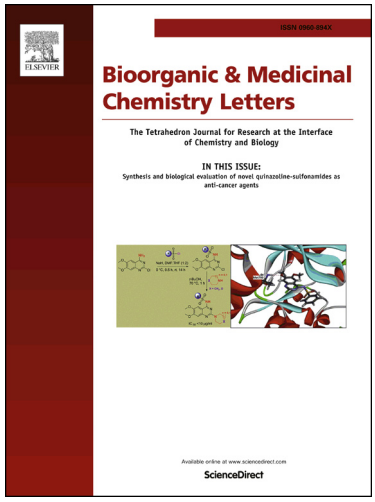


Pyrrolinone derivatives as a new class of P2X3 receptor antagonists. Part 1: Initial structure-activity relationship studies of a hit from a high throughput screening

Hiroyuki Tobinaga, Takayuki Kameyama, Miho Oohara, Naotake Kobayashi, Masahide Ohdan, Natsuki Ishizuka, Masaharu Kume, Maki Tomari, Yoshikazu Tanaka, Fumiyo Takahashi, Haruki Kinoshita, Shinji Shimada, Shunji Shinohara, Hiroyuki Kai

PII: S0960-894X(17)30434-1
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.04.060
Reference: BMCL 24913

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 22 February 2017
Revised Date: 12 April 2017
Accepted Date: 18 April 2017

Please cite this article as: Tobinaga, H., Kameyama, T., Oohara, M., Kobayashi, N., Ohdan, M., Ishizuka, N., Kume, M., Tomari, M., Tanaka, Y., Takahashi, F., Kinoshita, H., Shimada, S., Shinohara, S., Kai, H., Pyrrolinone derivatives as a new class of P2X3 receptor antagonists. Part 1: Initial structure-activity relationship studies of a hit from a high throughput screening, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.04.060

Pyrrolinone derivatives as a new class of P2X3 receptor antagonists. Part 1: Initial structure-activity relationship studies of a hit from a high throughput screening.

Hiroyuki Tobinaga[a,*], Takayuki Kameyama[b], Miho Oohara[a], Naotake Kobayashi[a], Masahide Ohdan[a], Natsuki Ishizuka[a], Masaharu Kume[a], Maki Tomari[c], Yoshikazu Tanaka[d], Fumiyo Takahashi[b], Haruki Kinoshita[b], Shinji Shimada[c], Shunji Shinohara[b], and Hiroyuki Kai[a,*]

[a] *Medicinal Chemistry Research Laboratory, Shionogi & Co., Ltd, 1-1 Futaba-cho, 3-chome, Toyonaka, Osaka 561-0825, Japan*
[b] *Drug Discovery & Disease Research Laboratory, Shionogi & Co., Ltd, 1-1 Futaba-cho, 3-chome, Toyonaka, Osaka 561-0825, Japan*
[c] *Shionogi Techno Advance Research Co., Ltd., 1-1 Futaba-cho, 3-chome, Toyonaka, Osaka 561-0825, Japan*
[d] *Global Innovation Office, Shionogi & Co., Ltd, 1-1 Futaba-cho, 3-chome, Toyonaka, Osaka 561-0825, Japan*

*Corresponding authors. Tel.: +81-6-6331-6155; fax: +81-6-6332-6385; E-mail address: hiroyuki.tobinaga@shionogi.co.jp (H. Tobinaga)
Tel.: +81-6-6331-6203; fax: +81-6-6332-6385; E-mail address: hiroyuki.kai@shionogi.co.jp (H. Kai)

Key words: Purinergic receptors, ion channels, P2X3 receptor antagonists, pyrrolinone derivatives, pain

Abstract

The P2X3 receptor is primarily expressed in the peripheral sensory nerves, and therefore, antagonists of this receptor may be useful for the treatment of chronic pain. Pyrrolinone derivatives have been identified as a novel class of P2X3 receptor antagonists. A lead structure with moderate activity was discovered through a high-throughput screening assay. A structure-activity study led to the discovery of several P2X3 receptor antagonists. Compound **34** showed potent and specific antagonistic activity and analgesic efficacy.

