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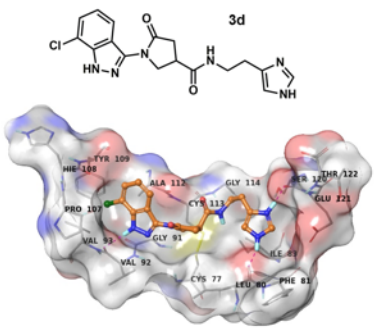
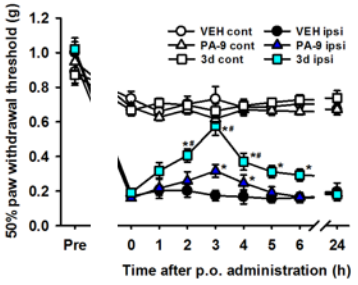


Novel PAC1 receptor antagonist



Orally available analgesic in the treatment of the neuropathic pain

Synthesis of a novel and potent small-molecule antagonist of PAC1 receptor for the treatment of neuropathic pain

Ichiro Takasaki [a, b, *], Haruna Ogashi [c], Takuya Okada [b, c], Ayaka Shimodaira [a], Daichi Hayakawa [d], Ai Watanabe [a], Atsuro Miyata [e], Takashi Kurihara [e], Hiroaki Gouda [d], Naoki Toyooka [b, c, *]

[a] Department of Pharmacology, Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan

[b] Graduate School of Innovative Life Science, University of Toyama, Toyama 930-0194 and 930-8555, Japan

[c] Department of Bio-functional Molecular Engineering, Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan

[d] Department of Analytical and Physical Chemistry, School of Pharmacy, Showa University, Tokyo 142-8555, Japan

[e] Department of Pharmacology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan

*** Corresponding authors;**

Ichiro Takasaki, Ph.D. (*in vitro/vivo* Pharmacology)
Department of Pharmacology, Graduate School of Science and Engineering,
University of Toyama, Toyama 930-8555, Japan
Tel: +81-76-445-6875
E-mail; takasaki@eng.u-toyama.ac.jp

Naoki Toyooka, Ph.D. (Bioinformatics and Organic Synthesis)
Department of Bio-functional Molecular Engineering, Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan
Tel: +81-76-445-6859
E-mail; toyooka@eng.u-toyama.ac.jp

ABSTRACT

We recently identified novel small-molecule antagonists of the PACAP type I (PAC1) receptor using docking-based *in silico* screening followed by *in vitro/vivo* pharmacological assays. In the present study, we synthesized 18 novel derivatives based on the structure of PA-9, a recently developed antagonist of the PAC1 receptor, with a view to obtain a panel of compounds with more potent antagonistic and analgesic activities. Among them, compound **3d** showed improved antagonistic activities. Intrathecal injection of **3d** inhibited both pituitary adenylate cyclase-activating polypeptide (PACAP) and spinal nerve ligation-induced mechanical allodynia. The effects were more potent than PA-9. Compound **3d** also showed anti-allodynic effects following oral administration. Hence, our results suggest that **3d** may become an orally available analgesic in the treatment of the neuropathic pain.

KEYWORDS

PACAP; PAC1 receptor; small-molecule antagonist; neuropathic pain; allodynia; analgesics

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from ovine hypothalamic extracts based on its ability to stimulate adenylate cyclase in rat anterior pituitary cell cultures [1]. PACAP exists as two variants, a 38-amino acid form (PACAP38) and a 27-amino acid C-terminal truncated form (PACAP27) [1, 2]. Both peptides share 68% homology with vasoactive intestinal polypeptide (VIP), indicating that PACAP belongs to the VIP/secretin/glucagon superfamily [1, 2, 3]. Three distinct G-protein-coupled receptors mediate the actions of PACAP and VIP. Unlike VIP, the two forms of PACAP bind to PACAP type I (PAC1) receptor, which is coupled mainly to adenylate cyclase/protein kinase A, with high affinity and selectivity. $VPAC_1$ and $VPAC_2$ receptors, also primarily coupled to adenylate cyclase, however, interact with PACAP and VIP with similar affinities [3, 4].

Previously, we demonstrated that a single intrathecal (i.t.) injection of PACAP or maxadilan (Max), a selective PAC1 receptor agonist [5, 6], induced transient nociceptive behaviors followed by a long-lasting mechanical allodynia in mice [7, 8]. Furthermore, we also demonstrated that the induction of PACAP- or Max-induced transient nociceptive behaviors and mechanical allodynia were prevented by spinal pretreatment with max.d.4, a peptide antagonist for the PAC1 receptor [7, 8]. These results suggest that the spinal PACAP-PAC1 receptor systems play an important role in the modulation of spinal nociceptive

transmission and induction of chronic pain. Our findings, together with the previous studies, indicate that the PAC1 receptor is a potential drug target for the treatment of both inflammatory pain and neuropathic pain.

Recently, we identified novel small-molecule antagonists of the PAC1 receptor, named PA-8, PA-9, and PA-10, using docking-based *in silico* screening followed by *in vitro/vivo* pharmacological assays [9]. The results of the *in vivo* pharmacological assays demonstrated that i.t. injection of these compounds blocked induction of PACAP-induced aversive responses and mechanical allodynia in mice. In contrast, the compounds themselves exerted neither agonistic nor algesic effects in the *in vitro/vivo* assays. We therefore concluded that PA-8, PA-9, and PA-10 could be used as seed compounds for developing novel analgesics. The current study focuses on the synthesis of 18 derivatives of PA-9 to obtain novel compounds exhibiting improved antagonistic and analgesic activities. Among the newly synthesized derivatives, we identified **3d** as the compound with more potent antagonistic and analgesic activities than PA-9.

2. Results and discussion

2.1. Synthesis of PA-9 derivatives

Based on the analysis of the PA-9 structure and the docking model of the compound with the PAC1 receptor (Fig. 1) [9], it was determined that the imidazole ring of the histamine moiety in PA-9 would be important for the antagonistic and analgesic activities. This was explained by the presence of hydrogen bonding between the S120 and L80 amino acid residues of the PAC1 receptor and the imidazole functionality.

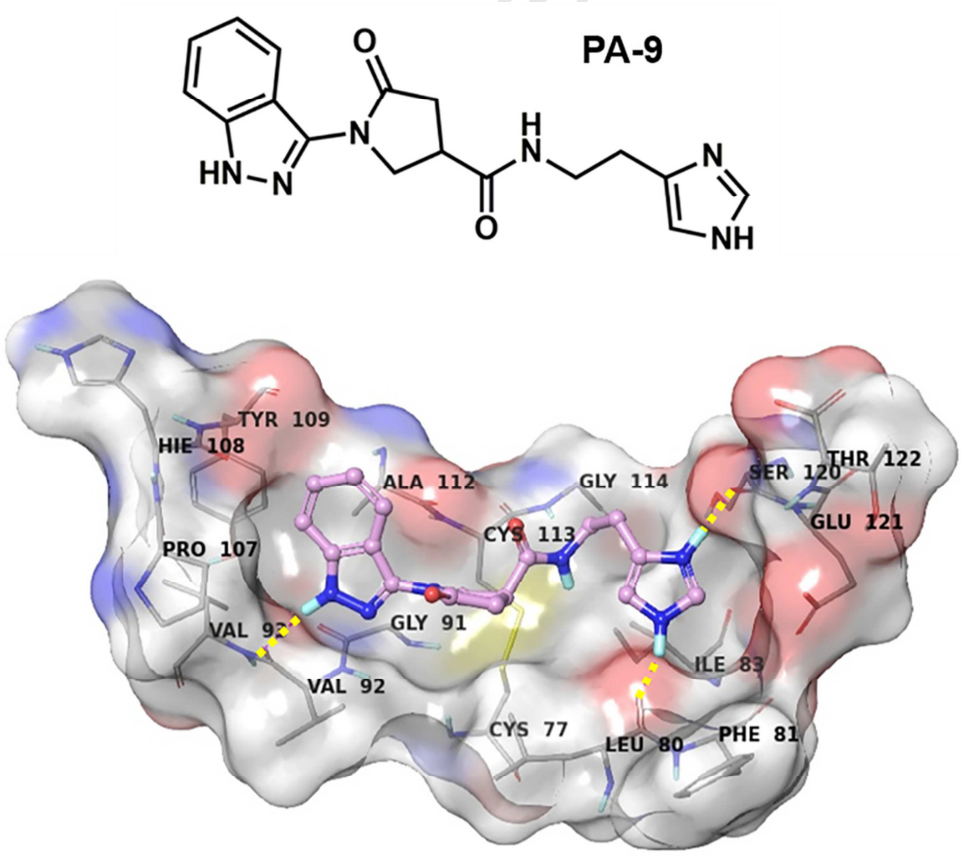
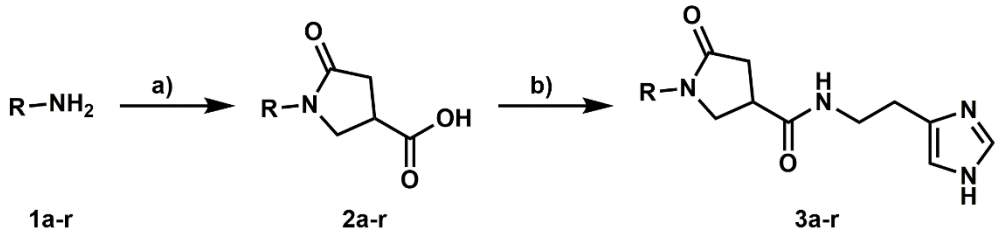


Fig. 1 Docking model of PA-9 with PAC1 receptor. Hydrogen-bonding interactions are indicated by yellow dashed lines.

