a protein. It would diminish the conformational ambiguity of Gly^2-Gly^3-Phe^4 peptide chain of the enkephalins. **ATI13N** has only straight alkyl chain ester. It is a reference compound to detect the influence of terminal phenyl portion. **BTI7** and **CTI7** are an *N,N*-dimethylated tyrosine and an *N*-monomethylated tyrosine congeners of **ATI7**, respectively. They were prepared by the same method as **ATI7**.

Opioid Receptor Binding

The opioid receptor affinities of the compounds were evaluated by the radioligand receptor assay.[15] The non-selective opioid receptor affinities of the compounds were evaluated by the displacement of ^{3}H-diprenorphine in rat brain membrane preparations.

Type selective receptor affinities were evaluated using selective radioligands as follows:

a) μ-Receptor: Displacement of ^{3}H-DAGO binding in rat brain P2 membrane preparations.

b) δ-Receptor: Displacement of ^{3}H-DPDPE binding in rat brain P2 membrane preparations.

c) κ-Receptor: Displacement of ^{3}H-U69533 binding in human placenta P3 preparations.

ATIn derivatives effected specific binding in rat brain P2 membrane preparations using the ^{3}H-diprenorphine (Fig. 1). Each **ATIn** derivative replaced the ^{3}H-diprenorphine dose-dependently, although it required high concentrations for the displacement (Fig. 1A). **ATI13N** having no terminal aromatic ring, displayed the weakest opioid affinity. The activities of **ATIn** derivatives were compared in the displacement of ^{3}H-diprenorphine binding under the definite concentration (10^{-5} *M*) of **ATIn** derivatives (Fig. 1B). The seven methylene (n=7) congener, **ATI7**, was the most effective in displacing the ^{3}H-diprenorphine. The congeners having fewer or more methylenes were less effective than **ATI7**, the congener with seven methylenes.

The opioid receptor selectivity of **ATIn** derivatives was studied. μ-Opioid receptor affinities of ATIn derivatives (10^{-5} *M*) were determined by the displacement of ^{3}H-DAGO, a μ-receptor selective ligand, specific binding. The **ATI7** inhibited the ^{3}H-DAGO more effectively than the other congeners. The displacement of ^{3}H-DAGO binding with **ATIn** derivatives was similar to but more apparent than that of ^{3}H-diprenorphine (Fig. 2).

ATI0 represents tyrosine itself, which was used to compare the effect of the alkyl ester moiety of **ATIn** on the receptor binding.

The δ-receptor binding affinities of **ATIn** derivatives were measured by the displacement of ^{3}H-DPDPE in the rat brain membrane preparations. ATIn derivatives (10^{-5} *M*) slightly replaced the ^{3}H-DPDPE at δ-opioid receptors. The difference of the δ-receptor affinities by the length of methylene chain was not so obvious as that of μ-receptor affinities (Fig. 3A).

The κ-receptor binding affinities of ATIn derivatives (10^{-5} *M*) were measured by the displacement of ^{3}H-U-69533 in the human placenta P3 fractions. Affinities of **ATIn** derivatives to κ-receptor was much weaker than those to μ- and δ-receptors. The difference of κ-receptor affinities corresponding to the number of methylene carbons was not observed (Fig. 3B).

As mentioned above, the compounds were shown to be more selective for μ-receptor than for the other opioid receptors. **ATI7** was most active in opioid receptor binding among the **ATIn** series.

The saturation displacement experiments of a definite concentration of **ATI7** with ^{3}H-DAGO was performed in rat brain membrane preparations. The open-circles represent the results of controls. The experiments of **ATI7** are represented by the closed-squares (10^{-6} *M*) and by the open-squares (10^{-5} *M*) (Fig. 4). The saturation curve of ^{3}H-DAGO was shifted to higher concentration in the

Fig. 1A. **Effect of ATI-derivatives on the ^{3}H-diprenorphine specific binding in rat brain P2 membranes**