Adenosine triphosphate (ATP), produced in the mitochondria in cells, is used as a source of energy for various physiological activities, as a phosphorylation substrate for proteins, and as an extracellular neurotransmitter. ATP is released into the extracellular fluid by a variety of stimuli including inflammatory or cell damage. Extracellular ATP acts as a neurotransmitter for P2 receptors at the nerve terminal. P2 receptors are classified into two groups, P2X includes the ligand-gated ion channels[1] and P2Y includes G protein-coupled receptors[2]. P2X3, a subtype of P2X receptors, was first cloned in 1995[3,4]. P2X3 receptors are primarily expressed in the peripheral sensory nerve. P2X3 receptor knockout mice were reported for suppression of pain behaviors[5]. In 2002, A-317491, which is a specific P2X3 receptor antagonist, showed an analgesic effect by subcutaneous administration to rats in both an inflammation pain model and neuropathic pain model[6]. Thus, in an effort to develop a new analgesic

drug, P2X3 receptor antagonists with various chemotypes were reported (Figure 1)[7-9]. Among them, AF-219 improved symptoms of pain and urinary urgency in patients with moderate to severe symptoms of interstitial cystitis/bladder pain syndrome (IC/BPS) in a Phase 2 clinical trial. Furthermore, AF-219 is in a Phase 2 clinical trial that examines its ability to improve refractory chronic cough[10].

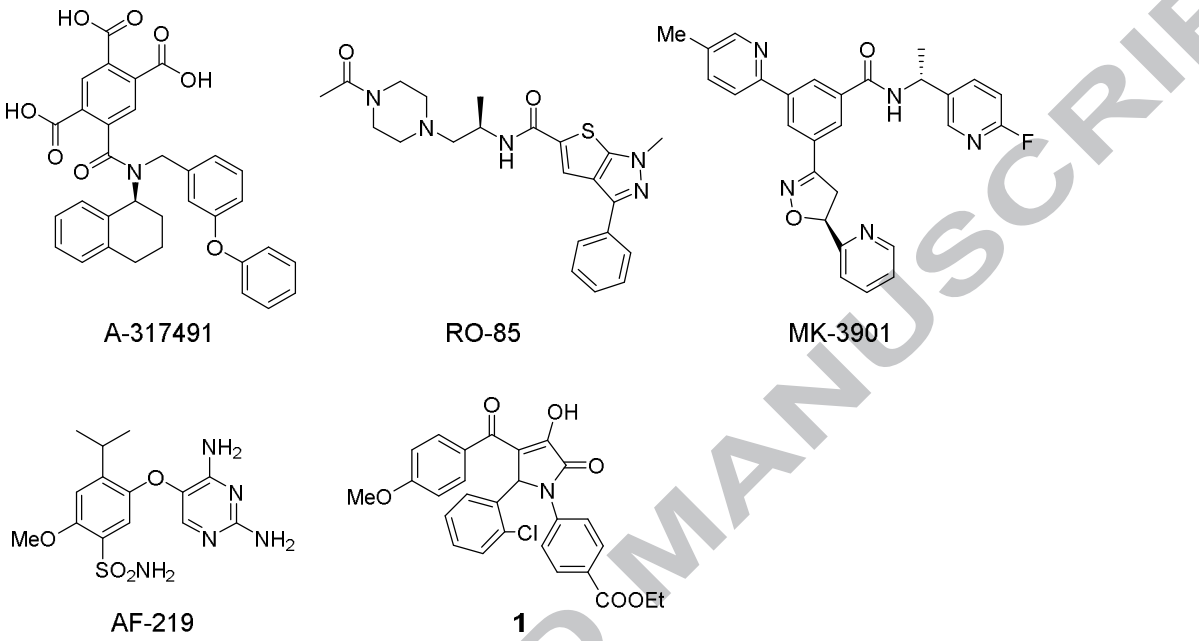
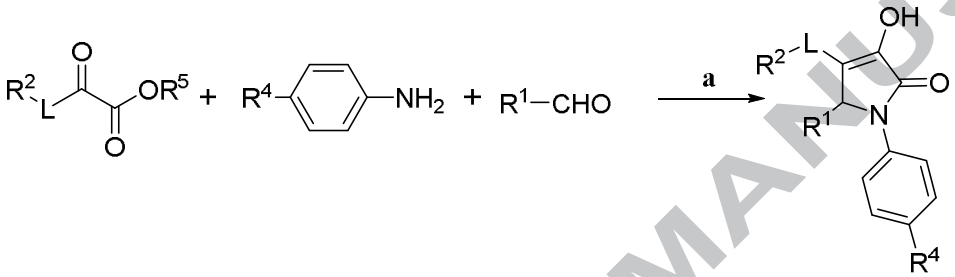