Hence, we designed a number of derivatives containing different aromatic substituents on the nitrogen atom of the central lactam ring system, as shown in **Scheme 1**. Treatment of itaconic acid with appropriate amines (**1a**-**r**) afforded lactams (**2a**-**r**). Condensation of **2a**-**r** with histamine resulted in the formation of the corresponding amides (**3a**-**r**).



1a-r	2a-r	3a-r
1a : R = 4-Cl-indazol	2a : R = 4-Cl-indazol	3a : R = 4-Cl-indazol (47% in 2 steps)
1b : R = 5-Cl-indazol	2b : R = 5-Cl-indazol	3b : R = 5-Cl-indazol (23% in 2 steps)
1c : R = 6-Cl-indazol	2c : R = 6-Cl-indazol	3c : R = 6-Cl-indazol (22% in 2 steps)
1b : R = 7-Cl-indazol	2b : R = 7-Cl-indazol	3b : R = 7-Cl-indazol (35% in 2 steps)
1e : R = Ph	2e : R = Ph	3e : R = Ph (63% in 2 steps)
1f : R = 4-Me-Ph	2f : R = 4-Me-Ph	3f : R = 4-Me-Ph (82% in 2 steps)
1g : R = 4-Cl-Ph	2g : R = 4-Cl-Ph	3g : R = 4-Cl-Ph (74% in 2 steps)
1h : R = 4-F-Ph	2h : R = 4-F-Ph	3h : R = 4-F-Ph (87% in 2 steps)
1i : R = 4-OMe-Ph	2i : R = 4-OMe-Ph	3i : R = 4-OMe-Ph (83% in 2 steps)
1j : R = 4-CN-Ph	2j : R = 4-CN-Ph	3j : R = 4-CN-Ph (47% in 2 steps)
1k : R = 2-OH-Ph	2k : R = 2-OH-Ph	3k : R = 2-OH-Ph (25% in 2 steps)
1l : R = 3-OH-Ph	2l : R = 3-OH-Ph	3l : R = 3-OH-Ph (47% in 2 steps)
1m: R = 4-OH-Ph	2m: R = 4-OH-Ph	3m: R = 4-OH-Ph (41% in 2 steps)
1n : R = pyrazol	2n : R = pyrazol	3n : R = pyrazol (28% in 2 steps)
1o : R = Bn	2o : R = Bn (64%)	3o : R = Bn (76%)
1p : R = 2-OH-Bn	2p : R = 2-OH-Bn (58%)	3p : R = 2-OH-Bn (30%)
1q : R = 3-OH-Bn	2q : R = 3-OH-Bn (77%)	3q : R = 3-OH-Bn (40%)
1r : R = 4-OH-Bn	2r : R = 4-OH-Bn (66%)	3r : R = 4-OH-Bn (29%)

Reagents and conditions: a) itaconic acid, heating at 150 °C with no solvent *or* reflux in H_2O
b) DCC, HOBt, histamine, CH_2Cl_2/DMF *or* EDC, DMAP, histamine, CH_2Cl_2/DMF

Scheme 1: Synthesis of PA-9 derivatives.

2.2. In vitro pharmacological assay

In order to evaluate the antagonistic activity of the 18 synthesized compounds (**3a-r**, **Scheme 1**) on the PAC1 receptors, we examined their effects on the PACAP-induced phosphorylation of CREB in PAC1 receptor-expressing CHO (PAC1/CHO) cells. PA-9 [9]

was used as a control antagonist. PACAP (10 pM to 1 μM) dose-dependently induced CREB phosphorylation (data not shown) and a submaximal dose of PACAP (1 nM) was employed for the pharmacological assays. Pretreatment with PA-9 (1 nM) significantly inhibited PACAP (1 nM)-induced CREB phosphorylation (Fig. 2), which is consistent with our previous report [9]. Among the 18 derivatives (**3a-3r**), compound **3d** (1 nM) considerably attenuated PACAP (1 nM)-induced CREB phosphorylation (Fig. 2). Moreover, the inhibitory effects of **3d** (11.9% ± 8.5%) were more potent than those of PA-9 (30.6% ± 11.2%).

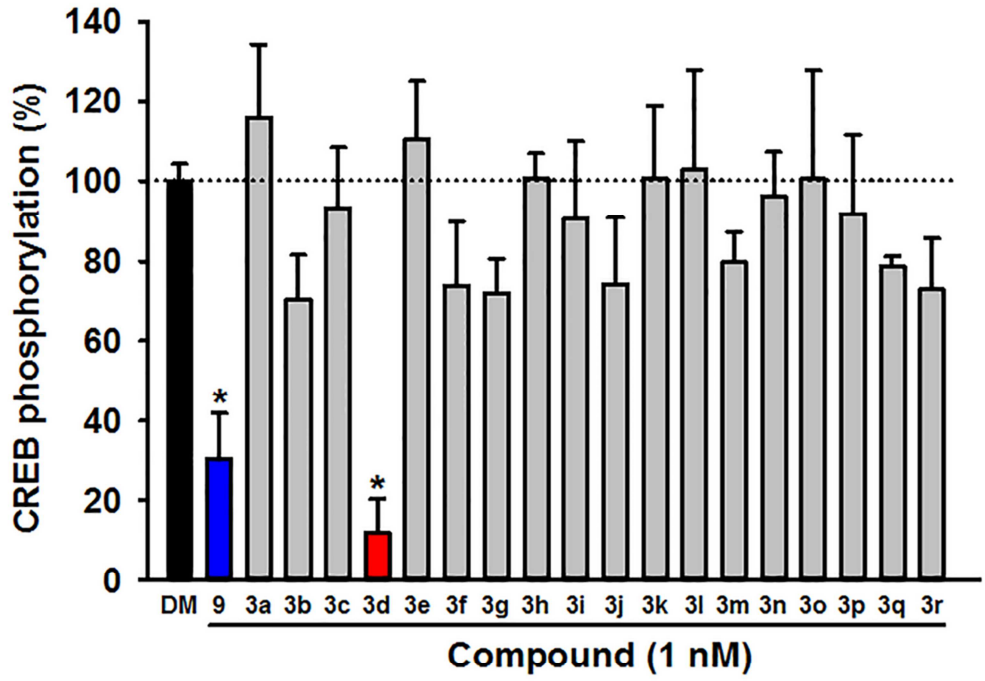


Fig. 2 Effects of 18 derivative compounds (**3a-3r**) on the PACAP-induced phosphorylation of CREB in the PAC1/CHO cells. The cells were incubated with PA-9 (1 nM) or each compound (1 nM) for 30 min, then the cells were stimulated with PACAP (1 nM) for 30 min and lysed. The lysates containing equivalent amounts of protein (20 μg) were subjected to SDS-PAGE. Immunoblots were probed with specific antibodies that recognize phosphorylated form of CREB, and total CREB. The data represent the mean ± SEM (n = 4-5). *P < 0.05 vs. DMSO (DM) control.

Next, we examined the dose-dependencies of PA-9 and **3d** (Fig. 3). Both PA-9 and **3d** (10 pM to 10 nM) dose-dependently inhibited PACAP (1 nM)-induced CREB phosphorylation. The inhibitory effects of **3d** (IC_{50} < 10 pM) in this case were also more potent than those of PA-9 (IC_{50} = 182 pM).

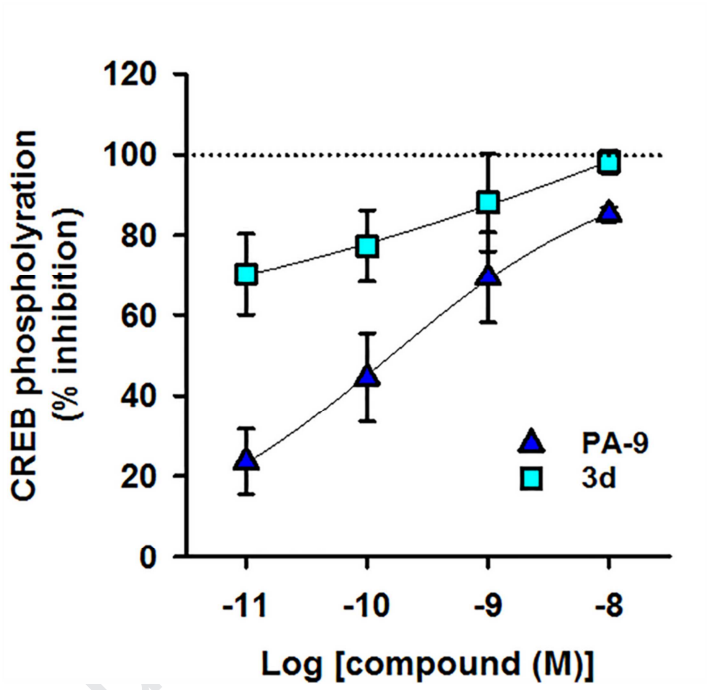


Fig. 3 Effects of **3d** or PA-9 on the PACAP-induced phosphorylation of CREB in the PAC1/CHO cells. The cells were incubated with **3d** or PA-9 compounds (10 pM to 10 nM) for 30 min, then the cells were stimulated with PACAP (1 nM). The data represent the mean ± SEM (*n* = 3-5).