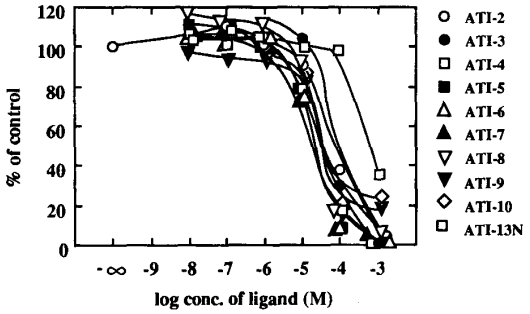
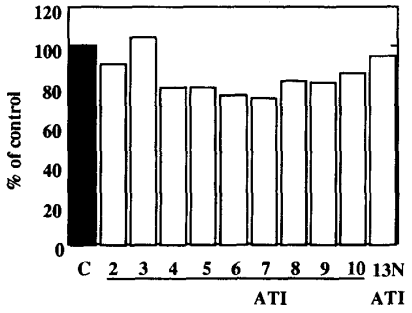


Fig. 1B. **^{3}H-Diprenorphine Binding in Rat Brain P2 membranes**



^{3}H-DAGO Binding in Rat Brain P2 membranes

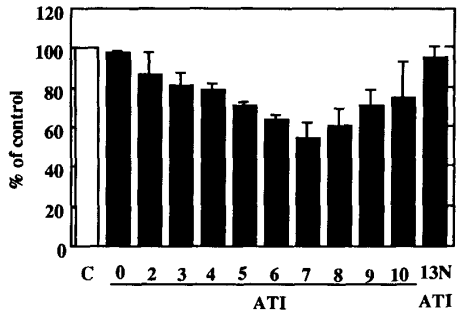


Fig. 2

^{3}H-DPDPE Binding in Rat Brain P2 membranes

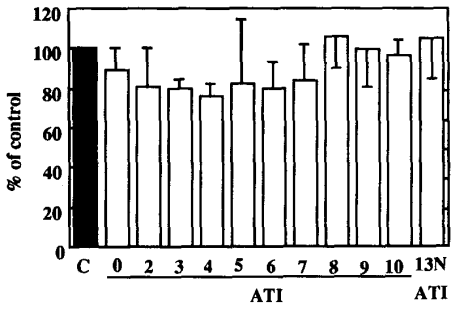


Fig. 3A

^{3}H-U-69593 Binding in human Placental P3

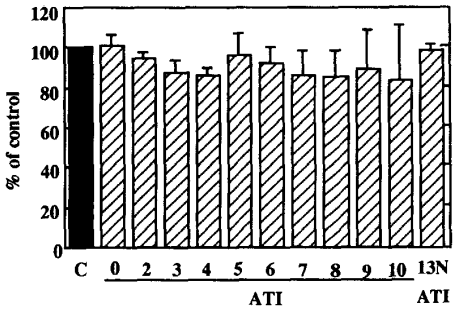


Fig.3B

Effects of ATI-7 on the ^{3}H-DAGO Binding in Rat Brain

Saturation

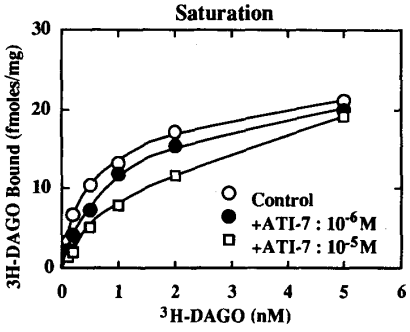


Fig. 4A

Scatchard Plot

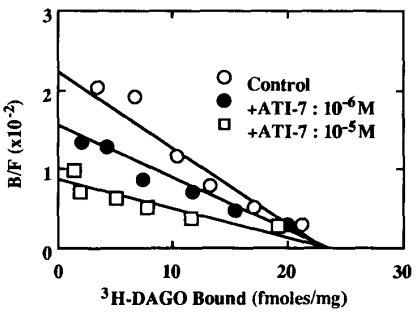


Fig. 4B

presence of **ATI7**. **ATI7** depressed the specific binding of ^{3}H-DAGO at the μ-opioid receptor. On the basis of the saturation experiment, a Scatchard analysis gave the result shown in Fig. 4B. All saturation experiments are linear plots and the lines gave the same B_{max} value. Consequently, the **ATI7** occupies the same binding site on the μ-receptor as ^{3}H-DAGO.