Figure 1. Structures of P2X3 receptor antagonists and hit compound **1**

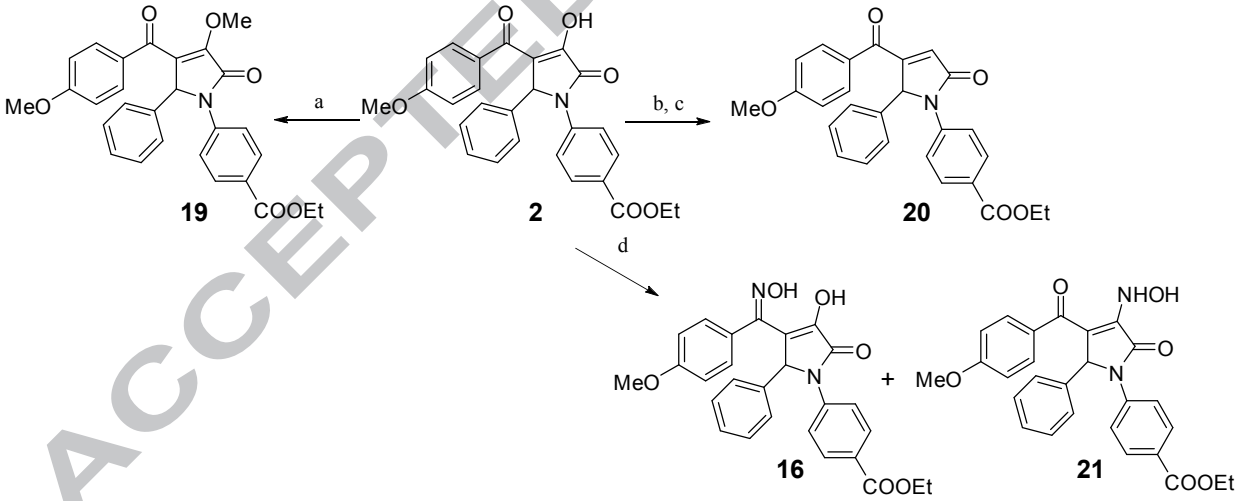
Building on the information gained from these previous studies, we aimed to develop a new P2X3 receptor antagonist as an analgesic drug free from the side effects observed for existing analgesics that act on the central nervous system. To discover novel chemotype P2X3 receptor antagonists, we performed high throughput screening of our chemical library using a FLIPR 384 Ca2+-influx assay system. Hit compound **1**, which has a pyrrolinone skeleton, was identified. The IC_{50} value of compound **1** showed the decrease by the increase in concentration of ATP. These results showed that compound **1** was orthosteric antagonist. In attempts to improve the antagonistic activity of compound **1**, a series of pyrrolinone derivatives were synthesized with various substituents to identify the structure exhibiting the strongest activity. Herein, we describe the synthesis and structure-activity relationships of pyrrolinone derivatives as P2X3 receptor antagonists.

Pyrrolinone skeletons were easily synthesized in a one-pot by condensation of an α-ketoester, aniline and aldehyde (Scheme 1)[11]. The other pyrrolinone derivatives were synthesized from compound **2** as a

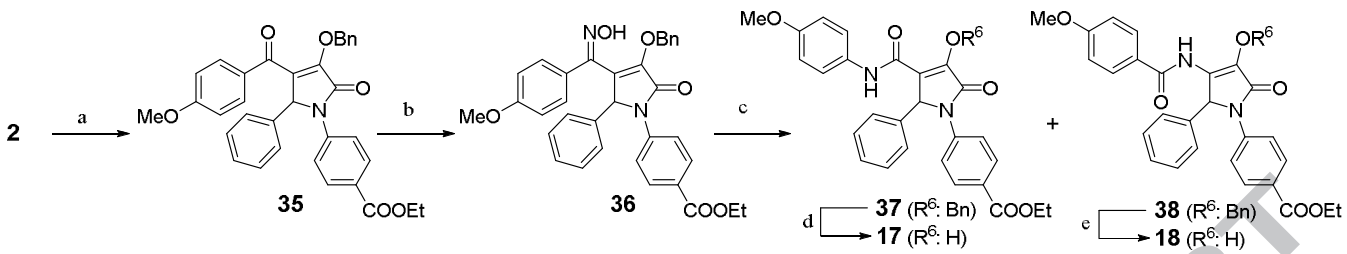
starting material according to Scheme 2. Compound **19** was prepared by methylation of compound **2** using iodomethane in the presence of potassium carbonate. Dehydroxylated compound **20** was synthesized by acetylation of the enolic alcohol **2** and hydrogenation using Pd/C. Compound **2** was reacted with hydroxylamine to give compound **16** and **21** in moderate yields. To change the linker L at position-4 of the pyrrolinone ring, the Beckmann rearrangement was applied to the pyrrolinone derivatives (Scheme 3). The enolic alcohol **2** was converted to the benzyl ether by alkylation with benzyl bromide to give **35**. Oxime **36** was obtained by using the same conditions as described for compound **16**, and then amide compounds **37** and **38** were synthesized by Beckmann rearrangement using cyanuric chloride-$ZnCl_2$[12]. Both amide compounds were debenzylated by hydrogenation to give **17** and **18**.



Scheme 1. Reagents and conditions for the synthesis of the pyrrolinone skeltons: (a) AcOH, dioxane, reflux.



Scheme 2. Reagents and conditions for the conversion of compound **2.**: (a) MeI, K_2CO_3, DMF, rt 3h, 83%; (b) Ac_2O, pyidine, rt 3h, 93%; (c) H_2, Pd/C, THF, rt, 3 days, 11%; (d) 50% NH_2OHaq, THF, reflux 7h, **16** (20%), **21** (25%).



Scheme 3. Reagents and conditions for the synthesis of amide derivatives. : (a) BnBr, K_2CO_3, DMF, rt overnight 84%; (b) $NH_2OH \cdot HCl$, pyridine, 80°C 6h, 64%; (c) cyanuric chloride, $ZnCl_2$, CH_3CN, reflux 2.5h, **32** (57%), **33** (31%); (d) H_2, Pd/C, dioxane, rt 2h, 83%; (e) H_2, Pd/C, dioxane, rt 2h, 83%.

Table 1 shows the effects of substitution of the pyrrolinone ring at each position and the ethoxycarbonyl moiety of the pyrrolinone derivatives on antagonistic activity[13].

Among compounds with a substituent (R^1) at position-5 of the pyrrolinone ring (**1** – **9**), the phenyl (**2**), 4-fluorophenyl (**5**), and cyclohexyl (**7**) derivatives were the most active, followed by the 3-fluorophenyl derivative (**4**). These compounds showed higher activity than compound **1** (R^1: 2-chlorophenyl). The 2-fluorophenyl (**3**), 4-chlorophenyl (**6**), and isopropyl (**8**) derivatives showed reduced antagonistic activities. Non-substituted compound (**9**) was inactive. These findings suggested that the presence of a bulky group and electron-donating substituent at position-5 on the pyrrolinone ring may contribute to the high activity.

By modification of substituents (R^2) at position-4 on the pyrrolinone ring (**10** – **13**), 4-methoxyphenyl (**2**) showed higher antagonistic activity than phenyl (**10**), and tert-butyl (**13**). The smaller substituents methyl (**11**) and n-butyl (**12**) led to over a 10-fold reduction in activity. Therefore, a bulky substituent was necessary at position-4 on the pyrrolinone ring for high activity.

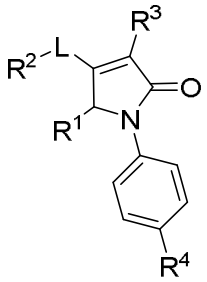
To explore the linker (L) at position-4 on the pyrrolinone ring, the carbonyl moiety was removed (**14**) or converted to a methylene from the carbonyl (**15**). Neither **14** nor **15** had activity. Additionally, oxime (**16**) maintained some activity, but amides (**17**, **18**) showed significant decreases in activity.

The enolic hydroxyl group (R^3) at position-3 of the pyrrolinone ring was converted to the methyl ether (**19**), non-substituted (**20**) or hydroxyamino (**21**) compound, all of which showed large activity decreases. From these results, it appears that the carbonyl moiety at position-4 and enolic hydroxyl group at position-3 were essential for the antagonistic activity.

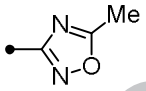
We also explored the substituent effects (R^4) at the para position on the benzene ring (**22** – **28**). The para-substituted trifluoromethyl compound (**27**) showed moderate activity, but the compounds with the other functional groups at this position showed significant decreases of activity (**22** – **26**). The ortho or

meta substituted compounds were also synthesized, and all showed significant decreased activities (data not shown).

Table 1. Structure activity relationship study of pyrrolinone derivatives



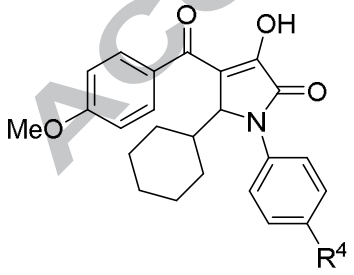
Compound	R^1	R^2	L	R^3	R^4	IC_{50} (μM)
1	2-Cl-Ph	4-MeO-Ph	CO	OH	COOEt	0.09
2	Ph	4-MeO-Ph	CO	OH	COOEt	0.02
3	2-F-Ph	4-MeO-Ph	CO	OH	COOEt	0.13
4	3-F-Ph	4-MeO-Ph	CO	OH	COOEt	0.03
5	4-F-Ph	4-MeO-Ph	CO	OH	COOEt	0.02
6	4-Cl-Ph	4-MeO-Ph	CO	OH	COOEt	0.13
7	c-Hexyl	4-MeO-Ph	CO	OH	COOEt	0.01
8	i-Pr	4-MeO-Ph	CO	OH	COOEt	0.14
9	H	4-MeO-Ph	CO	OH	COOEt	>10
10	Ph	Ph	CO	OH	COOEt	0.37
11	Ph	Me	CO	OH	COOEt	4.79
12	Ph	Bu	CO	OH	COOEt	1.02
13	Ph	t-Bu	CO	OH	COOEt	0.26
14	Ph	4-MeO-Ph	none	OH	COOEt	>10
15	Ph	4-MeO-Ph	CH_2	OH	COOEt	>10
16	Ph	4-MeO-Ph	C(=NOH)	OH	COOEt	0.56
17	Ph	4-MeO-Ph	NHCO	OH	COOEt	1.37
18	Ph	4-MeO-Ph	CONH	OH	COOEt	3.93
19	Ph	4-MeO-Ph	CO	OMe	COOEt	9.80
20	Ph	4-MeO-Ph	CO	H	COOEt	>10
21	Ph	4-MeO-Ph	CO	NHOH	COOEt	4.24

22	Ph	4-MeO-Ph	CO	OH	COOH	>10
23	Ph	4-MeO-Ph	CO	OH	H	>10
24	Ph	4-MeO-Ph	CO	OH	Ac	3.28
25	Ph	4-MeO-Ph	CO	OH	$CONMe_2$	5.92
26	Ph	4-MeO-Ph	CO	OH	CH_2OH	>10
27	4-F-Ph	4-MeO-Ph	CO	OH	CF_3	0.36
28	Ph	4-MeO-Ph	CO	OH		0.10

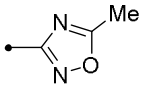
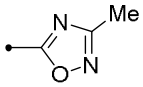
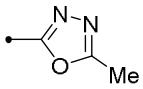
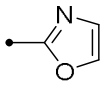
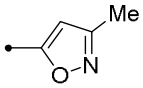
The ester group of R^4 contributed largely to the antagonistic activity, although the excretion rate *in vivo* would likely be large because of the metabolic instability of the ester group. To improve the metabolic stability while maintaining high activity, we attempted to incorporate 1,2,4-oxadiazole rings as a bioisostere for the ester groups[14]. The resulting oxadiazole compound (**28**) was highly active.

Pyrrolinone derivatives with an ester group or 1,3,4-oxadiazole ring at R^4 showed the highest activity, so other substitutions at R^4 were explored (Table 2). We introduced a 5-membered hetero aromatic ring at R^4 while R^1, R^2 and R^3 were maintained to ensure high activity (**29** – **34**). As a consequence, the IC_{50} values were less than 0.05 μM except for the 1,3,4-oxadiazole (**31**) and oxazole (**32**). We evaluated the rat pharmacokinetics for a series of these compounds. Ester (**5**) was rapidly excreted *in vivo* and was not orally absorbed. As expected, the change of R^4 from an ester to a 5-membered hetero aromatic ring greatly improved the total clearance. However, these compounds showed low oral bioavailability. This result suggested the possibility that the isoxazole ring was a bioisostere of the ester group.