2.3. In vivo pharmacological assay

We previously demonstrated that a single intrathecal (i.t.) injection of PACAP (100 pmol/5 µl) induced a long-lasting mechanical allodynia of the hind paw in mice [8, 9]. The mechanical allodynia was also induced by maxadilan, a PAC1 receptor-selective agonist, but not by VIP, a $VPAC_{1/2}$ receptor agonist. In addition, we also confirmed that the induction of

PACAP-induced mechanical allodynia was prevented by spinal pretreatment with max.d.4, a peptide antagonist for the PAC1 receptor [8]. These results suggest that i.t. PACAP-induced mechanical allodynia was mediated by stimulation of the spinal PAC1 receptors. With this in mind, we examined the effects of PA-9 and **3d** on the induction of PACAP-induced mechanical allodynia (Fig. 4).

In accordance with our previous report [8, 9], a single i.t. injection of PACAP (100 pmol) induced mechanical allodynia, which manifested one day after injection and persisted for a minimum of 7 days (Fig. 4). Co-injection of PA-9 or **3d** (100 pmol each) with PACAP (100 pmol), however, significantly blocked the induction of PACAP-induced mechanical allodynia (Fig. 4). The inhibitory effects of **3d** were more potent than those of PA-9 (Fig. 4).

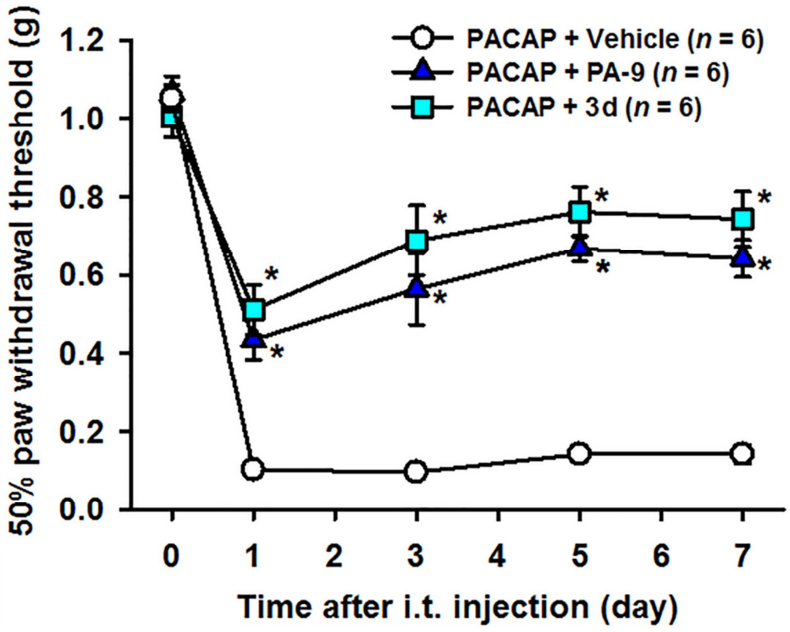


Fig. 4 Effects of **3d** or PA-9 on the PACAP-induced mechanical allodynia. A single i.t. injection of PACAP (100 pmol) induced long-lasting mechanical allodynia and the induction of allodynia was prevented by co-injection of **3d** or PA-9 (100 pmol each). The data represent the mean ± SEM (n = 6). *P < 0.05, when compared with vehicle.

With a view to evaluating these effects on neuropathic pain, we subsequently examined the impact of PA-9 and **3d** on spinal nerve ligation (SNL)-induced mechanical allodynia in mice (Fig. 5). Several lines of evidence indicate that spinal PACAP and its receptor systems could play an important role in the induction of peripheral nerve injury-induced neuropathic pain. For example, development of neuropathic pain induced by SNL was shown to be abrogated in PACAP-deficient mice [10], and SNL-induced mechanical allodynia was ameliorated by treatment with intrathecal PACAP 6–38, an antagonist of the PACAP receptor [11].

In this study, behavioral testing was performed with mice at 14 days after SNL. Compared with contralateral paws, the SNL induced marked mechanical hypersensitivity of the hind paw ipsilateral to the ligation (Fig. 5A and B). A single i.t. injection of PA-9 (100 pmol) or **3d** (100 pmol) produced a significant decrease in mechanical allodynia (Fig. 5A). Furthermore, the inhibitory effect peaked at 2-3 hours after the i.t. injection and persisted for at least 24 hours. It is noteworthy that at 3 hours after the injection, the inhibitory effect of **3d** was significantly stronger than that of PA-9. In contrast, both PA-9 and **3d** failed to influence paw withdrawal responses in contralateral paws (Fig. 5A).

Finally, we investigated whether systemic (p.o.) administration of PA-9 or **3d** is effective against the SNL-induced allodynia (Fig. 5B). We found that a single p.o. administration of both PA-9 (30 mg/kg) and **3d** (30 mg/kg) significantly ameliorated the SNL-induced

mechanical allodynia. The inhibitory effects of PA-9 and **3d** peaked at 3 hours after p.o. administration. At 2 to 4 hours after p.o. administration, the inhibitory effect of **3d** was notably stronger than that of PA-9. In contrast, both PA-9 and **3d** failed to influence paw withdrawal responses in contralateral paws.

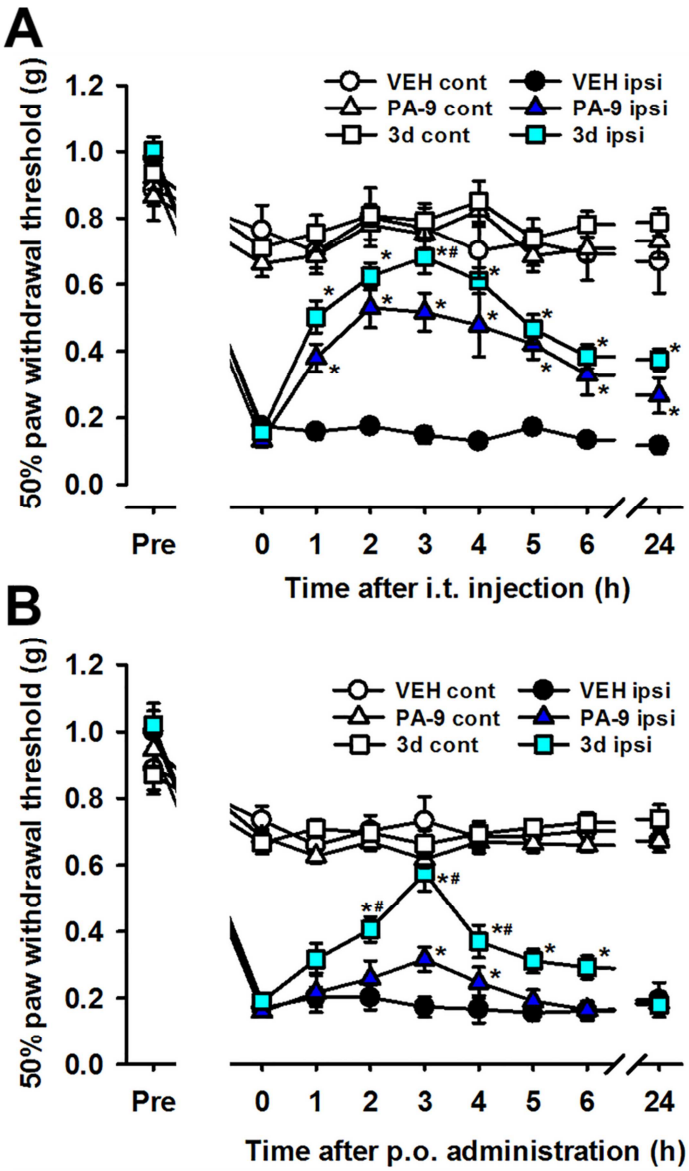


Fig. 5 Effects of **3d** or PA-9 on the SNL-induced mechanical allodynia. (A) **3d** (100 pmol, *n* = 6) or PA-9 (100 pmol, *n* = 6) was intrathecally injected 14 days after SNL. (B) **3d** (30 mg/kg, *n* = 6) or PA-9 (30 mg/kg, *n* = 6) was perorally administered 14 days after SNL. The data represent the mean ± SEM (*n* = 6). **P* < 0.05, when compared with vehicle. $^{\#}P$ < 0.05, when compared with PA-9.