Adrenergic receptor affinities of **ATIn** derivatives (10^{-5} *M*) were studied using ^{3}H-clonidine as the α_2 receptor radioligand and ^{3}H-dihydroalprenolol as the β-receptor radioligand in rat brain membrane preparations (Fig. 5).[16] A few **ATIn** derivatives bounds α_2 adrenoceptors, however, there was no significant correlation between the α_2 adrenoceptor affinities of **ATIn** derivatives and the numbers of methylene carbons. No **ATIn** derivatives had specific receptor affinity to the β-adrenergic receptors.

BTI7 is an *N*, *N*-dimethylated tyrosine 7-phenylheptyl ester, and **CTI7** is an *N*-monomethylated **ATI7**. Fig. 6 shows the specific receptor affinities of **ATI7**, **BTI7** and **CTI7** with the displacement of opioid radioligands. Methylation of the tyrosine amino nitrogen effected an increase in the affinities to each type of opioid receptor.

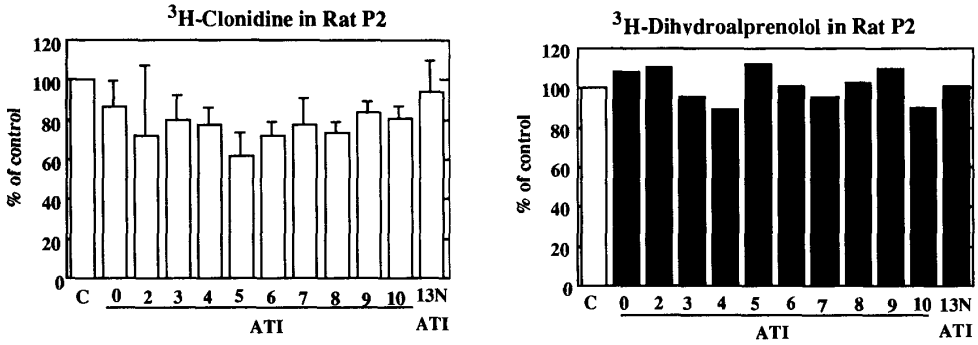


Fig. 5

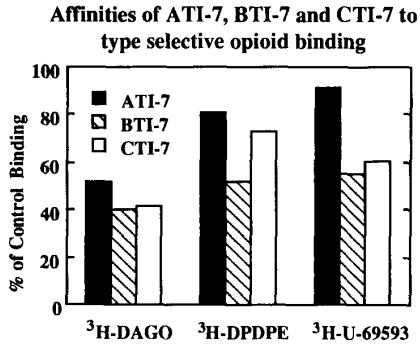


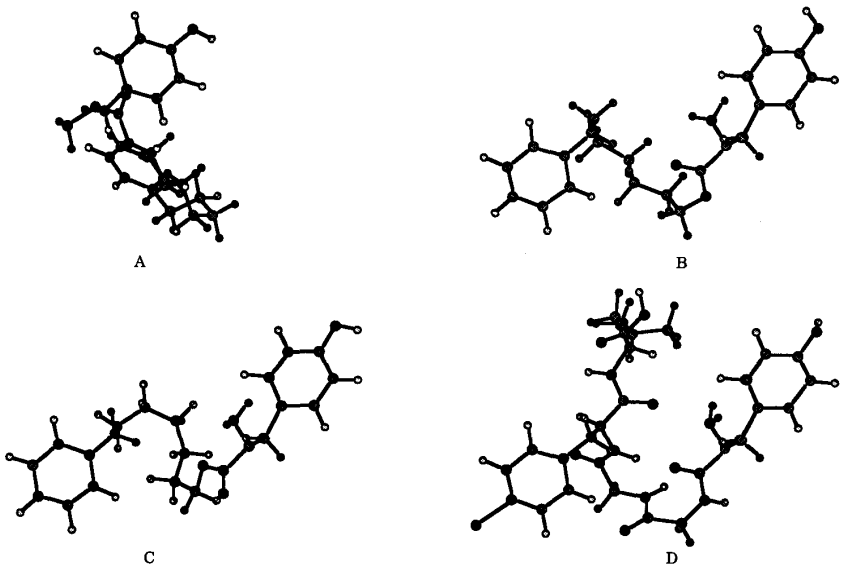
Fig. 6

Computational Chemistry

A conformation study of **ATI5-8** was done using the program, Discover CVFF force field by Molecular Simulation Inc. Alkyl chain took an antiperiplanary stretching conformation as that of lowest energy, however, MD simulation indicated a packing form was more stable than a fully extended chain form because that intramolecular hydrophobic interactions and solvent-cage effects outweigh steric repulsions in a polar solvent such as water. A partially conformationally

constrained model in which the tyramine and another phenyl ring were fixed, as well as the crystal structure of 4-bromoPhe[4]-Leu-enkephalin,[17] was simulated to give two local minimum conformers. The backbone shape of the higher energy one was similar to that of Leu-enkephalin. The carbon chain of the lower energy one was rather like to that of DPDPE, a δ-selective ligand. In the case of **ATI7**, MD simulation indicated that the energy of the partially constrained (lower en-

Fig. 7. Molecular Modeling and Simulation Study of ATI7



A. The lowest energy conformer of ATI7. B. The lower energy enkephalin-like conformer of ATI7.
C. The higher energy enkephalin-like conformer. D. The crystal structure of 4-BrPhe[4]-Leu-enkephalin.

Table 2. Conformational Energy of ATI5-ATI8 by MD Simulation.

Conformer	Energy (kcal/mol)	ATI5	ATI6	ATI7	ATI8