Table 2. Pharmacokinetics of selected compounds in rats



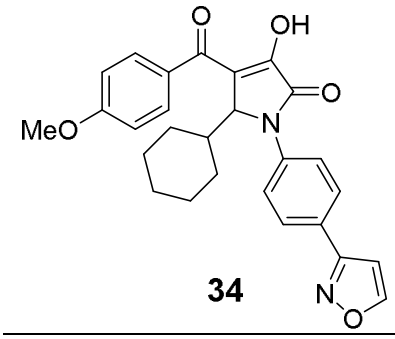
Compound	R^4	IC_{50} (μM)	t1/2 (hr)	CLt (ml/min/kg)*	BA (%)

5	COOEt	0.020	0.1	37	NC
29		0.019	0.8	5	7.1
30		0.021	1.0	3.4	13.5
31		0.072	1.7	5.5	2.9
32		0.135	NT	NT	NT
33		0.009	1.1	6.2	0.5
34		0.025	1.3	7.7	10.2

Compound **5**: Position-4 of the pyrrolinone ring has a 4-fluorophenyl group instead of a cyclohexyl.
CLt: total clearance; BA: oral bioavailability; NC: not calculated; NT: not tested.
a. All compounds were administered at 0.5 mg/kg iv and 1.0 mg/kg po.
These compounds were administered as a mixture of three to five compounds.

To evaluate the activity of the other purinergic receptors, isoxazole derivative (**34**) was selected from the above compounds for further study. Compound **34** was a specific and potent antagonist for the P2X3 receptor (Table 3). Additionally, compound **34** did not inhibit any of the 41 receptors and 17 enzymes tested at 10 μM (data not shown). To confirm the analgesic effect of our P2X3 antagonist, the sodium salt of compound **34** was prepared,[15] and evaluated for efficacy in an acetic acid induced writhing test[16] in mice by intravenous administration. The sodium salt of **34** showed good analgesic efficacy (ED_{50}; 2.6 mg/kg) in a dose-dependent manner (Figure 2).

Table 3. The selectivity of compound **34** for P2X receptors.



34

P2Xn	P2X1	P2X2	P2X3	P2X2/3	P2X4	P2X7
IC_{50} (μM)	>10	>10	0.025	4.32	>30	>10

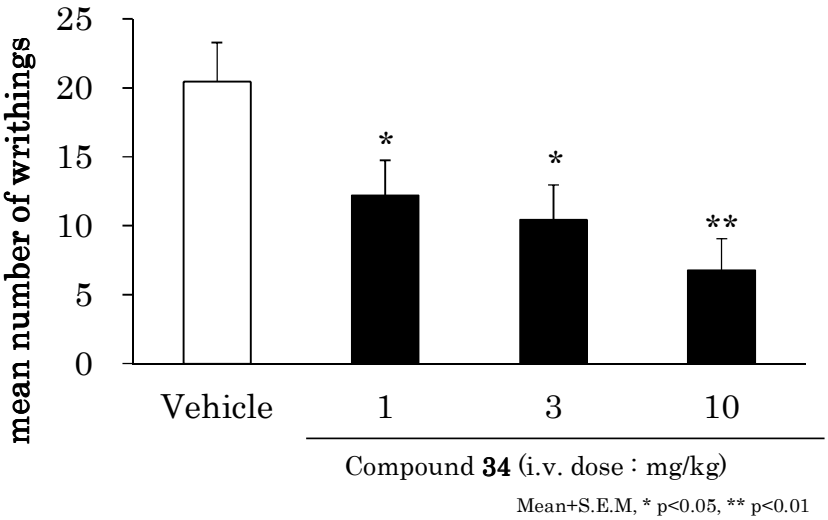


Figure 2. Analgesic effects of compound **34** by an acetic acid induced writhing test in mice

In conclusion, a high throughput screen was performed of our chemical library in an effort to identify a new P2X3 antagonist. Hit compound **1** was discovered, which had a novel pyrrolinone skeleton different from previously reported P2X3 antagonists. In a series of hit to lead structure activity relationship studies, we identified compound **34**, which showed potent and selective activity for the P2X3 receptor. Compound **34** showed analgesic efficacy in an acetic acid induced writhing test in mice, suggesting that a P2X3 antagonist may be useful as a new analgesic drug. It is expected that further improvement of the pharmacokinetics of compound **34** may lead to its development as a new analgesic drug. Further optimization of these pyrrolinone derivatives and evaluation of their optical isomers are ongoing.