*2.4. Models of **3d** binding to PAC1 receptor.*

Since **3d** showed potent and specific antagonistic activity toward the PAC1 receptor, we constructed a model of the compound binding to the *N*-terminal EC domain of PAC1 (Fig. 6). Similarly to PA-9 (Fig. 1), compound **3d** was predicted to be well accommodated within the PAC1 binding pocket (Fig. 6). Comparably to PA-9, **3d** was also envisaged to form three hydrogen bonds with the backbone carbonyl oxygen atoms of L80, V92, and S120 amino acid residues.

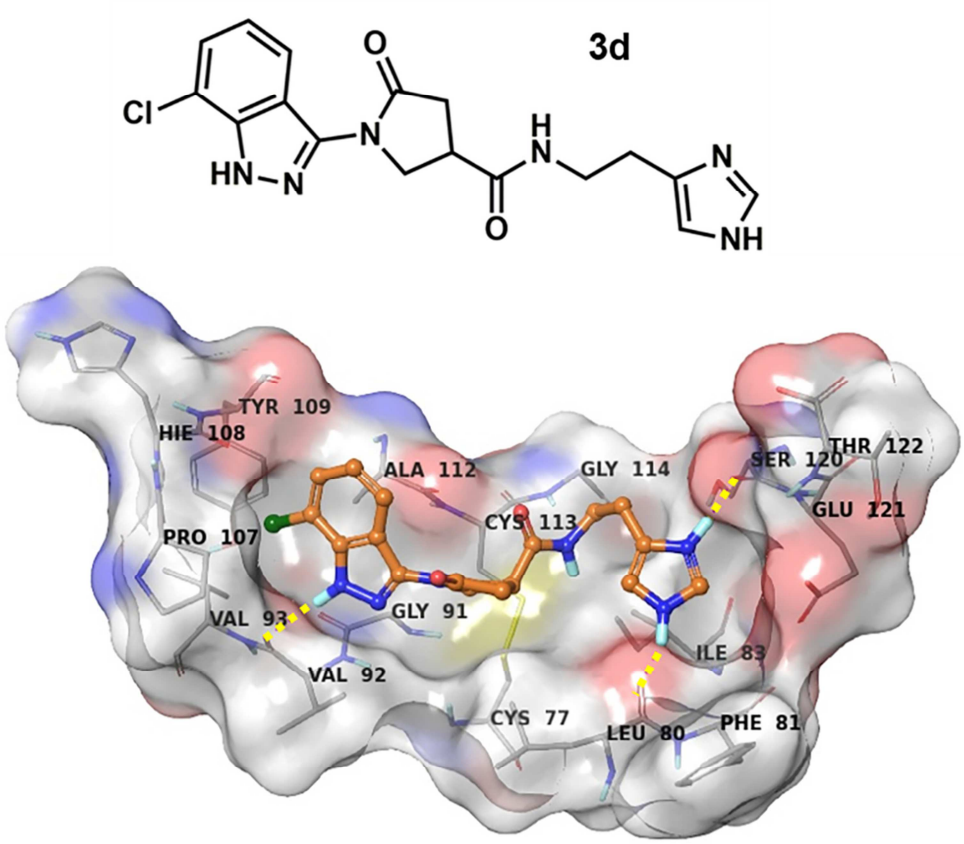


Fig. 6 Docking model of **3d** with PAC1 receptor. Hydrogen-bonding interactions are indicated by yellow dashed lines.

In order to explore the antagonistic activity of **3d** from the energetic perspective, we calculated binding free energy (ΔG_{bind}) and ligand efficiency (ΔG_{bind}/number of heavy atoms) [12] based on molecular mechanics generalized Born surface area (MM-GBSA) methods (Table 1). ΔG_{bind} and ligand efficiency values were also estimated for **3a**–**3c** (Scheme 1), which possess the same chlorine group at different positions with no or weak antagonistic activity (Fig. 2). The ranking of ligand efficiency was **3d** > PA-9 ≈ **3b** > **3a** > **3c**, which is consistent with the trend observed in the inhibitory activity studies (Fig. 2). This suggests that better ligand efficiency of **3d** would contribute its most potent antagonistic activity. Therefore, we next explored the source of better ligand efficiency of **3d**.

Table 1 Binding free energy (ΔG_{bind}) and ligand efficiency of PA-9 or its derivative compounds to the PAC1 receptor

Compounds	ΔG_{bind} (kcal/mol)	Ligand Efficiency (ΔG_{bind} /Number of heavy atoms)
PA-9	-46.397	-1.856
3a	-47.896	-1.842
3b	-48.237	-1.855
3c	-44.406	-1.708
3d	-49.456	-1.902

Comparing **3d** with PA-9, the ΔG_{bind} of **3d** was calculated to be approximately 3.06 kcal/mol more stable than that of PA-9. This difference seemed to be resulting from a more favorable van der Waals interaction energy of **3d** with the PAC1 receptor, which was

approximately 2.64 kcal/mol greater than that of PA-9. Although the interaction of **3d** with the PAC1 receptor was very similar to that of PA-9, the chloro group on the indazole ring of **3d** was suggested to be more deeply located in the sub-pocket formed by the V93, P107, H108, and Y109 amino acid residues of the PAC1 receptor (Fig. 7). The chloro functionality is likely to lead to more favorable van der Waals interactions of **3d**.

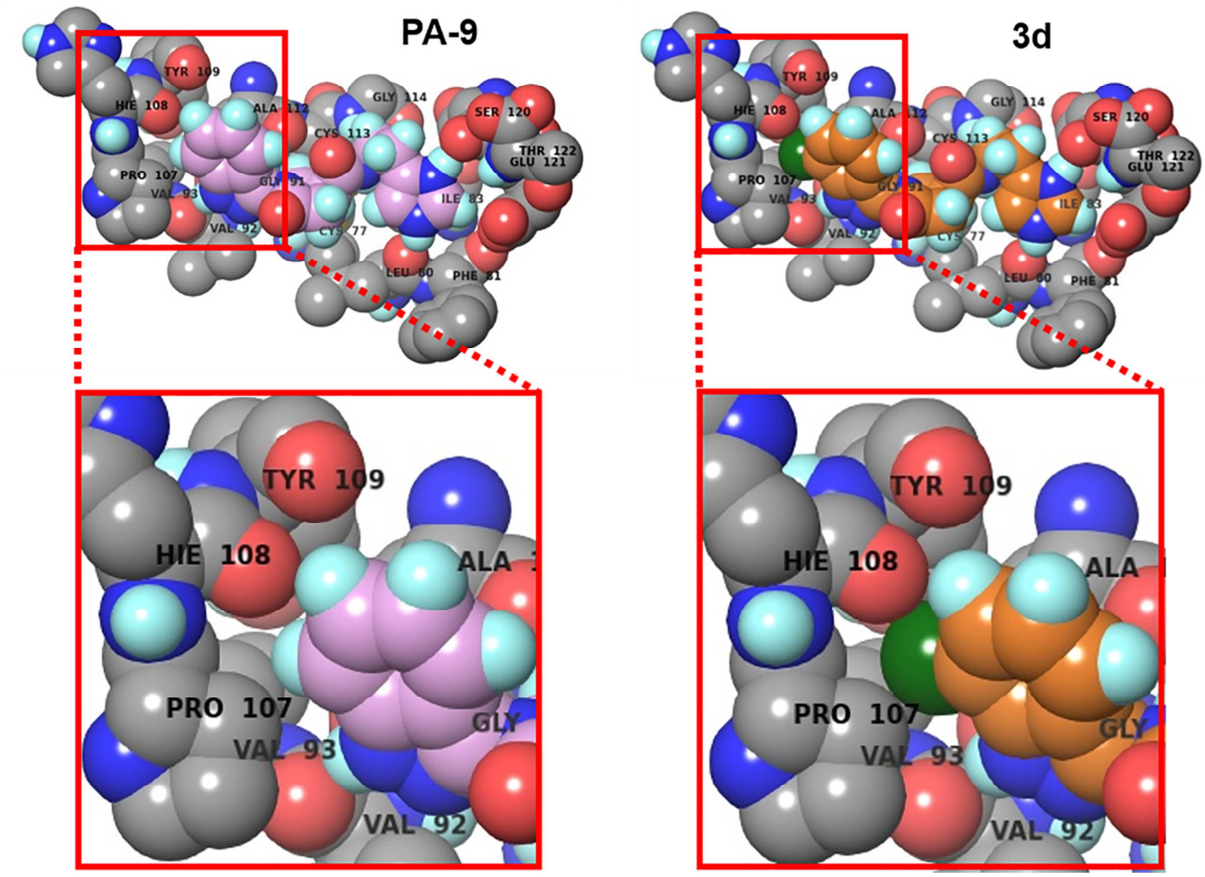


Fig. 7 Comparison of docking models of PA-9 or **3d** with PAC1 receptor.

3. Conclusion

In search of novel and potent PAC1 receptor antagonists for pharmacological studies and new analgesics, we synthesized and evaluated 18 derivative compounds of PA-9 which is identified as a new PAC1 receptor antagonist by *in silico* screening followed by pharmacological assays [9]. Among the synthesized compounds, **3d** showed more potent antagonistic action than PA-9 in an *in vitro* pharmacological assay. Furthermore, **3d** exibited more potent analgesic activities against both PACAP- and SNL-induced mechanical allodynia. Compound **3d** also displayed strong anti-allodynic activity by oral administration, therefore, showing potential to become an orally available analgesic against neuropathic pain.