Extended	steric	66.209	67.295	67.611	68.234
	electrostatic	3.982	5.832	2.815	17.018
	total	**70.191**	**73.127**	**70.425**	**85.252**
Minimum	steric	58.767	64.301	62.227	61.353
	electrostatic	2.495	2.551	3.182	12.775
	total	**61.262**	**66.851**	**65.409**	**74.129**
Fixed (lower)	steric	105.938	96.525	67.068	69.809
	electrostatic	6.359	7.049	5.200	20.353
	total	**112.296**	**103.573**	**72.268**	**90.162**
Fixed (higher)	steric	89.123	85.638	84.260	83.037
	electrostatic	20.210	20.157	18.942	20.387
	total	**109.332**	**105.795**	**103.220**	**103.424**

The extended conformer has all antiperiplanar methylenes.
The fixed conformer are fixed and arranged in its tyramine and phenyl groups as well as the crystal structure of enkephalin.

ergy) conformation was only 6.86 kcal/mol higher than the minimum energy conformation, and only 1.84 kcal/mol higher than the extended conformation (Fig. 7, Table 2).

Conclusion

We endeavored to elucidate the opioid receptor binding properties of tyrosine ω-phenylalkyl esters as the simple model analogs of opioid peptides. A certain distance between tyrosine and the phenyl aromatic ring of the tyrosine ester was required for the μ- or δ-opioid receptor binding. Molecular simulation study indicated the most active compound, **ATI7**, would be able to assume a similar conformation to the crystal structure of enkephalin, and the energy discrepancy between the enkephalin-like conformation and the lowest energy one was estimated by only 6.86 kcal/mol. It suggested the specific conformation of the tyramine part and the other aromatic moiety was arranged like the crystal structure of enkephalin for the designing of the μ selective ligand. On the basis of our results, at the μ-receptor ligand binding site, the conformational arrangement of the tyramine and the aromatic part of the enkephalins is essential, however the conformation of the spacer moiety connecting the two parts is not particular. On the contrary, δ-receptor binding site would request both conformational environments.

Experimental

Materials and Methods

L-Tyrosine, 2-phenylethanol, 3-phenylpropanol, and 4-phenylbutanol were purchased from Tokyo Kasei, Tokyo. The labeled opioids used were ^{3}H-DAGO (NEN, USA), ^{3}H-DPDPE (NEN, USA), ^{3}H-U-69593 (Amersham, UK), ^{3}H-naloxone (NEN, USA), and ^{3}H-diprenorphine (Amersham, USA).

