

## Summary

- RaQualia Pharma identified a potent and selective TRPM8 antagonist, RQ-00203078.
- RQ-00203078:
  - showed potent *in vitro* antagonistic activity against both human and rat TRPM8.
  - significantly attenuated cold allodynia in a rat chronic constriction injury (CCI) neuropathic pain model with 10 mg/kg s.c.
  - significantly reduced CCI-induced static allodynia in rats with 10 mg/kg s.c.
  - exhibited no significant effect on beam walking up to 100 mg/kg s.c.
  - demonstrated superior safety profile to pregabalin.
- TRPM8 antagonist, RQ-00203078 is a potential candidate for the treatment of neuropathic pain.

## Introduction

Transient receptor potential (TRP) channels are one of the largest groups of ion channels, and they are divided into 6 subfamilies (TRPV, TRPM, TRPA, TRPC, TRPP and TRPML), in which TRPM subfamily is known as the mammalian melastatin-related subfamily. TRPM8 channels are activated by cold temperature and menthol, and therefore named as cold menthol receptor-1 (CMR-1). This channel can sense temperature changes in the range of both innocuous cold (15–28°C) and noxious cold (<15°C) as well as by chemical agents such as menthol and icilin<sup>1,2</sup>. TRPM8 is mainly expressed in primary sensory neurons (A-delta and C-fibers in the dorsal root ganglia), which provide a basis for abnormal cold sensitivity in pathologic conditions, resulting in pain such as cold allodynia. TRPM8-null mice are deficient in temperature discrimination, sense of noxious cold temperatures, injury-evoked hypersensitivity to cold after nerve injury and inflammation<sup>3,4</sup>. These evidences suggest that TRPM8 is a useful target for treating cold allodynia in patients with neuropathic pain or for other indications. Recently, we discovered several potent and selective TRPM8 antagonists that inhibited menthol or icilin-induced Ca<sup>2+</sup> influx in HEK293 cells expressing human TRPM8 channels. These compounds are potential candidates for the treatment of pain, inflammation, and other disease states.

## References

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## In vitro Pharmacology

### Materials and Methods

#### *In vitro* human and rat TRPM8 functional assays:

30 μM Menthol or 3 μM icilin-induced Ca<sup>2+</sup> influx was monitored by the cell imaging technology by Hamamatsu Photonics's Functional Drug Screening System (FDSS) using a Ca<sup>2+</sup>-sensitive fluorescent dye, Fluo4 (Molecular Probes).

#### Cell information:

HEK293 cells stably expressing human TRPM8 or transiently expressing rat TRPM8 were used for functional assays.

#### Reference compound:

BCTC(N-(4-t-Butylphenyl)-4-(3-Chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide, BIOMOL #CA-231).