A growing body of evidence demonstrates that the PACAP signaling system plays a crucial role in pain transmission. For example, development of neuropathic pain was shown to be abrogated in *PACAP*-deficient mice [10]. However, since the mice exhibited whole-body PACAP deficiency, it was not clear which site of action (peripheral and/or central nervous system) for PACAP is involved in the development of neuropathic pain. It has also been reported that i.t. injection of PACAP 6-38, a peptide antagonist of the PACAP receptor, attenuated the spinal nerve injury-induced mechanical allodynia [11], suggesting the involvement of spinal PACAP receptors in the neuropathic pain. However, because PACAP 6-38 is recognized as a dual PAC1/$VPAC_2$ antagonist [13], it was not clear which of the PACAP receptor subtypes (PAC1, $VPAC_1$ and/or $VPAC_2$) is involved in the neuropathic pain

transmission. In the present study, i.t. injection of PA-9 and **3d**, specific antagonists of the PAC1 receptor, attenuated the spinal nerve injury-induced mechanical allodynia. Considering that a single i.t. injection of maxadilan, a specific agonist for the PAC1 receptor, induced long-lasting mechanical allodynia [8], it is strongly suggested that spinal PAC1 receptor is primarily involved in the development of peripheral nerve injury-induced neuropathic pain.

4. Experimental section

4.1. Chemistry

General melting points are uncorrected. Flash chromatography was performed on Kanto Kagaku silica gel 60N. NMR spectra were recorded on a JEOL a-GX 400 spectrometer using C_5D_5N as the solvent. Chemical shifts (δ) are given in ppm downfield from TMS and referenced to C_5H_5N (7.19 ppm) as an internal standard. Peak multiplicities are designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet; br, broad and coupling constants in (*J*) Hz. High-resolution mass spectral data were obtained on a JEOL MStation JMS-700. All commercial reagents were used as received unless otherwise noted.

*4.1.1. General procedure for the synthesis of lactams (**2a**-**r**)*

According to the literature's procedure, lactams (**2a**-**r**) were prepared by direct heating at 150 °C with no solvent [14] or heating with reflux in H_2O [15] as solid.

*4.1.2. General procedure for the synthesis of amides (**3a**-**r**)*

Method A: To a stirred solution of lactam (**2**, 1 mmol) in CH_2Cl_2 (1.5 mL) and DMF (1.5 mL) were added DCC (1.2 mmol), HOBt (1.2 mmol) and histamine (1.2 mmol) at room

temperature, and the resulting mixture was stirred at room temperature for 24 h. After evaporation of the solvent, the residue was chromatographed on silica gel (15 g, CH_2Cl_2:MeOH = 20:1-5:1) to give the corresponding amide.

Method B: To a stirred solution of lactam (**2**, 1 mmol) in CH_2Cl_2 (1.5 mL) and DMF (1.5 mL) were added EDC (1.2 mmol), DMAP (0.1 mmol) and histamine (1.2 mmol) at room temperature, and the resulting mixture was stirred at room temperature for 24 h. After evaporation of the solvent, the residue was chromatographed on silica gel (15 g, CH_2Cl_2:MeOH = 20:1-5:1) to give the corresponding amide.

4.1.2.1.

_N_-[2-(1_H_-imidazol-4-yl)ethyl]-1-(4-chloro-1_H_-indazol-3-yl)-5-oxo-3-pyrrolidine-carboxamide (3a)

Method A; Yield: 47 % in 2 steps; mp: 190-191 °C; IR (KBr): 3566, 3437, 3306, 1695, 1636, 1558, 1508 cm^{-1}; ^1H NMR (400 MHz, Pyridine-d5): δ 9.00 (1H, br s), 7.96 (1H, s), 7.61 (1H, d, *J* = 7.4 Hz), 7.14 (1H, t, *J* = 7.4 Hz), 7.15 (1H, s), 6.98 (1H, d, *J* = 7.4 Hz), 4.72 (1H, t, *J* = 9.1 Hz), 3.96-3.85 (3H, m), 3.26 (1H, dd, *J* = 15.6, 9.1 Hz), 3.11 (2H, t, *J* = 6.8 Hz), 2.78 (1H, dd, *J* = 15.6, 9.1 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 169.56, 168.62, 147.59, 134.59, 131.61, 126.70, 123.80, 119.28, 116.97, 115.67, 107.11, 48.23, 36.47, 32.61, 26.57; MS (EI): *m/z* 372 (M^+); HRMS (EI): clacd for $C_{17}H_{17}ClN_6O_2$: 372.1102 (M^+), found: 372.1099.

4.1.2.2.

_N_-[2-(1_H_-imidazol-4-yl)ethyl]-1-(5-chloro-1_H_-indazol-3-yl)-5-oxo-3-pyrrolidine-carboxa mide (3b)

Method A; Yield: 23% in 2 steps; mp: 208-209 °C; IR (KBr): 3735, 3649, 3097, 1684, 1653, 1558, 1508 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.04 (1H, br s), 8.05 (1H, s), 7.69 (1H, d, *J* = 9.6 Hz), 7.68 (1H, s), 7.27 (1H, d, *J* = 9.6 Hz), 7.71 (1H, s), 5.09 (1H, dd, *J* = 13.6, 8.4 Hz), 4.73 (1H, t, *J* = 8.4 Hz), 3.94-3.86 (3H, m), 3.28 (1H, dd, *J* = 14.8, 8.4 Hz), 3.11 (2H, t, *J* = 6.6 Hz), 2.84 (1H, dd, *J* = 14.8, 8.4 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 169.63, 168.21, 145.55, 134.50, 133.79, 131.92, 127.15, 123.19, 118.50, 118.39, 116.85, 108.13, 48.33, 38.75, 36.33, 32.92, 26.53; MS (EI): *m/z* 372 (M^+); HRMS (EI): clacd for $C_{17}H_{17}ClN_6O_2$: 372.1102 (M^+), found: 372.1099.

4.1.2.3.

_N_-[2-(1_H_-imidazol-4-yl)ethyl]-1-(6-chloro-1_H_-indazol-3-yl)-5-oxo-3-pyrrolidine-carboxa mide (3c)

Method A; Yield: 22% in 2 steps; mp: 196-198 °C; IR (KBr): 3290, 3213, 3101, 1683, 1636, 1558, 1508 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.12 (1H, br s), 8.30 (1H, s), 7.83 (1H, s), 7.66 (1H, d, *J* = 9.2 Hz), 7.25 (1H, s), 6.96 (1H, d, *J* = 9.2 Hz), 5.03 (1H, dd, *J* = 13.0, 8.3

Hz), 4.71 (1H, t, *J* = 8.3 Hz), 3.91-3.84 (3H, m), 3.27 (1H, dd, *J* = 15.2, 8.3 Hz), 3.12 (2H, t, *J* = 6.4 Hz), 2.87 (1H, dd, *J* = 15.2, 8.3 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 169.79, 168.28, 147.25, 134.03, 132.74, 132.42, 131.35, 121.77, 119.93, 116.53, 115.03, 106.65, 48.23, 38.21, 36.25, 32.92, 25.47; MS (EI): *m/z* 372 (M^+); HRMS (EI): clacd for $C_{17}H_{17}ClN_6O_2$: 372.1102 (M^+), found: 372.1099.

4.1.2.4.

_N_-[2-(1_H_-imidazol-4-yl)ethyl]-1-(7-chloro-1_H_-indazol-3-yl)-5-oxo-3-pyrrolidine-carboxamide (3d)

Method A; Yield: 35% in 2 steps; mp: 238-239 °C; IR (KBr): 3319, 3231, 3213, 1663, 1636, 1558, 1508 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.02 (1H, br s), 8.49 (1H, s), 7.76 (1H, s), 7.52 (1H, d, *J* = 7.5 Hz), 7.40 (1H, d, *J* = 7.5 Hz), 6.98 (1H, s), 6.88 (1H, t, *J* = 7.5 Hz), 5.13 (1H, dd, *J* = 14.6, 8.5 Hz), 4.81 (1H, t, *J* = 8.5 Hz), 3.95-3.86 (3H, m), 3.26 (1H, dd, *J* = 16.2, 8.5 Hz), 3.11 (2H, t, *J* = 6.8 Hz), 2.83 (1H, dd, *J* = 16.2, 8.5 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 169.56, 168.32, 144.32, 134.51, 133.83, 133.30, 125.80, 120.48, 119.42, 119.04, 116.84, 109.42, 48.36, 38.77, 36.32, 32.95, 26.57; MS (EI): *m/z* 372 (M^+); HRMS (EI): clacd for $C_{17}H_{17}ClN_6O_2$: 372.1102 (M^+), found: 372.1113.