All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were recorded on a JASO IRA-100 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured using a JEOL GX-270 spectrometer with tetramethylsilane as an internal standard. Mass spectra were obtained using JEOL JMS-D300 spectrometer with direct-insertion probe at 70 eV. Analytical and preparative TLC, and column chromatography were performed on Merck silica gel.

Preparation of Tyrosine Phenylalkyl Esters.

To tyrosine (40 mmol) and phenylalkylalcohol (110 mmol) in a 125℃ oil bath was added over 5 min methanesulfonic acid (4.0 ml, 62 mmol). The reaction was stirred at 125-130℃ for 2.5 h. The brown solution was poured cautiously into 800 ml of chilled ether (dry ice-acetone bath). The resultant suspension was stirred for 2 h at room temperature and filtered, and the insoluble solid was washed with ether (3 x 300 ml) and dried in vacuo to give a crude methanesulfonate salt. To 1.5 l of fresh 0.1 *M* $NaHCO_3$ solution was added over 10 min the crushed crude ester. The suspension was stirred for 18h at room temperature and filtered and the resulting solid dried in vacuo.

The crude, crushed free base was added to 1 l of ether and this was stirred for 20 min with gentle heating. The translucent solution was filtered, and to a clear filtrate ethereal HCl was added. The white slurry was filtered and the solid was dried in vacuo. The structure of each tyrosine phenylalkyl ester was confirmed by IR, ^{1}H- and ^{13}C-NMR, and Mass spectroscopies and the elemental analysis.

Opioid Receptor Binding Studies

Cerebella from male Mongolian gerbils (65-80 g), forebrains from male guinea pigs (250-300 g), and male wistar rats (180-220 g) brains without cerebella were dissected and membrane fractions were prepared as described before.[14] P3 fraction from human placenta was also separated as described previously.[13] Membranes and P3 fractions were suspended in 50 mM tris-HCl buffer (tris buffer, pH 7.4) and kept at -80℃ until use.

The binding of ^{3}H-opioids to receptors was determined by a modification of the method of Pert and Snyder. To evaluate type selective opioid receptor binding, ^{3}H-DAGO binding in gerbil cerebellar membranes, ^{3}H-DPDPE binding in guinea pig forebrain membranes and ^{3}H-U-69593 binding in human placenta P3 fraction were used for μ-type, δ-type and κ-type, respectively. ^{3}H-naloxone and ^{3}H-diprenorphine bindings were also performed for opioid antagonistic binding site. The membrane suspension or P3 fraction containing radioligand and test compounds was incubated at 25℃ for 40 min. Then, Bound/Free-separation was done by rapid filtration using Cell Harvester over G-10 glass filters (Inotech, Switzerland). The filters were washed with ice cold tris buffer, and were dried and counted in 4 ml scintillation cocktail using scintillation counter (Beckmann-LS6500, USA). Specific binding of each ^{3}H-opioid was calculated from difference between the counts in the presence and absence of 10 μM of levorphanol for ^{3}H-DAGO, DPDPE-Cl for ^{3}H-DPDPE, bremazocine for ^{3}H-U-69593, naloxone for ^{3}H-naloxone, and diprenorphine for ^{3}H-diprenorphine. Protein concentrations were determined using BCA kit (Pierce, USA).

Competition experiments of opioids were performed with a fixed concentration of ^{3}H-opioid ligand. Ki values were calculated from competition data with Ki = IC50/(1+L/Kd); IC50 was the concentration of unlabeled ligand that caused 50 % inhibition of binding of ^{3}H-opioid, L was the concentration of radioligand, and Kd was the equilibrium dissociation constant of ^{3}H-opioid.

Statistics : Results were expressed as the mean ± SD. Statistically significant differences were determined by using two tailed unpaired Student's *t*-test.

Molecular simulations were performed on IRIS indigo^{2} with Discover CVFF force field by Molecular Simulation Inc. Dynamics were made under the condition parameters as follows: temperature = 3000K, time = 1 fs, steps = 100 x 100 = 10 ps, sampling = 100 structures, each structure was minimized.

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