P-344 New Positive Allosteric Modulators for μ Opioid Receptor from Oxytocin

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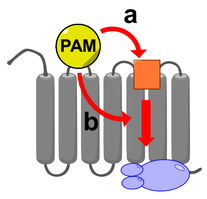
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Introduction

Positive allosteric modulators (PAMs) of G protein-coupled receptors (GPCRs) can increase the receptor signaling, the affinity of their orthosteric agonists, or both. Because PAMs can enhance the activity of an endogenous agonist which is not only secreted at an appropriate time and at a required part in our body but is also immediately metabolized, this class of modulators is expected to have fewer side effects than exogeneous orthosteric agonists. Therefore, PAMs are expected to have therapeutic advantages for the development of novel drug candidates. To date, **our group reported the first direct evidence that oxytocin (OT) functions as a PAM, but not an ago-PAM, toward the μ opioid receptor (MOR)**, which was a member of GPCR superfamily and a main target molecule of clinically used opioid analgesics. We also reported that **OT functions as a PAM at the κ opioid receptor** but not δ opioid receptor in the G protein signaling pathway[1]. Therefore, OT is expected to be a useful peptide to develop novel analgesic adjuvants. Our structure-activity relationship study of OT disclosed that the cyclic structure with an intramolecular disulfide bridge of OT was essential to exhibit the MOR-PAM activity as shown below. In addition, we found that the small Gly residue at the *C*-terminal would be a particularly critical structure for the MOR-PAM activity. **In this study, we attempted to identify side-chains of OT responsible for the MOR-PAM activity by using various OT mono-substituted analogs**. Their PAM activities for MOR agonistic activity induced by endmorphin-1 (EM-1) was evaluated by an electrical impedance-based receptor biosensor assay with CellKey™ system (label-free assay) using cells stably expressing human MOR[2].

1) Miyano, K., Uezono, Y. *et al. Cells* **2021**, *10*, 2651.
2) Miyano, K., Uezono, Y. *et al. J. Pharmacol. Sci.* **2014**, *126*, 302–309.

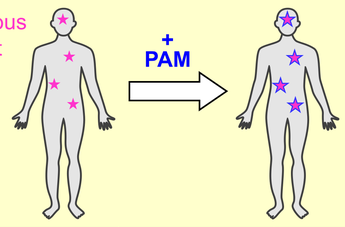
Positive allosteric modulator (PAM) of GPCRs[3]



Orthosteric agonist
a) **Binding affinity** ↑
b) **Efficacy** ↑

Signaling↑

【Merits of PAM versus synthetic agonist】

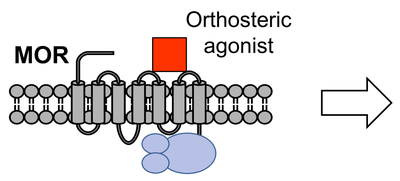


	Site of Action	During of Action
Endogenous Agonist	Localized	Transient
Exogenous Agonist	Widespread	Sustained
Endogenous Agonist + PAM	Localized	Transient

3) Burford, N. T., Alt, A. *et al.*, *Br. J. Pharmacol.*, **2015**, *172*, 277–286.

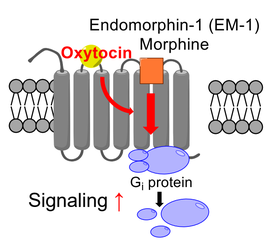
Oxytocin (OT) acts at μ opioid receptor (MOR) as a PAM[4].

μ opioid receptor (MOR) is the main target of clinically used opioid analgesics.

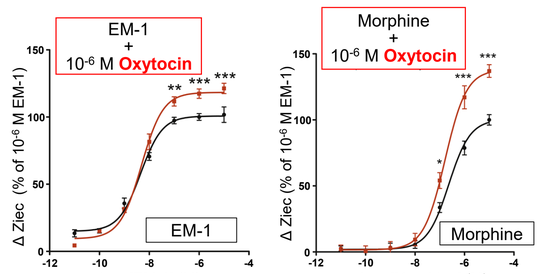


【Therapeutic efficacy】
✓ **Analgesia**

【Adverse effects】
✓ Constipation
✓ Nausea and Vomiting
✓ Respiratory depression
✓ Dependence



Oxytocin is expected to be a promising lead compound to develop novel analgesic adjuvants.