4.1.2.5.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-phenyl-5-oxo-3-pyrrolidinecarboxamide (3e)**

Method B; Yield: 63% in 2 steps; mp: 149-151 °C; IR (KBr): 3675, 3306, 1678, 1643, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.11 (1H, t, *J* = 5.8 Hz),7.90 (1H, s), 7.72 (2H, d, *J* = 8.4 Hz), 7.28 (2H, t, *J* = 8.4 Hz), 7.09 (1H, s), 7.06 (1H, t, *J* = 8.4 Hz), 4.13 (1H, dd, *J* = 9.6, 8.3 Hz), 3.93 (1H, t, *J* = 8.3 Hz), 3.83 (2H, q, *J* = 7.5 Hz), 3.47 (1H, quint, *J* = 8.3 Hz), 3.14 (1H, dd, *J* = 16.7, 8.3 Hz), 3.08 (2H, t, *J* = 7.5 Hz), 2.84 (1H, dd, *J* = 16.7, 8.3 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.16, 171.96, 139.22, 134.58, 133.95, 128.70, 124.00, 119.36, 116.83, 50.73, 38.66, 35.66, 35.43, 26.38; MS (EI): *m/z* 298 (M^{+}); HRMS (EI): clacd for $C_{16}H_{18}N_4O_2$: 298.1430 (M^{+}), found: 298.1437.

4.1.2.6.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(4-methylphenyl)-5-oxo-3-pyrrolidine-carboxamide (3f)**

Method B; Yield: 82% in 2 steps; mp: 176-178 °C; IR (KBr): 3306, 3088, 1675, 1639, 1556 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.10 (1H, t, *J* = 5.8 Hz), 7.91 (1H, s), 7.64 (2H, d, *J* = 7.2 Hz), 7.08 (2H, t, *J* = 7.2 Hz), 7.07 (1H, s), 4.14 (1H, dd, *J* = 9.6, 8.0 Hz), 3.92 (1H, t, *J* = 8.0 Hz), 3.83 (2H, q, *J* = 7.2 Hz), 3.46 (1H, quint, *J* = 8.0 Hz), 3.14 (1H, dd, *J* = 17.6, 8.0 Hz), 3.08 (2H, t, *J* = 7.2 Hz), 2.84 (1H, dd, *J* = 17.6, 8.0 Hz), 2.13 (3H, s); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.17, 172.10, 137.00, 134.86, 134.44, 133.26, 129.30, 119.58, 117.04, 51.00, 39.17, 36.02, 35.84, 27.01, 20.63; MS (EI): *m/z* 312 (M^{+}); HRMS (EI): clacd for

$C_{17}H_{20}N_4O_2$: 312.1586 (M^+), found: 312.1593.

4.1.2.7.

1-(4-chlorophenyl)-*N*-[2-(1*H*-imidazol-4-yl)ethyl]-5-oxo-3-pyrrolidine-carboxamide (3g)

[16]

Method B; Yield: 74% in 2 steps; mp: 196-198 °C; IR (KBr): 3119, 3017, 1695, 1647, 1558 cm^{-1}; ^1H NMR (400 MHz, Pyridine-d5): δ 9.16 (1H, t, *J* = 5.2 Hz), 7.95 (1H, s), 7.71 (2H, d, *J* = 8.4 Hz), 7.30 (2H, d, *J* = 8.4 Hz), 7.11 (1H, s), 4.10 (1H, dd, *J* = 9.4, 8.3 Hz), 3.92 (1H, t, *J* = 8.3 Hz), 3.83 (2H, q, *J* = 6.7 Hz), 3.50 (1H, quint, *J* = 8.3 Hz), 3.11 (1H, dd, *J* = 17.5, 8.3 Hz), 3.09 (2H, t, *J* = 5.7 Hz), 2.85 (1H, dd, *J* = 17.5, 8.3 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.20, 171.68, 137.90, 134.43, 133.95, 128.36, 127.49, 120.60, 116.62, 50.47, 38.74, 35.62, 35.30, 26.52; MS (EI): *m/z* 332 (M^+); HRMS (EI): clacd for $C_{16}H_{17}ClN_4O_2$: 332.1040 (M^+), found: 332.1039.

4.1.2.8.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(4-fluorophenyl)-5-oxo-3-pyrrolidine-carboxamide (3h)**

Method B; Yield: 87% in 2 steps; mp: 232-234 °C; IR (KBr): 3140, 3126, 1688, 1645, 1570 cm^{-1}; ^1H NMR (400 MHz, Pyridine-d5): δ 9.13 (1H, t, *J* = 4.8 Hz), 7.72-7.68 (2H, m), 7.92 (1H, s), 7.11 (1H, s), 7.09-7.04 (2H, m), 4.12 (1H, dd, *J* = 12.2, 8.7 Hz), 3.92 (1H, t, *J* = 8.7

Hz), 3.84 (2H, t, *J* = 7.0 Hz), 3.49 (1H, quint, *J* = 8.7 Hz), 3.14 (1H, dd, *J* = 17.2, 8.7 Hz), 3.09 (2H, t, *J* = 7.0 Hz), 2.85 (1H, dd, *J* = 17.2, 8.7 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.05, 171.90, 158.44 (d, *J* = 240.3 Hz), 135.64, 134.62, 134.14, 121.35 (d, *J* = 7.6 Hz), 116.81, 115.25 (d, *J* = 22.0 Hz), 50.92, 38.92, 35.68, 35.58, 26.72; MS (EI): *m/z* 316 (M^+); HRMS (EI): clacd for $C_{16}H_{17}FN_4O_2$: 316.1336 (M^+), found: 316.1335.

4.1.2.9.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(4-methoxyphenyl)-5-oxo-3pyrrolidine-carboxamide (3i)**

Method B; Yield: 83% in 2 steps; mp: 151-153 °C; IR (KBr): 3239, 3075, 1684, 1635, 1568 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.08 (1H, t, *J* = 5.2 Hz), 7.91 (1H, s), 7.68 (2H, d, *J* = 8.6 Hz), 7.10 (1H, s), 6.92 (2H, d, *J* = 8.6 Hz), 4.16 (1H, dd, *J* = 9.4, 8.4 Hz), 3.93 (1H, t, *J* = 8.4 Hz), 3.84 (2H, q, *J* = 7.6 Hz), 3.62 (3H, s), 3.46 (1H, quint, *J* = 8.4 Hz), 3.15 (1H, dd, *J* = 17.1, 8.4 Hz), 3.09 (2H, t, *J* = 7.6 Hz), 2.84 (1H, dd, *J* = 17.1, 8.4 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 171.97, 171.57, 155.80, 134.65, 134.26, 132.41, 121.20, 116.80, 113.82, 55.20, 51.03, 38.95, 35.68, 35.62, 26.81; MS (EI): *m/z* 328 (M^+); HRMS (EI): clacd for $C_{17}H_{20}N_4O_3$: 328.1535 (M^+), found: 328.1530.

4.1.2.10.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(4-cyanophenyl)-5-oxo-3-pyrrolidine-carboxamide (3j)**

Method B; Yield: 47% in 2 steps; mp: 211-212 °C; IR (KBr): 3151, 3019, 2231, 1703, 1646, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.17 (1H, t, *J* = 5.2 Hz), 7.92 (1H, s), 7.83 (2H, d, *J* = 8.8 Hz), 7.60 (2H, d, *J* = 8.8 Hz), 7.11 (1H, s), 4.12 (1H, dd, *J* = 9.6, 8.4 Hz), 3.96 (1H, t, *J* = 8.4 Hz), 3.84 (2H, t, *J* = 7.0 Hz), 3.51 (1H, quint, *J* = 8.4 Hz), 3.16 (1H, dd, *J* = 17.2, 8.4 Hz), 3.10 (2H, t, *J* = 7.0 Hz), 2.88 (1H, dd, *J* = 17.2, 8.4 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 173.28, 171.75, 143.03, 134.67, 133.06, 119.03, 118.93, 105.53, 51.51, 38.98, 36.03, 35.39, 26.85; MS (EI): *m/z* 323 (M^+); HRMS (EI): clacd for $C_{17}H_{17}N_5O_2$: 323.1382 (M^+), found: 323.1377.

4.1.2.11.

_N_-[2-(1_H_-imidazol-4-yl)ethyl]-1-(2-hydroxyphenyl)-5-oxo-3-pyrrolidine-carboxamide (3k)

Method B; Yield: 25% in 2 steps; mp: 120-122 °C; IR (KBr): 3651, 3265, 3213, 1684, 1670, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 9.08 (1H, br s), 7.91 (1H, s), 7.42 (1H, d, *J* = 8.8 Hz), 7.21-7.15 (2H, m), 7.10 (1H, s), 6.92-6.88 (1H, m), 4.30 (1H, dd, *J* = 9.2, 7.5 Hz), 4.10 (1H, t, *J* = 7.5 Hz), 3.85 (2H, q, *J* = 6.4 Hz), 3.47 (1H, quint, *J* = 7.5 Hz), 3.13 (1H, dd, *J* = 16.4, 7.5 Hz), 3.06 (2H, t, *J* = 6.4 Hz), 2.83 (1H, dd, *J* = 16.4, 7.5 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.52, 172.22, 152.77, 134.68, 128.30, 128.24, 125.48, 119.12, 118.74, 116.87, 116.74, 51.68, 38.98, 37.09, 34.39, 26.85; MS (EI): *m/z* 314 (M^+); HRMS (EI): clacd for

$C_{16}H_{18}N_4O_3$: 314.1379 (M^+), found: 314.1382.