References and Notes

1. North, R. A. *Physiol. Rev.* **2002**, *82*, 1013

2. Abbracchio, M. P.; Burnstock, G.; Boeynaems, J. A.; Barnard. E. A.; Boyer, J. L.; Kennedy. C.; Knight, G. E.; Fumagalli, M.; Gachet, C.; Jacobson, K. A.; Weisman, G. A. *Pharmacol. Rev.* **2006**, *58*, 281

3. Chen, C.; Akopian, A. N.; Sivilottit, L.; Colquhoun, D.; Burnstock, G.; Wood, J. N. *Nature*, **1995**, *377*, 428,

4. Lewis, C.; Neidhart, S.; Holy, C.; North, R. A.; Buell, G.; Surprenant, A. *Nature*, **1995**, *377*, 432

5. Cocckayne, D. A.; Hamilton, S. G.; Zhu, Q. M.; Dunn, P. M.; Zhong, Y.; Novakovic, S.; Malmberg, A. B.; Cain, G.; Berson, A.; Kassotakis, L.; Hedley, L.; Lachnit, W. G.; Burnstock, G.; McMahon, S. B.; Ford, A. P. D. W. *Nature*, **2000**, *407*, 1011

6. Jarvis, M. F.; Burgard, E.C.; McGaraughty, S.; Honore, P.; Lynch, K.; Brennan, T. J.; Subieta, A.; Biesen, T. V.; Cartmell, J.; Bianchi, B.; Niforatos, W.; Kage, K.; Yu, H.; Mikusa, J.; Wismer, C. T.; Zhu, C. Z.; Chu, K.; Lee, C.; Stewart, A. O.; Polakowski, J.; Cox, B. F.; Kowaluk, E.; Williams, M.; Sullivan, J.; Faltynek, C. *PNAS*, **2002**, *99*, 17179

7. Brotherton-Pleiss, C. E.; Dillon, M. P.; Ford, A. P. D. W.;Gever, J. R.; Carter, D. S.; Gleason, S. K.; Lin, C. J.; Moore, A. G.; Thompson, A. W.; Villa, M.; Zhai, Y. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1031

8. Burgey, C. S. 241st ACS Natl Meet (March 27-30, Anaheim) **2011** Abst MEDI 15

9. Szántó, G.; Makó, A.; Bata, I.; Farkas, B.; Kolok, S.; Vastag, M.; Cselenyák, A. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 3896

10. Abdulgawi, R.; Dockry, R.; Holt, K.; Layton, G.; McCarthy, B. G.; Ford, A. P.; Smith, J. A. *Lancet*, **2015**, *385*, 1198

11. Andreichikov, Yu. S.; Gein, V.; Anikina, I. N. *Zh. Org. Khim.* **1986**, *22*, 1749-1756

12. Furuya, Y.; Ishihara, K.; Yamamoto, H. *J. Am. Chem. Soc.* **2005**, *127*, 11240-11241

13. Evaluation of P2X3 receptor inhibitory activity: Stably expressing cell line (C6BU-1 cell transfected with human P2X3 receptor gene (GenBank accession number Y07683)) was used. The cells were seeded in a 96-well microtiter plate at a concentration of 8000 cells/well and cultured in the medium (8.3% fetal bovine serum, 8.3% horse serum, 1% antibiotic and antifungal in DMEM) for one day at 37 °C under 5 % carbon dioxide atmosphere. The medium was replaced with 4 μM Fluo-3-AM solution (20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.9 mM MgCl2, 5.0 mM CaCl2, 5.6 mM D-glucose, 2.5 mM probenecid, 10% BSA, and 0.08% Pluronic F-127, pH 7.5) and incubated at 37°C under 5% dioxide carbon atmosphere for one hour. The Fluo-3-AM solution was replaced with

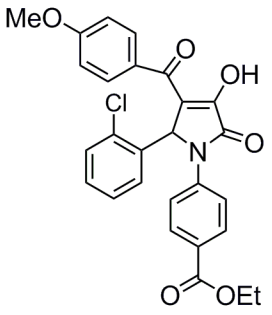
washing buffer (20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.9 mM MgCl2, 5.0 mM CaCl2, 5.6 mM D-glucose, 2.5mM probenecid, pH7.5). Inhibitory activity of the test compound was measured by High-Throughput Screening System FDSS 3000 (Hamamatsu Photonics K.K.). The test compoundsolution was prepared with dilution buffer (washing buffer with 0.1% Pluronic F-127) and dispensed to each well. Five minutes after starting the measurement in FDSS 3000, 50 nM ATP solution prepared by dilution buffer was dispensed through the built-in automatic dispenser, and the measurement of fluorescence intensity was continued for 3 min. For each well, the fluorescence ratio was calculated as the ratio of the maximum fluorescence intensity to the fluorescence intensity at the starting of the measurement. The 50% inhibitory concentration (IC50) of the test compound was calculated using Microsoft Excel (Microsoft Corporation) and XLfit (ID Business Solutions Ltd.). The known P2X3 antagonist, A-317491 showed the IC_{50} value of 92 nM in house.

14. Meanwell, N. A. *J. Med. Chem.*, **2011**, *54,* 2529–2591

15. Experimental procedure for preparation of sodium salt of compound **34**: The synthesis method and data of compound 34 are as follows. To a solution of methyl 4-(4-methoxyphenyl)-2,4-dioxobutanone (236 mg, 1 mmol), 4-(isoxazol-3-yl)aniline (160 mg, 1 mmol) in AcOH (1 ml) was added cyclohexanecarbaldehyde (0.12 ml, 1 mmol) and stirred at 95 °C for 20 minutes, then yellow precipitate was observed. The pricipitate was filtered and washed with Et_2O, EtOAc and dried in vacuo to give compound **34** (190 mg, yield 41%) as an yellow solid; 1H NMR (300 MHz, DMSO-d_6) δ: 0.71-1.04 (5H, m), 1.38-1.51 (2H, m), 1.53-1.61 (2H, m), 1.69-1.81 (2H, m), 3.90 (3H, s), 5.48 (1H, d, J = 2.5 Hz), 7.13 (2H, d, J = 8.9 Hz), 7.23 (1H, d, J = 1.7 Hz), 7.84 (2H, d, J = 8.7 Hz), 7.91 (2H, d, J = 8.7 Hz), 8.03 (3H, d, J = 8.6 Hz), 9.06 (1H, t, J = 1.6 Hz).; MS-ESI (m/z) = 459 $[M+H]^+$.

To a suspension of compound **34** (183 mg, 0.4 mmol) in MeOH (10 ml), H_2O (10 ml) was added 0.1 M NaOH aqueous solution and stirred at room temprature for 1 hr. To a reaction mixture was added THF (5 ml) then turned to clear solution. The reaction mixtures were evaporated and lyophilized, which gave sodium salt of **34** (205 mg, yield 93%) as an yellow powder; ^{1}H-NMR (300 MHz, DMSO- d_6) δ: 0.78-1.05 (5H, m), 1.38-1.76 (5H, m), 1.82-1.97 (1H, m), 3.82 (3H, s), 5.25 (1H, s), 6.88 (2H, d, J = 8.5 Hz), 7.20 (1H, d, J = 1.6 Hz), 7.76 (2H, d, J = 8.5 Hz), 7.86 (2H, d, J = 8.8 Hz), 7.97 (2H, d, J = 8.5 Hz), 9.03 (1H, d, J = 1.6 Hz).; Anal. Found: C, 62.34; H, 5.63; N, 5.40; Na, 4.80, Calcd. for $C_{27}H_{25}N_2O_5Na \cdot 2H_2O$: C, 62.78; H, 5.66; N, 5.42; Na, 4.45

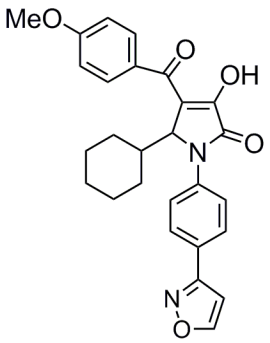
16. Koster, R.; Anderson, M.; De Beer, E. J. *Federation Proc.*, **1959**, *18*, 412



1

hP2X3_IC_{50} : 0.09 μM

Hit to Lead SAR



34

hP2X3_IC_{50} : 0.025 μM

Analgesic efficacy_ED_{50} : 2.6 mg/kg