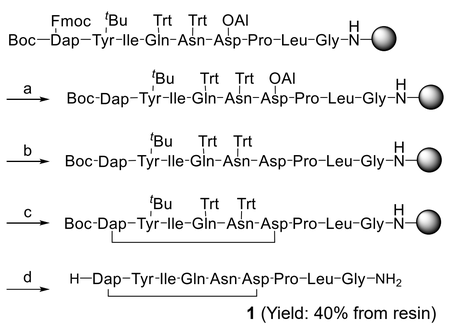
Concentration-response curves for opioid-induced ΔZiec in the absence and presence of 10^{-6} M oxytocin. All data are presented as means ± S.E.M. of 3–7 independent experiments (n = 9–21), *P < 0.05, **P < 0.01, ***P < 0.001, compared to each opioid treatment alone.

4) Meguro, Y., Uezono, Y. *et al.*, *J. Pharmacol. Sci.*, **2018**, *137*, 67–75.

Our previous SAR study[5]

Analog 1 having an amide bridge instead of the disulfide linkage

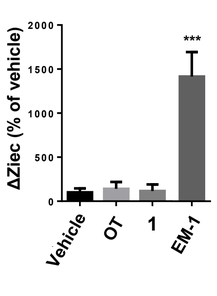
Synthesis of analog 1



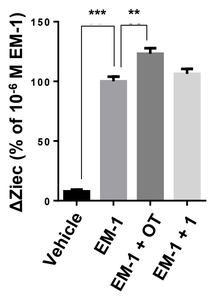
1 (Yield: 40% from resin)

Reagents and Conditions: a) 20% Piperidine/DMF, b) $Pd[P(C_6H_5)_3]_4$ (0.25 eq.), Phenylsilane (48 eq.), DCM, c) HATU (4 eq.), HOAt (4 eq.), DIPEA (8 eq.), DMF, d) TFA/TIS/H_2O (40:1:1, v/v)

MOR agonistic activity



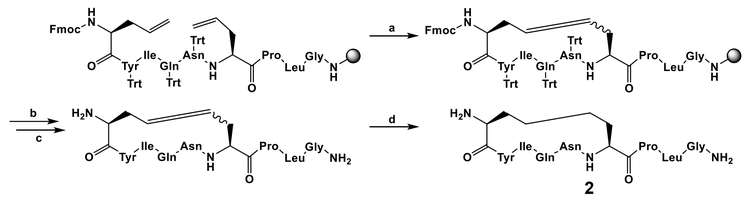
MOR-PAM activity



Flag-MOR-expressing HEK293 cells were treated with EM-1 and analog **1** at the concentration of 10^{-6} M. The data are presented as means ± S.E.M. of three to five independent experiments (n = 9–15). One-way ANOVA followed by Tukey's post hoc test. **, *** indicated $p < 0.01$, 0.001, respectively.

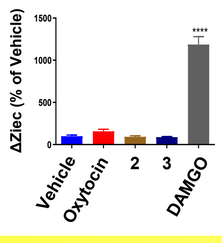
The intramolecular disulfide bridge of OT was a key structure to exert the MOR-PAM activity.

Analog 2 having an ethylene tether

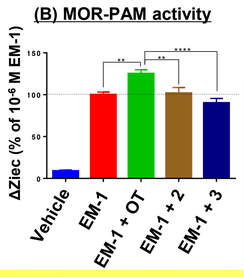


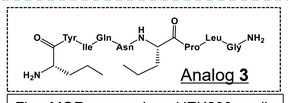
Reagents and Conditions: a) Hoveyda-Grubbs Catalyst® 2nd Generation, CH_2Cl_2, 40 °C, Ar, 12 h; b) 20% Piperidine/DMF; c) TFA, H_2O, TIS, 3 h; d) 10% Pd/C, H_2, MeOH, 20 h

(A) MOR agonistic activity



(B) MOR-PAM activity



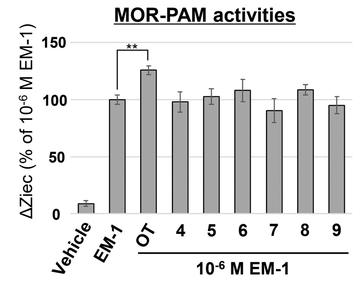


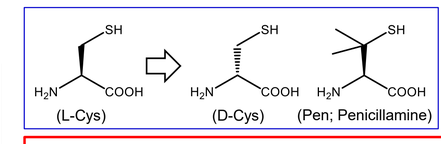
Flag-MOR-expressing HEK293 cells were treated with test analogs at the concentration of 10^{-6} M. The data are presented as means ± S.E.M. of three independent experiments (n = 6–10 and n = 12–15 in A and B, respectively). One-way ANOVA followed by Tukey's post hoc test. **, **** indicated $p < 0.01$, 0.001, respectively.

Configurational and sterical effects of cysteines

Analog	Amino-acid sequence	Yield
4	H-Cys-Gly-Ile-Gln-Asn-D-Cys-Pro-Leu-Gly-NH_2	25%
5	H-D-Cys-Tyr-Gly-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	19%
6	H-D-Cys-Tyr-Ile-Gly-Asn-D-Cys-Pro-Leu-Gly-NH_2	16%
7	H-Pen-Tyr-Ile-Gln-Gly-Cys-Pro-Leu-Gly-NH_2	30%
8	H-Cys-Tyr-Ile-Gln-Asn-Pen-Gly-Leu-Gly-NH_2	25%
9	H-Pen-Tyr-Ile-Gln-Asn-Pro-Gly-Gly-NH_2	22%

MOR-PAM activities





Flag-MOR-expressing HEK293 cells were treated with test analogs at the concentration of 10^{-6} M. The data are presented as means ± S.E.M. of two independent experiments (n = 5), one-way ANOVA, followed by Tukey's post hoc test. ** indicated $p < 0.01$.

The configurations of two cysteine residues in OT presumably affect its PAM activity at the MOR. The sterically unhindered linker containing the disulfide bond would be necessary for exerting the MOR-PAM activity of OT.

5) Mizuguchi, T., Fujii, H. *et al.*, *Peptides* **2023**, *159*, 302–309.

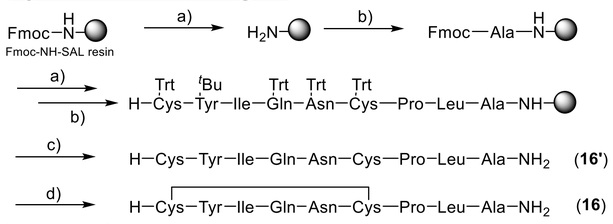
Results and Discussion

1. Investigation of responsible side-chains of oxytocin for MOR-PAM activity

List of analogs 10–24

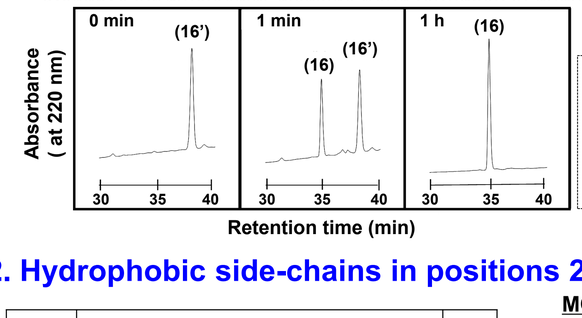
Analog	Amino-acid sequence	Yield
OT	H-Cys[1]-Tyr[2]-Ile[3]-Gln[4]-Asn[5]-Cys[6]-Pro[7]-Leu[8]-Gly[9]-NH_2	38%
10	H-Cys-Ala-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	26%
11	H-Cys-Tyr-Ala-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	37%
12	H-Cys-Tyr-Ile-Ala-Asn-Cys-Pro-Leu-Gly-NH_2	37%
13	H-Cys-Tyr-Ile-Gln-Ala-Cys-Pro-Leu-Gly-NH_2	25%
14	H-Cys-Tyr-Ile-Gln-Asn-Cys-Ala-Leu-Gly-NH_2	50%
15	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Ala-Gly-NH_2	36%
16	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Ala-NH_2	34%
17	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-OH	30%
18	Ac-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Ala-NH_2	23%
19	H-Cys-Gly-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	17%
20	H-Cys-Tyr-Gly-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	39%
21	H-Cys-Tyr-Ile-Gly-Asn-Cys-Pro-Leu-Gly-NH_2	27%
22	H-Cys-Tyr-Ile-Gln-Gly-Cys-Pro-Leu-Gly-NH_2	54%
23	H-Cys-Tyr-Ile-Gln-Asn-Cys-Gly-Leu-Gly-NH_2	31%
24	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Gly-Gly-NH_2	38%

Synthesis of analog 16



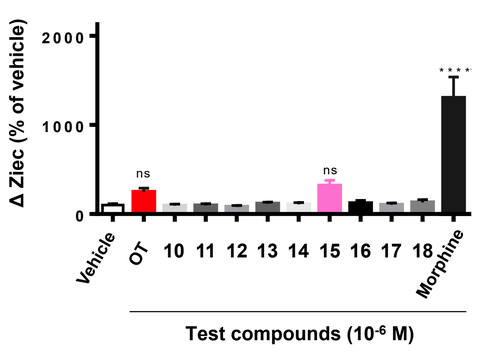
Reagents and Conditions:
a) 20% piperidine/DMF, b) Fmoc-AA-OH (3 eq.), DIPCDI (3 eq.), HOBt·H_2O (3 eq.), DMF, c) TFA/TIS/EDT/H_2O (40:1:1:1, v/v) , d) 0.1 M NH_4HCO_3 aq. (pH ~8.0)

*<Oxidative intramolecular disulfide bond formation in analog **16**>*



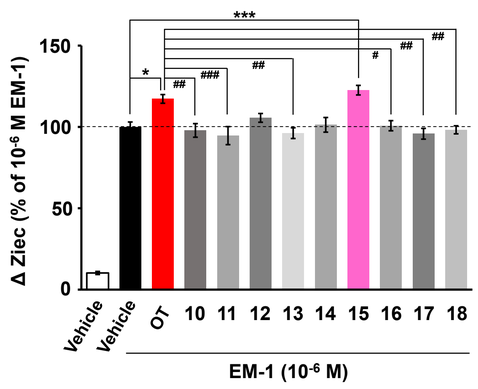
The cyclization reaction was monitored by analytical HPLC [$5C_{18}$-AR-II (4.6 mm I.D. × 150 mm); solvent, 0.1% TFA/CH_3CN-H_2O (gradient from 5:95 to 25:75 over a period of 40 min) at a flow rate of 1.0 mL/min, detected at 220 nm].

MOR agonistic activities of 10–18



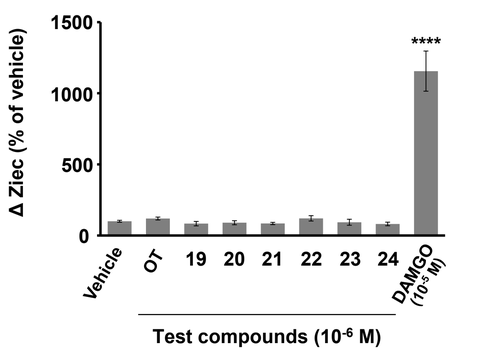
Flag-MOR-expressing HEK293 cells were treated with test analogs at the concentration of 10^{-6} M. The data are presented as means ± S.E.M. of three independent experiments (n = 9), One-way ANOVA followed by Tukey's multiple-comparison test. ****$p < 0.0001$ vs. vehicle alone. ns: not significant.

MOR-PAM activities of 10–18



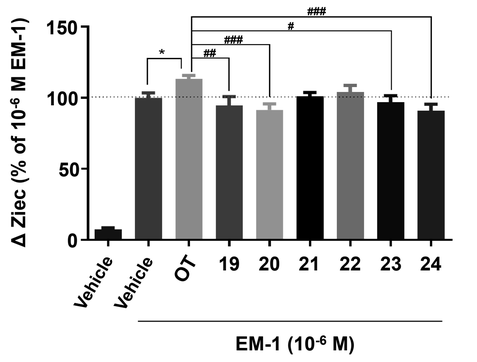
The data are presented as the means ± S.E.M. of three independent experiments (n = 9). One-way ANOVA followed by Tukey's multiple-comparison test. *$p < 0.05$, ***$p < 0.001$ vs. EM-1; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. EM-1 + OT.

MOR agonistic activities of 19–24



The data are presented as means ± S.E.M. of three independent experiments (n = 9–13), One-way ANOVA followed by Bonferroni's multiple comparison test, ****$p < 0.0001$ vs. vehicle alone.

MOR-PAM activities of 19–24



The data are presented as means ± S.E.M. of three independent experiments (n = 6–7), One-way ANOVA followed by Bonferroni's multiple comparison test. *$p < 0.05$ vs. EM-1; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. EM-1 + OT.

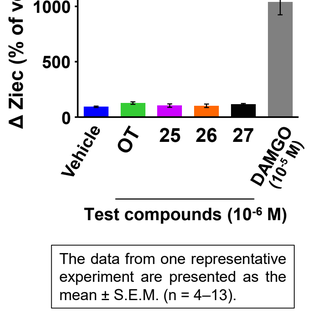
The assay results of analogs **10**–**15** and **19**–**24** indicated that side-chains of Tyr^2, Ile^3, Gln^4, Asn^5, and Pro^7 would be necessary for the MOR-PAM effect of OT. The results of analogs **17** and **18** suggested that the charge states of both termini of OT may contribute to its MOR-PAM effect. The results of analogs **16** supported the importance of Gly in position 9 for the MOR-PAM activity of OT.