4.1.2.12.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(3-hydroxyphenyl)-5-oxo-3-pyrrolidine-carboxamide (3l)**

Method B; Yield: 47% in 2 steps; mp: 102-104 °C; IR (KBr): 3790, 3439, 3337, 1684, 1653, 1558 cm^{-1}; ^1H NMR (400 MHz, Pyridine-d5): δ 8.97 (1H, br s), 8.09 (1H, s), 7.93 (1H, s), 7.28 (1H, t, *J* = 8.2 Hz), 7.20 (1H, d, *J* = 8.2 Hz), 7.13 (1H, s), 6.98 (1H, d, *J* = 8.2 Hz), 4.29 (1H, dd, *J* = 9.0, 8.3 Hz), 3.98 (1H, t, *J* = 8.3 Hz), 3.89 (2H, q, *J* = 6.9 Hz), 3.36 (1H, quint, *J* = 8.3 Hz), 3.21 (1H, dd, *J* = 17.1, 8.3 Hz), 3.09 (2H, t, *J* = 6.9 Hz), 2.82 (1H, dd, *J* = 17.1, 8.3 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.07, 171.93, 157.55, 140.28, 134.68, 129.38, 111.17, 109.78, 106.63, 50.84, 39.00, 35.99, 35.54, 26.89; MS (EI): *m/z* 314 (M^+); HRMS (EI): clacd for $C_{16}H_{18}N_4O_3$: 314.1379 (M^+), found: 314.1382.

4.1.2.13.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(4-hydroxyphenyl)-5-oxo-3-pyrrolidine-carboxamide (3m)**

Method B; Yield: 41% in 2 steps; mp: 104-105 °C; IR (KBr): 3585, 3251, 3190, 1684, 1653, 1558 cm^{-1}; ^1H NMR (400 MHz, Pyridine-d5): δ 11.49 (1H, br s), 8.97 (1H, br s), 7.93 (1H, s),

7.76 (2H, dd, *J* = 8.6, 2.4 Hz), 7.15 (2H, dd, *J* = 8.6, 2.4 Hz), 7.14 (1H, s), 4.29 (1H, td, *J* = 8.4, 2.1 Hz), 3.95 (1H, td, *J* = 8.4, 2.1 Hz), 3.90 (2H, q, *J* = 6.3 Hz), 3.39 (1H, quint, *J* = 8.4 Hz), 3.22 (1H, ddd, *J* = 16.7, 8.4, 2.1 Hz), 3.10 (2H, t, *J* = 6.3 Hz), 2.84 (1H, ddd, *J* = 16.7, 8.4, 2.1 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.52, 171.86, 154.61, 135.20, 131.48, 122.05, 115.61, 51.67, 39.49, 36.23, 36.09, 27.40; MS (EI): *m/z* 314 (M^+); HRMS (EI): clacd for $C_{16}H_{18}N_4O_3$: 314.1379 (M^+), found: 314.1382.

4.1.2.14.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-(1*H*-pyrazol-3-yl)-5-oxo-3-pyrrolidine-carboxamide (3n)**

Method A; Yield: 28% in 2 steps; mp: 213-211 °C; IR (KBr): 3676, 3320, 3203, 1689, 1652, 1635, 1557 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 12.24 (1H, br s), 9.00 (1H, br s), 8.03 (1H, s), 7.88 (1H, s), 7.56 (1H,s), 7.10 (1H, s), 4.78 (1H, dd, *J* = 12.6, 8.1 Hz), 4.26 (1H, t, *J* = 8.1 Hz), 3.82 (2H, q, *J* = 6.7 Hz), 3.69 (1H, quint, *J* = 8.1 Hz), 3.22 (1H, dd, *J* = 15.3, 8.1 Hz), 3.06 (2H, t, *J* = 6.7 Hz), 2.72 (1H, dd, *J* = 15.3, 8.1 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 169.67, 168.26, 162.33, 138.77, 138.73, 134.64, 134.53, 89.01, 47.04, 38.87, 36.72, 33.00, 26.97; MS (EI): *m/z* 288 (M^+); HRMS (EI): clacd for $C_{13}H_{16}N_6O_2$: 288.1335 (M^+), found: 288.1336.

4.1.2.15.

***N*-[2-(1*H*-imidazol-4yl)ethyl]-5-oxo-1-(phenylmethyl)-3-pyrrolidine-carboxamide (3o)**

[17]

Method A; Yield: 76%; mp: 61-63 °C; IR (KBr): 3271, 3155, 1670, 1652, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 8.85 (1H, br), 7.92 (1H, s), 7.33-7.25 (5H, m), 7.08 (1H, s), 4.56 (1H, d, *J* = 14.6 Hz), 4.48 (1H, d, *J* = 14.6 Hz), 3.85 (2H, q, *J* = 6.5 Hz), 3.67 (1H, t, *J* = 8.0 Hz), 3.39 (1H, t, *J* = 8.0 Hz), 3.25 (1H, quint, *J* = 8.0 Hz), 3.10 (1H, dd, *J* =17.0, 8.0 Hz), 3.05 (2H, t, *J* = 6.5 Hz), 2.72 (1H, dd, *J* = 17.0, 8.0 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.44, 172.25, 136.77, 134.58, 133.72, 128.59, 127.59, 127.28, 125.39, 123.57, 118.69, 116.81, 110.25, 49.13, 45.31, 38.71, 35.87, 33.93, 26.42; MS (EI): *m/z* 312 (M^{+}); HRMS (EI): clacd for $C_{17}H_{20}N_4O_2$: 312.1586 (M^{+}), found: 312.1593.

4.1.2.16.

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-1-[(2-hydroxyphenyl)methyl]-5-oxo-3-pyrrolidinecarboxamide (3p)**

Method A; Yield: 30%; mp: 88-90 °C; IR (KBr): 3748, 3738, 3651, 1684, 1653, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 11.36 (1H, br s) 8.81 (1H, t, *J* = 5.8 Hz), 7.84 (1H, d, *J* = 1.2 Hz), 7.34 (1H, dd, *J* = 7.5, 1.3 Hz), 7.13 (1H, td, *J* = 7.5, 1.3 Hz), 7.08 (1H, dd, *J* = 7.5, 1.3 Hz), 7.03 (1H, s), 6.81 (1H, td, *J* = 1.3, 7.5 Hz), 4.74 (1H, d, *J* = 15.2 Hz), 4.65 (1H, d, *J* = 15.2 Hz), 3.82 (1H, dd, *J* = 9.6, 8.2 Hz), 3.77 (2H, q, *J* = 6.6 Hz), 3.57 (1H, t, *J* = 8.2 Hz),

3.22 (1H, quint, *J* = 8.2 Hz), 3.03 (1H, dd, *J* = 16.5, 8.2 Hz), 2.99 (2H, t, *J* = 6.6 Hz), 2.63 (1H, dd, *J* = 16.5, 8.2 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.57, 172.18, 155.26, 134,67, 128.78, 128.38, 122.58, 119.03, 115.21, 49.63, 40.57, 38.94, 35.93, 33.91, 26.87; MS (EI): *m/z* 328 (M^+); HRMS (EI): clacd for $C_{17}H_{20}N_4O_3$: 328.1535 (M^+), found: 328.1533.

4.1.2.17.

_N_-[2-(1*H*-imidazol-4-yl)ethyl]-1-[(3-hydroxyphenyl)methyl]-5-oxo-3-pyrrolidinecarboxamide (3q)

Method A; Yield: 40%; mp: 104-106 °C; IR (KBr): 3734, 3647, 3623, 1684, 1653, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 8.83 (1H, br s), 7.93 (1H, s), 7.24 (2H, d, *J* = 7.9 Hz), 7.22 (1H, s), 7.09 (1H, s), 7.08 (1H, d, *J* = 7.9 Hz), 6.88 (1H, d, *J* = 7.9 Hz), 4.57 (1H, d, *J* = 14.6 Hz), 4.48 (1H, d, *J* = 14.6 Hz), 3.84 (2H, q, *J* = 6.1 Hz), 3.72 (1H, t, *J* = 8.1 Hz), 3.45 (1H, t, *J* = 8.1 Hz), 3.23 (1H, quint, *J* = 8.1 Hz), 3.09 (1H, dd, *J* = 16.0, 8.1 Hz), 3.05 (2H, t, *J* = 6.1 Hz), 2.67 (1H, dd, *J* = 16.0, 8.1 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.31, 172.05, 157.63, 138.14, 134.66, 134.18, 129.55, 118.17, 116.90, 114.41, 114.30, 49.14, 45.31, 38.88, 35.89, 26.78; MS (EI): *m/z* 328 (M^+); HRMS (EI): clacd for $C_{17}H_{20}N_4O_3$: 328.1535 (M^+), found: 328.1533.

4.1.2.18.