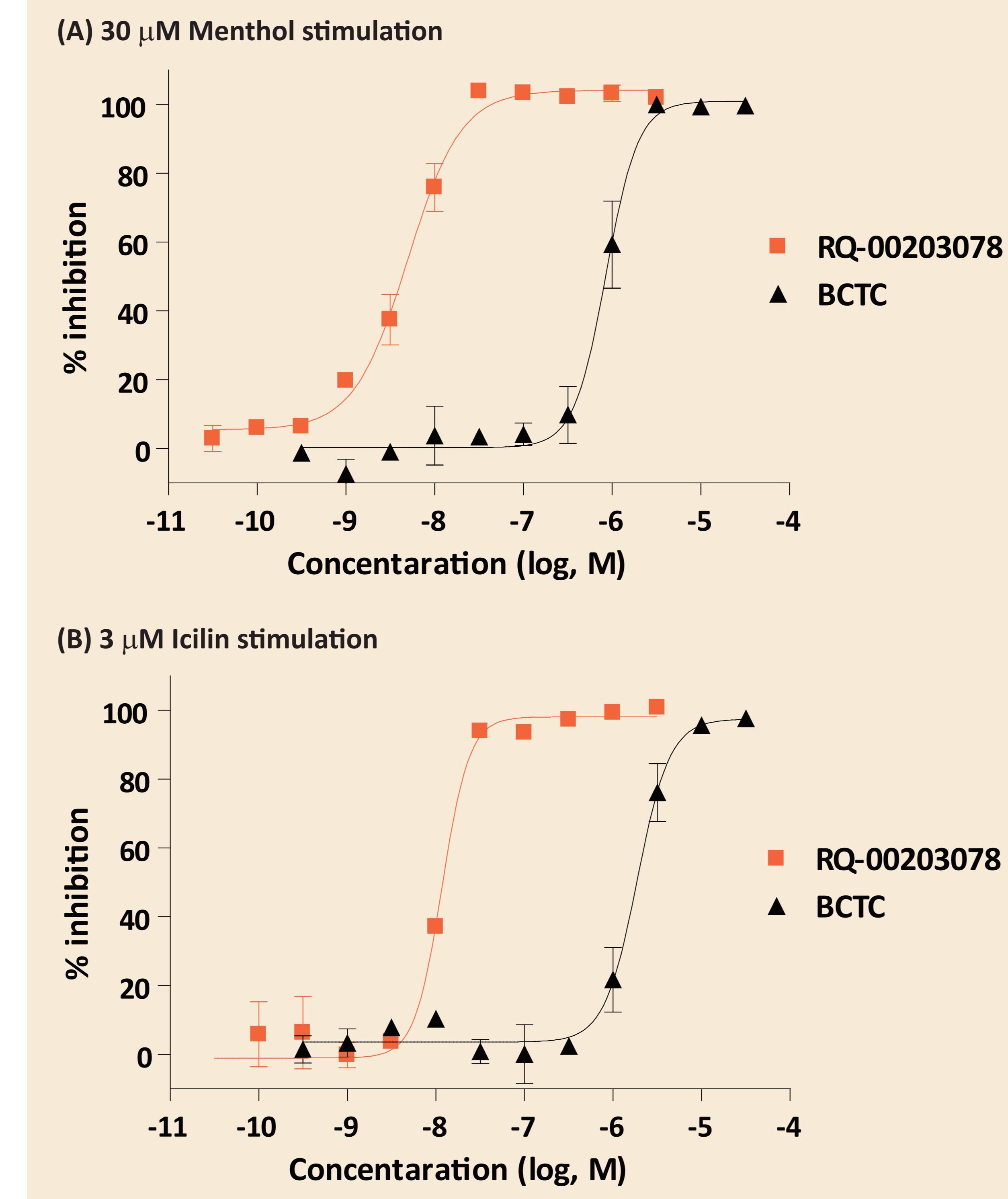
### Results

Table 1 Antagonistic activities of RQ-00203078 and BCTC in 30 μM menthol or 3 μM icilin-induced Ca<sup>2+</sup> influx using HEK293 cells expressing the human or rat TRPM8 receptors.

Compound	human TRPM8 IC <sub>50</sub> (nM)		rat TRPM8 IC <sub>50</sub> (nM)	
	Menthol	Icilin	Menthol	Icilin
RQ-00203078	4.8	18	3.5	18*
BCTC	760	2100	400	2000*

\* n = 1  
Values are geometric mean (n = 3 or 4).

Figure 1 Effect of RQ-00203078 (■) and BCTC (▲) on 30 μM menthol (A) or 3 μM icilin (B)-induced Ca<sup>2+</sup> influx in HEK293 cells expressing the human TRPM8 receptors. Curve fitting analysis was performed with GraphPad Prism version 4.02. Results are from one representative experiment in three or four experiments.



## In vivo Pharmacology

### Materials and Methods

#### Animals

Male Sprague-Dawley rats were purchased from Charles River Japan and housed in groups of 2 under a 12 hour light/dark cycle with food and water *ad libitum*. All experimental procedures were approved by the animal ethic committee of RaQualia Pharma Inc.

#### CCI surgery

The CCI surgery was performed according to the method of Bennett and Xie<sup>5</sup>. Briefly, the left common sciatic nerve was exposed and 4 ligatures were loosely tied around it by using 4-0 silk (Ethicon, Inc.). Sham operation was performed in the same manner except of sciatic nerve ligation.

#### Cold allodynia

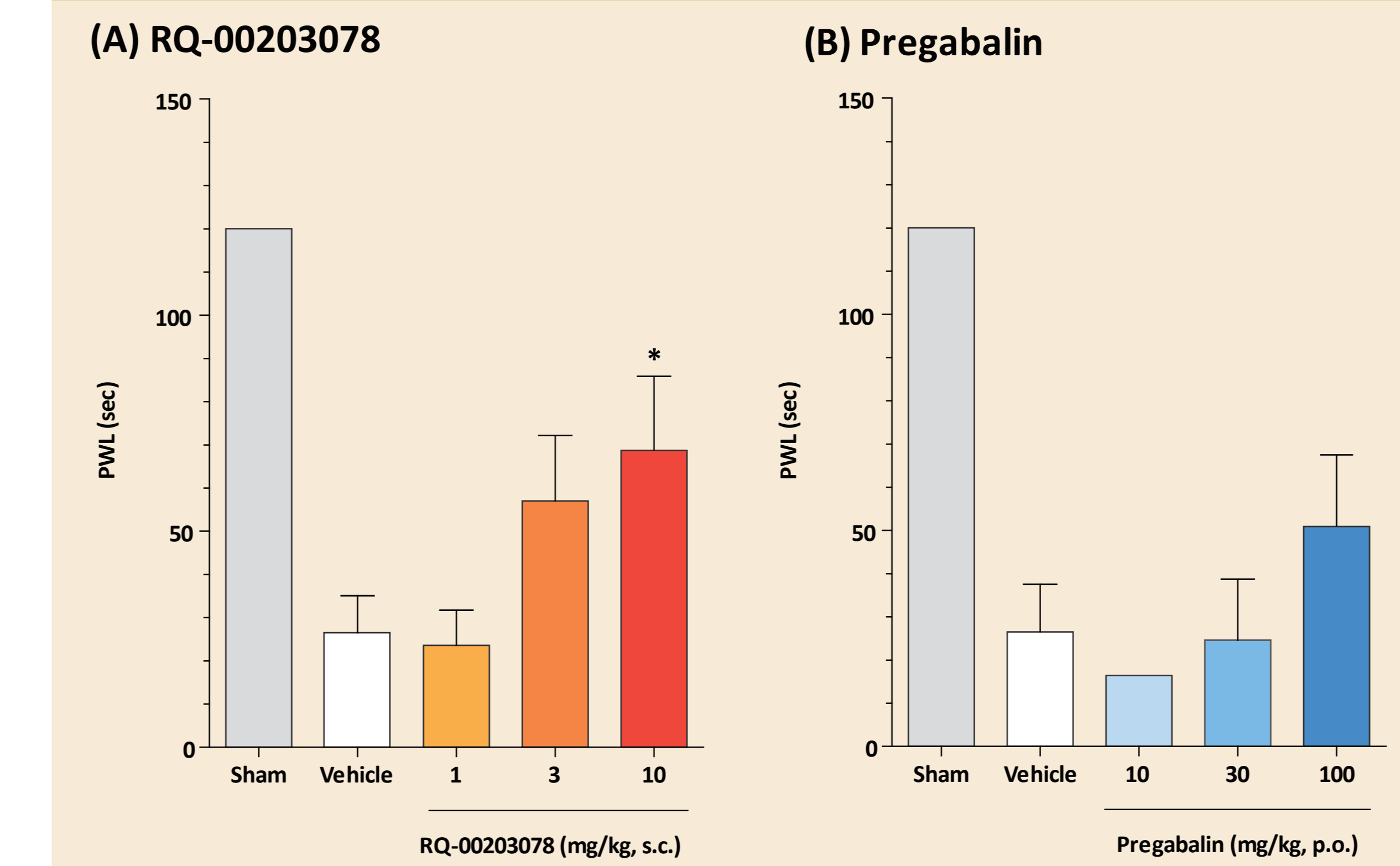
Cold allodynia at 10°C was assessed using a cold plate (LHP-1700CP, TECA) as described previously<sup>6</sup>. The rat was placed on the cold plate and the paw withdrawal latency (PWL) was measured (cut-off; 120 sec). Evaluation was performed on the 7th post-operative days (POD) in a non-blind manner.

#### Static allodynia

Static allodynia was evaluated by application of von Frey hairs (VFHs) in ascending order of force (0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15 and 26 gram) to the plantar surface of the hind paw as described previously<sup>7</sup>. Each VFH was applied to the paw for 6 sec, or until a withdrawal response occurred. The lowest amount of force required to elicit a response was recorded as paw withdrawal threshold (PWT). Evaluation was performed on the 14th POD in a non-blind manner.

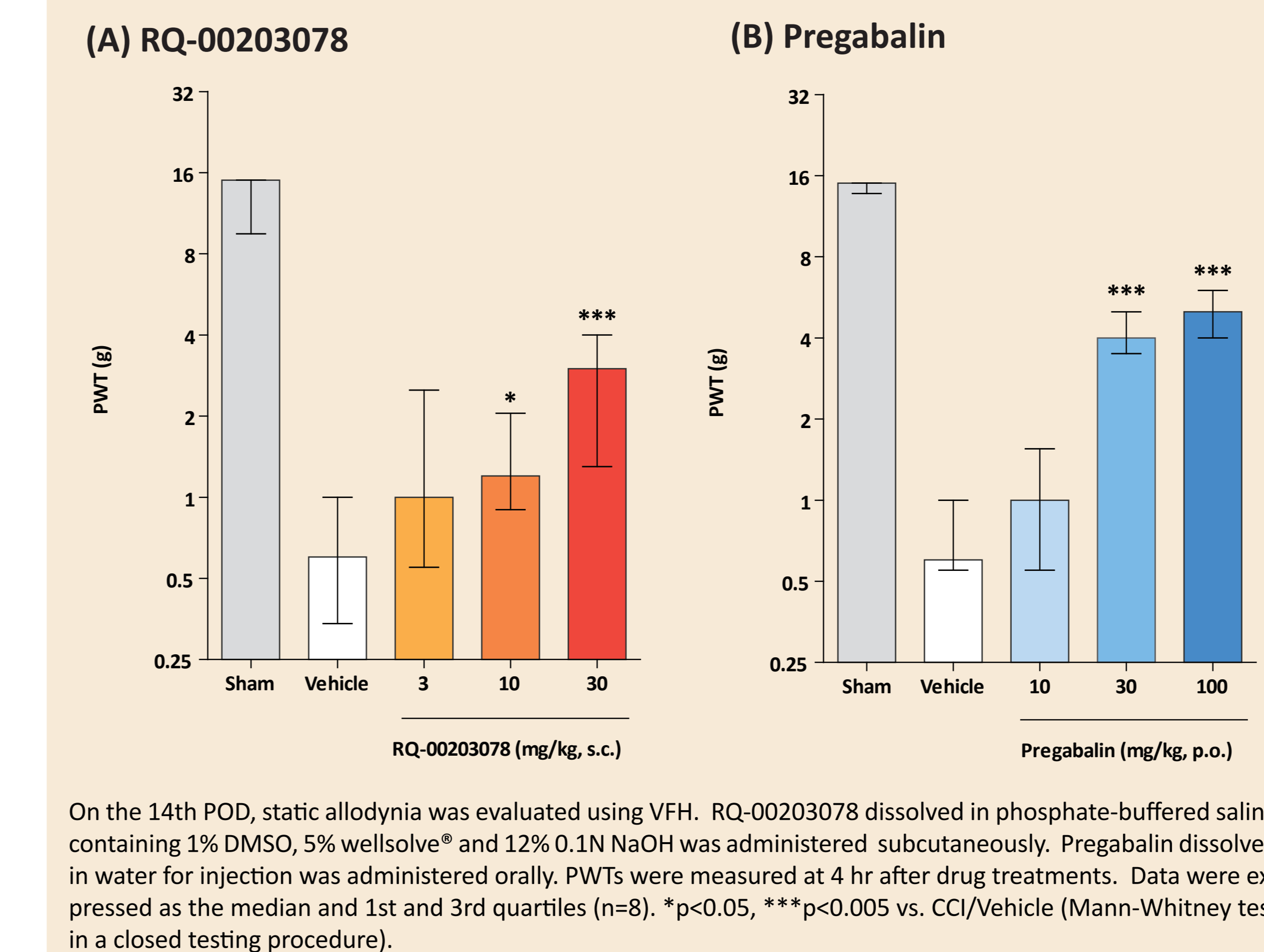
### Results

Figure 2 Effect of (A) RQ-00203078 and (B) Pregabalin on CCI-induced cold allodynia in rats



On the 7th POD, cold allodynia was evaluated using cold plate apparatus. RQ-00203078 dissolved in phosphate-buffered saline containing 1% DMSO, 5% wellove<sup>®</sup> and 12% 0.1N NaOH was administered subcutaneously. Pregabalin dissolved in water for injection was administered orally. PWLs at 10°C were measured at 4 hr after drug treatments. Data were expressed as the mean ± S.E.M. (n=7-8). \*p<0.05 vs. CCI/Vehicle (unpaired t-test in a closed testing procedure).

Figure 3 Effect of (A) RQ-00203078 and (B) Pregabalin on CCI-induced static allodynia in rats



On the 14th POD, static allodynia was evaluated using VFH. RQ-00203078 dissolved in phosphate-buffered saline containing 1% DMSO, 5% wellove<sup>®</sup> and 12% 0.1N NaOH was administered subcutaneously. Pregabalin dissolved in water for injection was administered orally. PWTs were measured at 4 hr after drug treatments. Data were expressed as the median and 1st and 3rd quartiles (n=8). \*p<0.05, \*\*\*p<0.005 vs. CCI/Vehicle (Mann-Whitney test in a closed testing procedure).

## Safety Evaluation

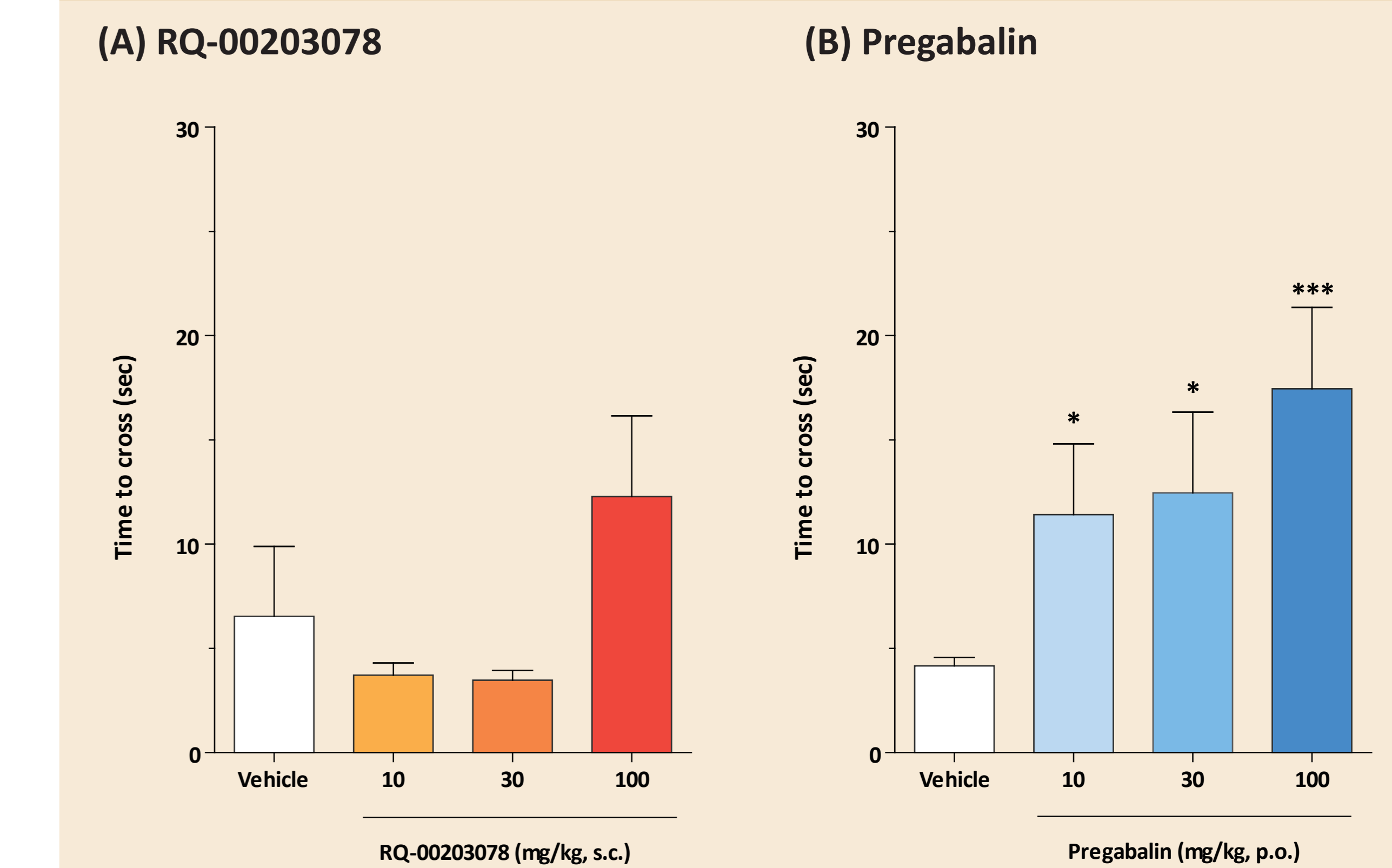
### Materials and Methods

#### Beam walking

Beam walking apparatus consists of a 1.5 m long beam with a 2.7x2.7 cm square cross section, elevated 0.8 m above the floor. The test was performed in a dim light condition. A light source was placed at the start-end of the beam and served as avoidance stimuli while a dark box at the other side represented a goal box to reach. Rats were trained to cross the beam over 2 days. On the day of test, baseline recordings were taken and selected rats were those crossing the beam in less than 10 seconds and with 1 foot slip or less. Cut-off of 30 sec and 5 foot slips were taken for those rats that did not cross or fell off the beam.

### Results

Figure 4 Effect of (A) RQ-00203078 and (B) Pregabalin on motor performance in rats



In the normal rats, motor performance was evaluated using beam walking. RQ-00203078 dissolved in phosphate-buffered saline containing 4% DMSO, 11% wellove<sup>®</sup> and 35% 0.1N NaOH was administered subcutaneously. Pregabalin dissolved in water for injection was administered orally. Time to cross was measured at 4hr after drug treatment. Data were expressed as the mean ± S.E.M. (n=8). \*p<0.05, \*\*\*p<0.005 vs. Vehicle (Mann-Whitney test in a closed testing procedure).

Table 2 Selectivity to TRP family and Pain-related ion channels.

Target	Species	Stimulant	IC <sub>50</sub> (μM)
<b>In-house TRP channel assays</b>			
TRPA1	human	300 μM Arylisothiocyanate	>10
TRPV1	human	300 μM Capsaicin	>30
TRPV4	human	Hypotonic solution	10
TRPM2	human	500 μM H <sub>2</sub> O <sub>2</sub>	>10
<b>In-house pain-related ion channel assays</b>			
Nav1.3	human	Veratridine/Na <sup>+</sup>	>30
Nav1.5	human	Veratridine/Na <sup>+</sup>	>30
Nav1.7	human	Veratridine/Na <sup>+</sup>	13
T-type Ca	human	90 mM KCl	3.1
N-type Ca	human	117 mM KCl	>10
ASIC1a	human	H <sup>+</sup> (pH6.0)	7.8
ASIC3	human	H <sup>+</sup> (pH6.0)	19

### Cerep broad profiling

- Total 64 targets were tested at 10 μM
- No inhibition except for:
  - Cl<sup>-</sup> channel (GABA-gated): 79% inhibition at 10 μM
  - PPARγ (agonist ligand): 56% inhibition at 10 μM

### Others

- hERG channel (dofetilide binding): 3% inhibition at 30 μM
- I<sub>HERG</sub>: No inhibition at 30 μM
- In vitro* micronucleus: negative with/without S9