2. Hydrophobic side-chains in positions 2 and 8

Analog	Amino-acid sequence	Yield
25	H-Cys-Phe-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	17%
26	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Phe-Gly-NH_2	39%
27	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Cha-Gly-NH_2	27%

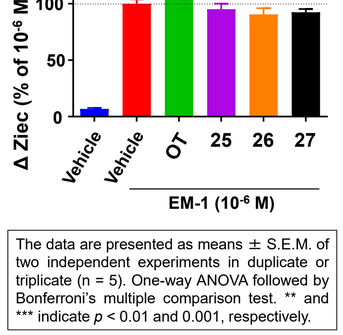
Small-sized hydrophobic amino acids in the range from Ala to Leu would be suitable for position 8 of OT. In addition, the phenolic hydroxy group of Tyr in position 2 may play a role in exerting the MOR-PAM activity.

MOR agonistic activities



The data from one representative experiment are presented as the mean ± S.E.M. (n = 4–13).

MOR-PAM activities

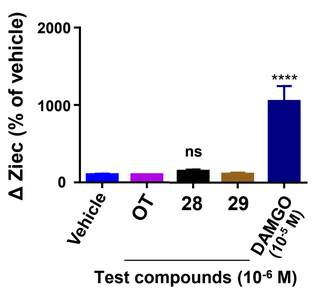


The data are presented as means ± S.E.M. of two independent experiments in duplicate or triplicate (n = 5). One-way ANOVA followed by Bonferroni's multiple comparison test. ** and *** indicate $p < 0.01$ and 0.001, respectively.

3. MOR-PAM activities of oxypressin and vasotocin

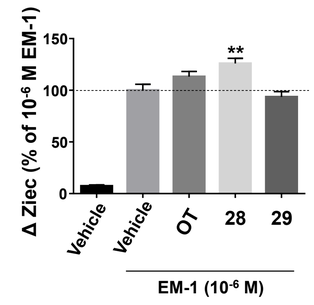
Analog	Amino-acid sequence	Yield
28 (Oxypressin)	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	43%
29 (Vasotocin)	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2	39%

MOR agonistic activities



The data are presented as means ± S.E.M. of three independent experiments (n = 8–9), One-way ANOVA followed by Tukey's test. ****$p < 0.0001$ vs. vehicle. ns: not significant.

MOR-PAM activities



The data are presented as means ± S.E.M. of three independent experiments (n = 9), One-way ANOVA followed by Tukey's test. **$p < 0.01$ vs. vehicle + EM-1.

These findings suggest that the size of the hydrophobic moiety at position 3 of OT would be a critical determinant of MOR-PAM activity.

4. Concentration-dependent MOR-PAM activity

The data are presented as means ± S.E.M. of three independent experiments (n = 6–15). One-way ANOVA followed by Dunnet's test. ***P < 0.005 vs. EM-1.

Analog **15** possessing Ala instead of Leu in the position 8 concentration-dependently showed almost comparable MOR-PAM activity to OT.

Conclusion

In this study, we synthesized and evaluated various OT analogs to identify side-chains of OT responsible for the MOR-PAM activity. Among analogs **10**–**16** and **19**–**24**, only analog **15** containing Ala instead of Leu in position 8 exhibited the MOR-PAM activity. These results suggested that other side-chains of Tyr^2, Ile^3, Gln^4, Asn^5, and Pro^7 would be necessary for the PAM effect of OT. On the other hand, analogs **24**, **26** and **27** containing Gly, Phe and Cha, respectively, in the position 8 did not enhance the agonistic activity of EM-1 for the MOR. Therefore, small-sized hydrophobic amino acids in the range from Ala to Leu would be suitable for the position 8. In addition, the results of analogs **17** and **18** indicated that the uncharged *C*-terminal amide and the positively charged *N*-terminal primary amine of OT under physiological conditions are likely to contribute to the MOR-PAM activity. Furthermore, we found that analog **28** having Phe instead of Ile in position 3 exerted the MOR-PAM activity approximately at the same level as OT. This result indicated that hydrophobicity in position 3 of OT would also play an important role in the MOR-PAM activity. Analogs **15** and **28** are clearly new peptide MOR-PAMs identified following OT.

Acknowledgement

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We have no conflicts of interest with regard to the presentation.