_N_-[2-(1_H_-imidazol-4-yl)ethyl]-1-[(4-hydroxyphenyl)methyl]-5-oxo-3-pyrrolidine-carboxamide (3r)

Method A; Yield: 29%; mp: 58-60 °C; IR (KBr): 3651, 3271, 3213, 1663, 1653, 1558 cm^{-1}; ^{1}H NMR (400 MHz, Pyridine-d5): δ 8.85 (1H, br s), 7.91 (1H, s), 7.28 (2H, d, *J* = 7.6 Hz), 7.10 (2H, d, *J* = 7.6 Hz), 4.55 (1H, d, *J* = 14.6 Hz), 4.44 (1H, d, *J* = 14.6 Hz), 3.91-3.71 (2H, m), 3.70 (1H, dd, *J* = 8.8, 7.9 Hz), 3.45 (1H, t, *J* = 7.9 Hz), 3.26 (1H, quint, *J* = 7.9 Hz), 3.11 (1H, dd, *J* = 15.0, 7.9 Hz), 3.06 (2H, t, *J* = 7.4 Hz), 2.71 (1H, dd, *J* = 15.0, 7.9 Hz); ^{13}C NMR (100 MHz, DMSO-d6): δ 172.15, 156.66, 134.68, 129.07, 126.80, 115.30, 48.93, 44.83, 38.94, 35.83, 34.03, 26.89; MS (EI): *m/z* 328 (M^+); HRMS (EI): clacd for $C_{17}H_{20}N_4O_3$: 328.1535 (M^+), found: 328.1533.

4.2. Materials.

PACAP (38 amino acid form) was purchased from Peptide Institute Inc. (Osaka, Japan). These drugs were made up as concentrated stock solutions in phosphate-buffered saline (PBS) or physiological saline, and stored at -30°C. An aliquot was diluted to the desired concentration in PBS or artificial cerebrospinal fluid (ACSF: NaCl 138 mM, KCl 3 mM, $CaCl_2$ 1.25 mM, $MgCl_2$ 1 mM, D-glucose 1 mM) immediately prior to use.

4.3. Cell culture

PAC1 receptor-expressing CHO cells (PAC1/CHO) cells were cultured in DMEM/F12 (Invtrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 200 µg/ml G418 or 5 µg/ml blasticidin S. The cells were propagated in a humidified 37°C incubator in 5% CO_2.

4.4. Western blot analysis

Cells were seeded onto 12-well plates at 1×10^5 cells/well and incubated for 48 h. After treatment with candidate compounds or 0.1% DMSO (vehicle control) for 30 min, the cells were stimulated with PACAP for 30 min.

Cellular materials were lysed in lysis buffer (150 mM NaCl, 1% NP-40, 10% glycerol, protease inhibitor mix (Nacalai Tesque, Kyoto, Japan), and 20 mM Tris-HCl (pH 7.4)). The lysate (20 µg total protein per lane) were subjected to SDS-polyacrylamide gel electrophoresis (10% gel) and then were blotted onto PVDF membranes (GE healthcare, Buckinghamshire, UK). The membranes were blocked with Blocking One (Nacalai Tesque) at room temperature for 1 h. The membranes were then incubated with primary antibodies in TBS containing 0.1% Tween 20 (TBST) and 5% Blocking One at 4°C for 16-18 h, washed three times with TBST, and exposed to peroxidase-conjugated secondary antibody in TBST containing 5% Blocking One at room temperature for 1-2 h. Immunoreactive proteins were visualized by a luminescent image analyzer (LAS-1000 plus, Fujifilm, Tokyo, Japan) using an enhanced

chemiluminescence detection system (Chemi-Lumi One L, Nacalai Tesque). The following primary antibodies were used; anti-phospho-CREB (Ser133) and anti-CREB (Cell Signaling Technology, Danvers, MA). The phosphorylation level of CREB was calculated as the ratio of the intensity of the band relative to that of total CREB protein in each sample.

4.5. Animals

Male ddY mice (six weeks old at the start of experiments) were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in standard polycarbonate cages (four mice/cage) under controlled temperature (24 ± 1°C) and humidity (55% ± 10%) with a 12-h light–dark cycle (lights on at 07:00 h) with food and water freely available. Mice were habituated to the animal facility for at least one week before experimentation. The animal experiments were approved by the Animal Care Committee of University of Toyama (approval no. 2014ENG-2) and were conducted in accordance with the ethical guidelines for the study of experimental pain in conscious animals of the International Association for the Study of Pain [18].

4.6. Intrathecal injection and behavioral observation

Intrathecal (i.t.) injection was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the fifth or sixth lumbar vertebra, according to a previously reported procedure [19].

Mechanical sensitivity of the hind paw was evaluated with calibrated von Frey hairs (Stoelting Co., Wood Dale, IL) by measuring the tactile stimulus producing a 50% likelihood of hind paw withdrawal response (50% gram threshold), which was determined using the up-down paradigm [8, 20].

All behavioral tests were done in double-blinded manner.

4.7. Spinal nerve ligation model

To induce peripheral nerve injury, we used spinal nerve ligation (SNL) model [21, 22] with a slight modification. Briefly, under anesthesia, the right L5 spinal nerve was exposed by removing a small piece of the paravertebral muscles and a part of the right spinous process of the L5 lumbar vertebra. The L5 spinal nerve were then carefully isolated and tightly ligated with 8-0 silk thread. After nerve ligation, the muscle, the adjacent fascia and the skin were closed with sutures.

4.8. Statistical analysis

Experimental data are expressed as mean ± standard error of the mean (SEM). Single comparisons were made using Student's two-tailed unpaired t-test. One- or two-way analysis of variance followed by the Dunnett's test was used for multiple comparisons. For the analyses of mechanical thresholds, we employed the Mann–Whitney U-test for single

comparisons or the Friedman test followed by the Steel test for multiple comparisons. $P < 0.05$ was considered statistically significant.

*4.9. Modeling of binding mode of **3d** with PAC1 receptor*

All calculations were performed using the Small-Molecule Drug Discovery Suite 2016-3 (Schrödinger, LLC, New York, NY, 2016). The 2D structure of **3d** was first converted into 3D structure using the LigPrep program. The docking of generated-structures of **3d** against PAC1 receptor was performed using SP mode in Glide program. The structure of PAC1 receptor complexed with PA-9, which was obtained in the previous study [9], was used as a receptor for docking. The option of "Restrict docking to reference position" was used to generate docking poses of **3d** with a similar mode to the interaction model of PA-9 with PAC1 receptor. We selected a pose with the lowest docking score as the interaction model of **3d** with PAC1 receptor. Finally, the binding free energies (ΔG_{bind}s) of PA-9 and **3d** were estimated by the MM-GBSA method using the Prime program.

Conflict of interest statement

The authors indicated no potential conflicts of interest.

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Figure legends

Fig. 1 Docking model of PA-9 with PAC1 receptor. Hydrogen-bonding interactions are indicated by yellow dashed lines.

Fig. 2 Effects of 18 derivative compounds (**3a**-**3r**) on the PACAP-induced phosphorylation of CREB in the PAC1/CHO cells. The cells were incubated with PA-9 (1 nM) or each compound (1 nM) for 30 min, then the cells were stimulated with PACAP (1 nM) for 30 min and lysed. The lysates containing equivalent amounts of protein (20 μg) were subjected to SDS-PAGE. Immunoblots were probed with specific antibodies that recognize phosphorylated form of CREB (pCREB), and total CREB. The data represent the mean ± SEM (n = 4-5). *P < 0.05 vs. DMSO (DM) control.

Fig. 3 Effects of **3d** or PA-9 on the PACAP-induced phosphorylation of CREB in the PAC1/CHO cells. The cells were incubated with **3d** or PA-9 compounds (10 pM to 10 nM) for 30 min, then the cells were stimulated with PACAP (1 nM). The data represent the mean ± SEM (n = 3-5).

Fig. 4 Effects of **3d** or PA-9 on the PACAP-induced mechanical allodynia. A single i.t. injection of PACAP (100 pmol) induced long-lasting mechanical allodynia and the induction of allodynia was prevented by co-injection of **3d** or PA-9 (100 pmol each). The data represent the mean ± SEM (n = 6). *P < 0.05, when compared with vehicle.

Fig. 5 Effects of **3d** or PA-9 on the SNL-induced mechanical allodynia. (A) **3d** (100 pmol, n = 6) or PA-9 (100 pmol, n = 6) was intrathecally injected 14 days after SNL. (B) **3d** (30 mg/kg, n = 6) or PA-9 (30 mg/kg, n = 6) was perorally administered 14 days after SNL. The data represent the mean ± SEM (n = 6). *P < 0.05, when compared with vehicle. $^{\#}P$ < 0.05, when compared with PA-9.

Fig. 6 Docking model of **3d** with PAC1 receptor. Hydrogen-bonding interactions are indicated by yellow dashed lines.

Fig. 7 Comparison of docking models of PA-9 or 3d with PAC1 receptor.

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Highlights

- A novel and potent small-molecule antagonist of PAC1 receptor was synthesized.
- We identified compound **3d** as a novel and potent PAC1 receptor antagonist.
- Intrathecal injection of **3d** inhibited PACAP- and nerve injury-induced allodynia.
- Compound **3d** also showed anti-allodynic effects following oral administration.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: