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Corticotropin releasing hormone receptor 2 antagonist, RQ-00490721, for the prevention of pressure overload-induced cardiac dysfunction

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ABSTRACT

Background: G protein-coupled receptors (GPCRs) regulate the pathological and physiological functions of the heart. GPCR antagonists are widely used in the treatment of chronic heart failure. Despite therapeutic advances in the treatments for cardiovascular diseases, heart failure is a major clinical health problem, with significant mortality and morbidity. Corticotropin releasing hormone receptor 2 (CRHR2) is highly expressed in cardiomyocytes, and cardiomyocyte-specific deletion of the genes encoding *CRHR2* suppresses pressure overload-induced cardiac dysfunction. This suggests that the negative modulation of CRHR2 may prevent the progression of heart failure. However, there are no systemic drugs against CRHR2.

Findings: We developed a novel, oral, small molecule antagonist of CRHR2, RQ-00490721, to investigate the inhibition of CRHR2 as a potential therapeutic approach for the treatment of heart failure. *In vitro*, RQ-00490721 decreased CRHR2 agonist-induced 3′, 5′-cyclic adenosine monophosphate (cAMP) production. *In vivo*, RQ-00490721 showed sufficient oral absorption and better distribution to peripheral organs than to the central nervous system. Oral administration of RQ-00490721 inhibited the CRHR2 agonist-induced phosphorylation of cAMP-response element binding protein (CREB) in the heart, which regulates a transcription activator involved in heart failure. RQ-00490721 administration was not found to affect basal heart function in mice but protected them from pressure overload-induced cardiac dysfunction.

Interpretation: Our results suggest that RQ-00490721 is a promising agent for use in the treatment of chronic heart failure.

1. Introduction

Heart failure is a critical disease caused by various factors leading to myocardial damage, with a 4-year mortality rate of 60% in developed countries [1,2]. In developing countries, the incidence of heart failure is expected to increase to a level similar to that of developed countries as socio-economic development progresses [3]. Although the treatment of cardiovascular diseases, such as coronary artery disease and hypertension, has improved, the prognosis for heart failure remains poor [4]. Therefore, there is a need for new treatment options for the management of heart failure. G protein-coupled receptors (GPCRs) are the largest superfamily of cell surface receptors; these proteins are involved in various transmembrane signaling systems and play a role in numerous physiological and pathological processes [5]. The GPCR family includes seven highly-conserved transmembrane receptors, which are considered good targets for drug therapy [6]. GPCRs in the heart are involved in the regulation of cardiac function in response to extracellular stimuli, such as catecholamines and angiotensin II, and play a role in cardiac dysfunction and fibrosis [7]. Therefore, GPCR antagonists are commonly used to treat patients with chronic heart failure [8,9]. Several GPCRs are

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expressed in the heart, but only β-adrenergic and angiotensin II receptor blockers are clinically targeted for the long-term treatment of patients with chronic heart failure [10]. Recently, angiotensin receptor–neprilysin inhibitors, which increase the activity of natriuretic peptides, in addition to functioning as angiotensin II receptor blockers, have been spread worldwide [11]. Despite the availability of these effective treatments, mortality and hospitalization rates have remained high for over 10 years, suggesting that additional uncharacterized GPCRs may also be involved in mediating the pathophysiology of the disease [12].

A non-biased quantitative RT-PCR (qRT-PCR) analysis, which determined the gene copy numbers of 475 GPCRs in adult murine cardiomyocytes, revealed that corticotropin releasing hormone receptor 2 (CRHR2) was the 4th most abundantly expressed GPCR in cardiomyocytes [13]. Western blot analysis indicated that CRHR2 is exclusively expressed in the heart and is undetectable in other tissues. The continuous infusion of urocortin 2 (Ucn2), an endogenous CRHR2 agonist, has been reported to reduce the left ventricular ejection fraction in mice, suggesting that constitutive CRHR2 activation may be responsible for cardiac dysfunction [13]. The decline of cardiac function is suppressed in CRHR2-deficient mice as a result of Ucn2 loading and transverse aortic constriction (TAC). These findings suggest that negative modulators of CRHR2 could form a new oral treatment option for heart failure. In this study, we investigated the *in vitro* and *in vivo* pharmacological profiles of the CRHR2 antagonist, RQ-00490721, and found it to be a safe and promising oral treatment option for chronic heart failure.

2. Materials and methods

2.1. Study design

To examine whether the CRHR2 antagonist, RQ-00490721, could be a novel treatment for chronic heart failure, we conducted pharmacological and pharmacokinetic studies, and investigated the efficacy in pressure overload-induced heart failure. Mice were randomized into groups before TAC surgery and RQ-00490721 was continuously administered twice a day for 4 weeks.

2.2. Chemicals

RQ-00490721, a fused cyclic urea derivative claimed in WO2019198692, was synthesized by RaQualia Pharma Inc. Human and murine Ucn2 peptides were purchased from Bachem (Bubendorf, Switzerland) and Peptide Institute (Osaka, Japan), respectively. For the *in vitro* study, RQ-00490721 was dissolved in dimethyl sulfoxide (DMSO), and the lyophilized Ucn2 peptides were dissolved in distilled water. For the *in vivo* study, RQ-00490721 was dissolved in saline containing 4% DMSO and 10% Cremophor EL (Sigma, St. Louis, MI, USA) for intravenous administration and suspended in distilled water containing 0.5% methylcellulose 4000 (Wako Chemicals, Richmond, VA, USA) for oral administration. Lyophilized murine Ucn2 was dissolved in saline.

2.3. Animals

Male C57BL/6J mice (7–9 weeks old) were obtained from Charles River Laboratories Japan Inc. All procedures of animal care and animal use in this study were approved by the Animal Ethics Review Board of Nagoya University School of Medicine. A standard 6-lead electrocardiogram was performed with ECG Processor System (SP-2000; Softron, Tokyo, Japan), measuring I, II, and III induction. Blood pressure was measured in an awake state by the tail-cuff method using an automatic sphygmomanometer (BP98A; Softron).

To examine the Ucn2-induced phosphorylation of CREB in mice, Ucn2 (25, 50, 100 μg/kg) or vehicle was subcutaneously injected into male C57BL/6J mice. Thirty minutes after administration, the heart was sampled, and immunoblot analysis of phosphorylated CREB (pCREB) was performed. Antibodies for CREB (#9197) and pCREB (Ser133, #9198) were obtained from Cell Signaling Technology (Danvers, MA, USA). RQ-00490721 (10, 30, and 100 mg/kg) was orally administered to the mice. Murine Ucn2 (100 μg/kg) was subcutaneously injected at 90 min after the oral administration of RQ-00490721. Total protein was extracted from the heart at 30 min after the subcutaneous injection of Ucn2.

Total RNA was extracted from left ventricular tissue with the ReliaPrep™ RNA Tissue Miniprep System (Promega, Fitchburg, WI, USA), followed by reverse transcription to cDNA with SuperScript™ III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. qRT-PCR was performed with TaqMan Gene Expression Assays (Thermo Fisher Scientific) for Nppb (Mm01255770_g1) and Gapdh (Mm99999915_g1) using 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific).

2.4. cAMP assay

CHO-K1 cells stably expressing human CRHR2 (NM_001883.4; DiscoveRX, Eurofins Scientific, Luxembourg) or murine CRHR2 (NM_001288618; generated by RaQualia Pharma Inc., Japan) were harvested after serum starvation in culture media containing 0.1% fetal bovine serum. Intracellular 3′,5′-cyclic adenosine monophosphate (cAMP) levels were measured using a LANCE Ultra cAMP kit (Perkin Elmer, Waltham, MA, USA), as per the manufacturer's protocol. Briefly, the cells were thawed and washed with Hanks' balanced salt solution (HBSS) before being resuspended in assay buffer. The cell suspensions were plated in 384-well microplates at a density of 4000 cells/well. The cells were then pre-incubated with various concentrations of RQ-00490721 for 30 min at 25 °C. Human or murine Ucn2 was added to stimulate the production of cAMP, and the mixtures were incubated at 25 °C for 30 min. Detection reagent containing europium-labeled cAMP tracer and ULight dye-labeled cAMP antibodies were added, and the mixtures were incubated at 25 °C for 60 min. Time-resolved fluorescence energy transfer signals were measured using an EnVision plate reader (Perkin Elmer) with the recommended settings. The IC_{50} values of RQ-00490721 were calculated by fitting to an equation for a non-linear regression four-parameter dose response curve using the EC_{90} concentrations of human and murine Ucn2. Six independent experiments were performed. Schild plots were created by the calculated data obtained from concentration response curves of Ucn2, and the corresponding pA2 values were calculated. Three independent experiments were performed.

2.5. In vitro selectivity and safety profiling

To investigate *in vitro* selectivity profiling, RQ-00490721 was tested at a concentration of 10 μM in the lead profiling screen 2 panel (Eurofins Cerep, Celle-Lévescault, France). The affinities of RQ-00490721 to hERG and human Nav1.5 were assessed using an automated patch clamp QPatch HTX system (Sophion Bioscience, Ballerup, Denmark). The hERG assay was performed using HEK293 cells stably expressing human Kv11.1, with an extracellular solution (145 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4 adjusted with NaOH) and an intracellular solution (5.374 mM $CaCl_2$, 1.75 mM $MgCl_2$, 10 mM egtazic acid, 10 mM HEPES, 120 mM KCl, 10 mM sucrose, 4 mM Na_2-ATP; pH 7.2 adjusted with KOH). The cells were clamped at − 80 mV, and the currents were elicited every 5 s by depolarizing the membrane potential to + 40 mV for 1000 ms; this was followed by a 240 ms voltage step to − 80 mV to allow the determination of IC_{50} against tail current amplitude. The Nav1.5 assay was performed using CHO cells stably expressing human Nav1.5 with an extracellular solution (137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4 adjusted with NaOH) and an intracellular solution (135

mM CsF, 5 mM egtazic acid, 10 mM HEPES, 10 mM NaCl; pH 7.3 adjusted with CsOH). The cells were clamped at − 110 mV, and the currents were elicited every 30 s, using a conventional two-pulse protocol. An 8 s conditioning pulse, at which approximately 50% of the channels were inactivated, was applied following the first test pulse. After a 20 ms recovery period at − 110 mV, the second test pulse at − 10 mV was applied for 10 ms. The IC_{50} value was calculated based on the reduction in current amplitude evoked by the second test pulse.

The CYP inhibition assay was conducted by Sumika Chemical Analysis Service, Ltd (Japan). Briefly, the inhibitory potency of RQ-00490721 was assessed at concentrations ranging from 0.4 to 50 μM using pooled human liver microsomes (0.05 mg/mL; SEKISUI XenoTech, LLC, Kansas City, KS, USA) in a cocktail format for 7 major CYPs. Probe substrates (phenacetin 50 μM for 1A2, bupropion 3 μM for 2B6, amodiaquine 2 μM for 2C8, diclofenac 10 μM for 2C9, S-mephenytoin 40 μM for 2C19, bufuralol 5 μM for 2D6, and midazolam 2.5 μM for 3A4) were used for the assay.

2.6. Pharmacokinetics of RQ-00490721 in mice

For single dosing, RQ-00490721 (1 mg/kg) was administered intravenously or orally to male C57BL/6J mice ($n = 2$). For multiple dosing, RQ-00490721 (100 mg/kg) was administered orally to mice ($n = 3$) for 5 days. Blood samples were drawn from the tail vein at the indicated time points after dosing. Brain and plasma samples were collected at 1 h after the administration of a single oral dose of 10 mg/kg, and brain samples were homogenized. After centrifugation at 4000*g* for 5 min at 4 °C, the extraction step was conducted. The concentrations of RQ-00490721 in plasma and brain tissues were measured using liquid chromatography-tandem mass spectrometry (Triple Quad 5500; SCIEX, Framingham, MA, US) and calculated using Analyst Software 1.6.3. (SCIEX). Pharmacokinetic parameters were determined *via* non-compartmental analysis using Phoenix WinNonlin version 6.2 (Pharsight Corporation, Mountain View, CA, USA).

2.7. TAC surgery

Eight-week-old male mice were anesthetized with hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg). TAC was performed under anesthesia *via* intraperitoneal administration with intubation. The aortic arch was visualized by opening the chest, and the transverse aorta was ligated between the right innominate and left common carotid arteries using a blunted 24-gauge needle with an 8–0 suture. The sham procedure was performed in a similar manner, except for the ligation of the aorta. Treatment with oral RQ-00490721 was started one week after performing a sham procedure or TAC surgery. RQ-00490721 (100 mg/kg) was orally administered twice daily for 4 weeks. Transthoracic echocardiography was performed using a Vevo 1100 imaging system (FUJIFILM VisualSonics, Inc., Toronto, Canada) equipped with an MS400 (18–38 MHz) phased-array transducer. The left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured to calculate the left ventricular fractional shortening (%FS): %FS = [LVEDD-LVESD]/LVEDD × 100. Five weeks after the TAC surgery, the animals were sacrificed, and organs were isolated. Sections of the left ventricles were stained with hematoxylin and eosin and Picrosirius red as per standard protocols, and specimens were prepared. Cardiomyocyte size and cardiac fibrosis were observed using a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan). Sixteen random images were quantified using the ImageJ software.

2.8. Statistical analysis

Data are presented as the mean ± SEM for all experiments. Statistical analysis for the animal experiments was performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). The means of two experimental groups were compared using the Student's unpaired *t*-test. One-way analysis of variance with Tukey's *post hoc* test was used to compare the means of more than two groups. "n" refers to the number of independent experiments or mice per group. Statistical significance was defined as $p < 0.05$; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. In vitro antagonistic activity

The potency of CRHR2 antagonist was determined by blocking Ucn2-induced 3′,5′-cyclic adenosine monophosphate (cAMP) production in CHO-K1 cells stably expressing human CRHR2. We discovered RQ-00490721 as a CRHR2 antagonist by examining its ability to inhibit Ucn2-induced cAMP production in a screen of over 100,000 compounds. The antagonistic effect of RQ-00490721, determined based on the 90% effective concentration (EC_{90}) of human (45 nM) or murine (0.5 nM) Ucn2, was found to be a pIC_{50} value of 7.09 ± 0.03 ($n = 6$) in humans (Fig. 1A) and 6.33 ± 0.07 ($n = 6$) in mice (Fig. 1B). The concentration response curves of human Ucn2 were shifted rightward, with no change in the maximum response (Fig. 1C). Moreover, the Schild slope was calculated as 1.00 (Fig. 1D), suggesting that RQ-00490721 competes with Ucn2 for binding to CRHR2. The pA2 value of RQ-00490721 was found to be 7.12 ± 0.02 ($n = 3$) for human CRHR2. We also examined the antagonistic activity of RQ-00490721 in CHO-K1 cells stably expressing murine CRHR2 (Supplemental Fig. 1A). The pA2 value of RQ-00490721 for murine CRHR2 was found to be 6.61 ± 0.16 ($n = 3$, Supplemental Fig. 1B). The variation in pA2 showed that the potency of RQ-00490721 against human CRHR2 was approximately 3-fold higher than that against murine CRHR2.

3.2. In vitro evaluation of safety

To examine potential off-target binding and selectivity issues of RQ-00490721, the inhibitory activity of the radioactively labeled ligand against 80 targets, including GPCRs, ion channels, and transporters, was assessed. RQ-00490721 showed less than 50% inhibition at a concentration of 10 μM against most of the targets, except for the human M1 muscarinic acetylcholine receptor (60% inhibition) and human glucocorticoid receptor (70% inhibition) (Supplemental Table 1). The cardiovascular and drug–drug interactions of RQ-00490721 were also assessed *in vitro*. No inhibition of human ether-a-go-go-related gene (hERG) potassium channels or human cardiac sodium channels (hNav1.5), which are associated with cardiac safety, was noted (half maximal inhibitory concentration [IC_{50}] > 10 μM). Although induction or inhibition of cytochrome P450 (CYP), a critical drug-metabolizing enzyme, has the potential to cause drug–drug interactions, RQ-00490721 did not significantly inhibit the levels of CYP isoforms 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 ($IC_{50} > 10$ μM).

3.3. Pharmacokinetic profile of RQ-00490721

RQ-00490721 (1 mg/kg) was administered intravenously to mice. It exhibited moderate clearance (0.18 L/h/kg) and volume of distribution at steady state (0.73 L/kg, Fig. 2A). After administering the same dose orally, the maximum plasma concentration (C_{max}) of RQ-00490721 was found to be 0.816 μg/mL, and the moderate-terminal elimination half-life was recorded as 3.8 h. The bioavailability was close to 100%, suggesting that RQ-00490721 was well absorbed upon oral administration (Fig. 2B). Upon administration of multiple oral doses of RQ-00490721 (100 mg/kg), no changes in the plasma C_{max} and area under the curve (AUC) were observed between day 1 and day 5 (Fig. 2C and D); the unbound or free concentration of RQ-00490721 was approximately 4.3–5.3 μM. The brain-to-plasma ratio of RQ-00490721 concentration was found to be 0.038 (Supplemental Table 2), suggesting that RQ-00490721 showed higher distribution to the peripheral organs than to

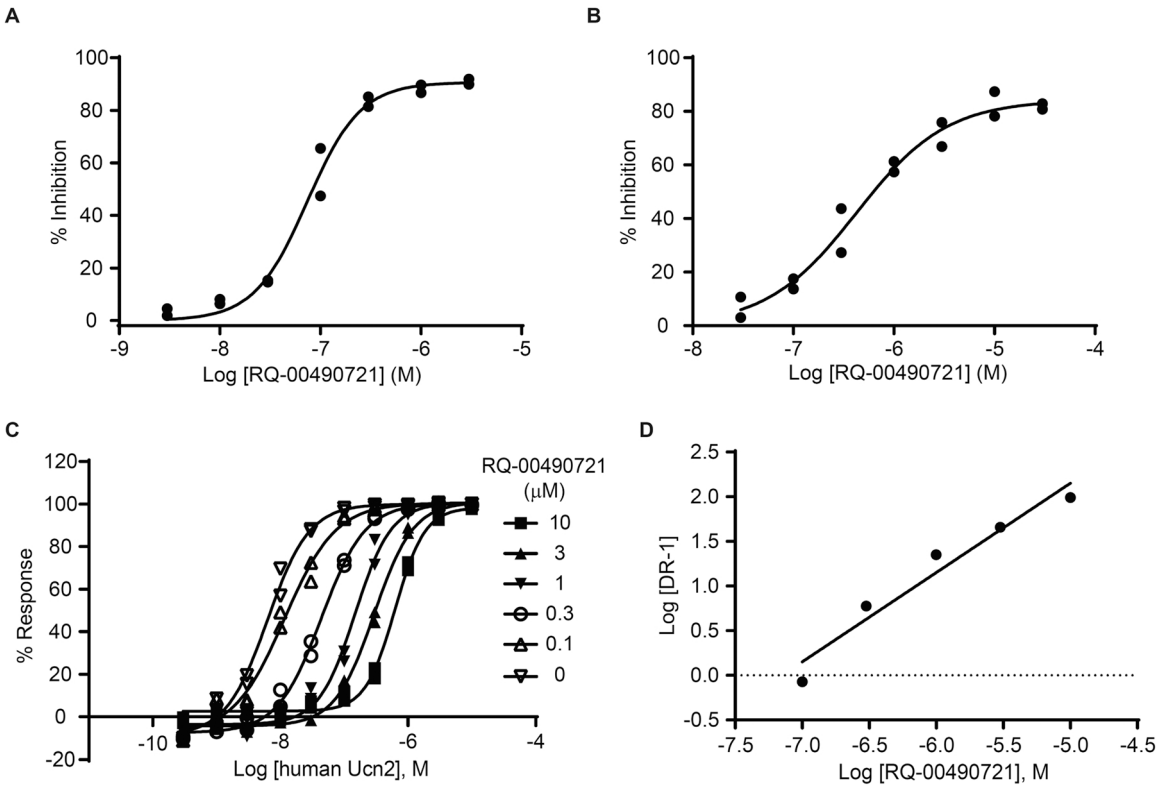


Fig. 1. RQ-00490721 suppressed corticotropin-releasing hormone receptor 2 (CRHR2) agonist-induced 3′, 5′-cyclic adenosine monophosphate (cAMP) production *in vitro*. (A and B) The inhibitory concentration–response curves of RQ-00490721, based on data from the cAMP assay using human (A) or murine (B) CRHR2-expressing cells. Data indicate the percentage of maximal inhibition of cAMP production provided by the assay buffer without Ucn2 or antagonist. Representative data from six independent experiments. (C) Concentration–response curves of human Ucn2 in the absence and presence of RQ-00490721, based on data obtained from the cAMP assay using human CRHR2-expressing cells. Representative data from three independent experiments conducted in duplicate. (D) Schild regression plots calculated from the concentration–response curve (C). The pA2 value was 7.15 and the slope was 1.00. Representative data from three independent experiments. Abbreviations: Ucn2, urocortin 2.

the central nervous system.

3.4. Inhibitory effect on Ucn2-induced phosphorylation

The CRHR2-mediated phosphorylation of cAMP-response element binding protein (CREB) at Ser133 enhances the expression of genes associated with heart failure [14]. Immunoblot analysis showed that Ucn2 induced the phosphorylation of CREB at Ser133 in the heart in a dose-dependent manner (Fig. 3A, B). Next, we examined the ability of RQ-00490721 to suppress CRHR2-mediated CREB phosphorylation *in vivo*. The Ucn2-induced phosphorylation of CREB in the heart was suppressed by RQ-00490721 in a dose-dependent manner (Fig. 3C and D), suggesting that orally administered RQ-00490721 suppresses the Ucn2-induced activation of CRHR2 in the heart.

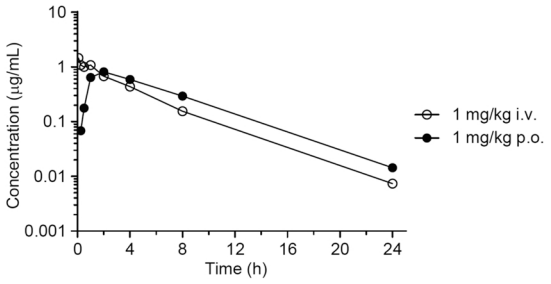
3.5. Consecutive oral administration of RQ-00490721

RQ-00490721 (100 mg/kg) was orally administered twice daily for 4 weeks (Table 1). No significant differences were noted in the body weight and blood pressure of the mice treated with the vehicle or those treated with RQ-00490721. Next, we performed an electrocardiogram analysis. PR interval, the interval between the onset of the P wave and the onset of the QRS complex, represents the intra-atrial or atrioventricular conduction [15]. A long QT interval due to prolonged ventricular repolarization is associated with polymorphic ventricular tachycardia known as *torsades de pointes* [16]. Electrocardiogram analysis showed no significant differences in the heart rate, PR interval, or QT interval of mice between the control and RQ-00490721-treated groups. Echocardiography also revealed no significant differences in fractional shortening between the two groups. These findings suggest that the repetitive administration of RQ-00490721 does not affect the physiological cardiac function in normal mice.

3.6. Effect on pressure overload-induced cardiac dysfunction

We investigated the effect of RQ-00490721 treatment on the attenuation of cardiac hypertrophy progression in mice. Mice with pressure overload due to TAC are expected to develop cardiac hypertrophy within 1–2 weeks [17,18]. Echocardiography was performed before and after performing TAC surgery, and left ventricular fractional shortening was measured (Fig. 4A). Oral treatment with RQ-00490721 was started one week after the sham procedure or TAC surgery. Macroscopically, TAC showed a strong tendency to cause cardiac hypertrophy, and treatment with RQ-00490721 was found to suppress TAC-induced cardiac hypertrophy (Fig. 4B). In addition, RQ-00490721 inhibited the TAC-induced increase in the left ventricular weight-to-tibia length ratio (Fig. 4C). qRT-PCR analysis showed that RQ-00490721 decreased the TAC-induced gene expression of brain natriuretic peptide, which is secreted by cardiomyocytes in response to pressure and volume overload (Fig. 4D) [19]. Although TAC decreased cardiac fractional shortening at three weeks after the surgery, RQ-00490721 was found to protect mice from further cardiac dysfunction at 3 and 5 weeks after TAC surgery (Fig. 4E). Sustained pressure overload causes adverse cardiac remodeling with hypertrophy and fibrosis, leading to heart failure [20,21]. We evaluated cardiomyocyte size by measuring the cross-sectional area in hematoxylin and eosin-stained heart sections (Fig. 4F). Four-week treatment with RQ-00490721 significantly reduced the TAC-induced increase in cardiomyocyte size (Fig. 4G). To investigate the

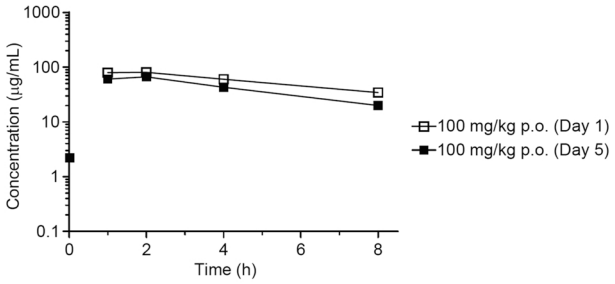
A



B

Route of administration	Dose (mg/kg)	C0 or Cmax (μg/mL)	t1/2 (h)	AUC0-24h (μg/hr/mL)	Vdss (L/kg)	CLtotal (L/hr/kg)	BA (%)
Intravenous administration	1	1.7	3.5	5.61	0.729	0.177	-
oral administration	1	0.816	3.8	6.62	-	-	119

C



D

Route of administration	Dose (mg/kg)	Days	Cmax (μg/mL)	t1/2 (h)	AUC0-8h (μg/hr/mL)
oral administration	100	1	82.9	5.3	452
	100	5	66.8	3.6	332

Fig. 2. Pharmacokinetic parameters of orally administered RQ-00490721 in mice. (A and B) Plasma concentrations of RQ-00490721 (1 mg/kg) following single intravenous and oral administration in mice. (C and D) Plasma concentrations of RQ-00490721 (100 mg/kg) following multiple oral administrations in mice. Abbreviations: i.v., intravenous; p.o., per os; C_{max}, peak plasma concentration; $t_{1/2}$, half life; AUC, area under the plasma concentration-time curve; Vdss, steady-state volume of distribution; CL, clearance; BA, bioavailability.

fibrotic changes induced by TAC, we performed Picrosirius red staining. Four-week treatment with RQ-00490721 significantly reduced TAC-induced cardiac fibrosis in mice (Fig. 4H and I). CRHR2 is coupled to the G-proteins, Gαs and Gβγ, resulting in the activation of respective cellular signaling pathways. Because CRHR2 activates Gαs-cAMP-dependent protein kinase A (PKA)-CREB and Gβγ-AKT signaling, we investigated the effect of RQ-00490721 treatment on the attenuation of TAC-induced phosphorylation of CREB at Ser133 and AKT at Ser473 (Supplemental Fig. 2A). Treatment with RQ-00490721 decreased TAC-induced CREB and AKT phosphorylation in the heart, suggesting that RQ-00490721 suppressed pressure-overload-induced Gαs/PKA/CREB, and Gβγ/AKT pathway activity (Supplemental Fig. 2B). These data indicate that RQ-00490721 has the potential to suppress heart failure induced by pressure overload.

4. Discussion

Pharmaceutical research has developed novel and unique therapies for various diseases. GPCRs, ion channels, kinases, nuclear receptors, and proteases are major targets for therapeutic drugs [22]. GPCRs constitute the largest family of targets for established drugs, and a large number of GPCRs are being explored as new drug targets in clinical trials [6]. Our previous work showed that the sustained stimulation of CRHR2 induces chronic heart failure [13]. Therefore, in order to evaluate negative modulation of CRHR2 as a therapeutic approach for the treatment of heart failure, we developed an orally available, novel, small molecule antagonist of CRHR2, RQ-00490721, and evaluated the pharmacological activity of RQ-00490721. The patent of RQ-00490721 and its analogs have been filed in WO2019198692 along with their drug-like structure, physicochemical properties, biological activities, and *in vitro* ADMET properties. RQ-00490721 is a lead compound for human clinical studies from the viewpoint of its chemical and biological properties and sufficient systemic exposure *via* oral administration in chronic treatment, as shown in this manuscript and discussed below.

GPCRs modulate physiological and pathological intracellular signaling through direct interactions with heterotrimeric G proteins on the inner surfaces of plasma membranes [23]. Gα subunits are classified into four families. CRHR2 increases intracellular cAMP levels through the activation of Gαs in monkey kidney cells and human embryonic kidney cell lines expressing exogenous CRHR2 [13,24]. *In vitro* analysis examining Ucn2-induced cAMP production showed that RQ-00490721 is a potent antagonist of CRHR2 (Fig. 1). The Schild slope suggested that RQ-00490721 may act as a competitive antagonist of CRHR2 (competing with Ucn2) rather than as an allosteric antagonist. Although oral administration of drugs is the most common route, absorption is often affected by various factors associated with the physiochemical properties of the drug and gastrointestinal physiology [25]. Continuous infusion of antisauvagine-30, a peptide antagonist of CRHR2, protects mice from cardiac dysfunction induced by pressure-overload [13,26]; however, the pharmacokinetic profiles of peptides do not allow for oral absorption and chronic administration [27]. In this study, the pharmacokinetic analysis of RQ-00490721 showed good oral absorption and reasonable properties to allow long-term studies by oral dosing.

Oral administration of RQ-00490721 (100 mg/kg) in mice achieved a C_{max} value of 82.9 μg/mL (Fig. 2). The unbound plasma concentration of RQ-00490721 was 10-fold greater than the cellular IC_{50} determined by the murine CRHR2 cAMP assay (Fig. 1C and D); therefore, a dose of 100 mg/kg was considered adequate for pharmacological evaluation. Furthermore, no changes in the plasma C_{max} or AUC were noted between day 1 and day 5 (Fig. 2C and D); this further warranted the long-term testing of RQ-00490721 in animal studies.

A non-biased qRT-PCR analysis revealed that CRHR2 was an abundantly expressed GPCR in adult murine cardiomyocytes [13]. The acute intravenous administration of urocortin in sheep has been reported to increase the heart rate and cardiac contractility [28]. To investigate whether RQ-00490721 affected electrophysiological characteristics of the heart, we performed patch clamp and electrocardiogram analyses. *In vitro*, automated patch clamp analysis indicated that RQ-00490721 did not inhibit hERG potassium channels (which are associated with long QT syndrome and life-threatening *torsade de pointes* arrhythmia) [29], or hNav1.5 (which is associated with PR and QT prolongation) [30]. *In vivo*, electrocardiography suggested that the heart rate, atrioventricular conduction, and ventricular repolarization were unaffected by multiple oral doses of RQ-00490721 in normal mice. Treatment with RQ-00490721 did not cause cardiac dysfunction, as shown by echocardiography. Moreover, basal cardiac function remained unaffected in conventional CRHR2-deficient mice and mice with cardiomyocyte-specific CRHR2 deficiency [13,31]. These data suggest that CRHR2 inhibition did not cause cardiac dysfunction in the normal heart. Western blot analysis of human tissues has indicated that the CRHR2 protein is exclusively expressed in the heart and is undetectable in other tissues; however, CRHR2 mRNA has been detected in the brain [14]. In this study, the brain to plasma ratio of RQ-00490721 concentration indicated low central nervous system penetration (Supplemental Table 2). Although the intravenous administration of Ucn2 reduced basal mean arterial pressure in rats [32], continuous oral administration of RQ-00490721 did not affect basal blood pressure in mice. These results indicated that RQ-00490721 did not cause any significant cardiotoxicity in mice. However, further studies are required to examine cardiotoxicity and physiological adverse effects of RQ-00490721 before

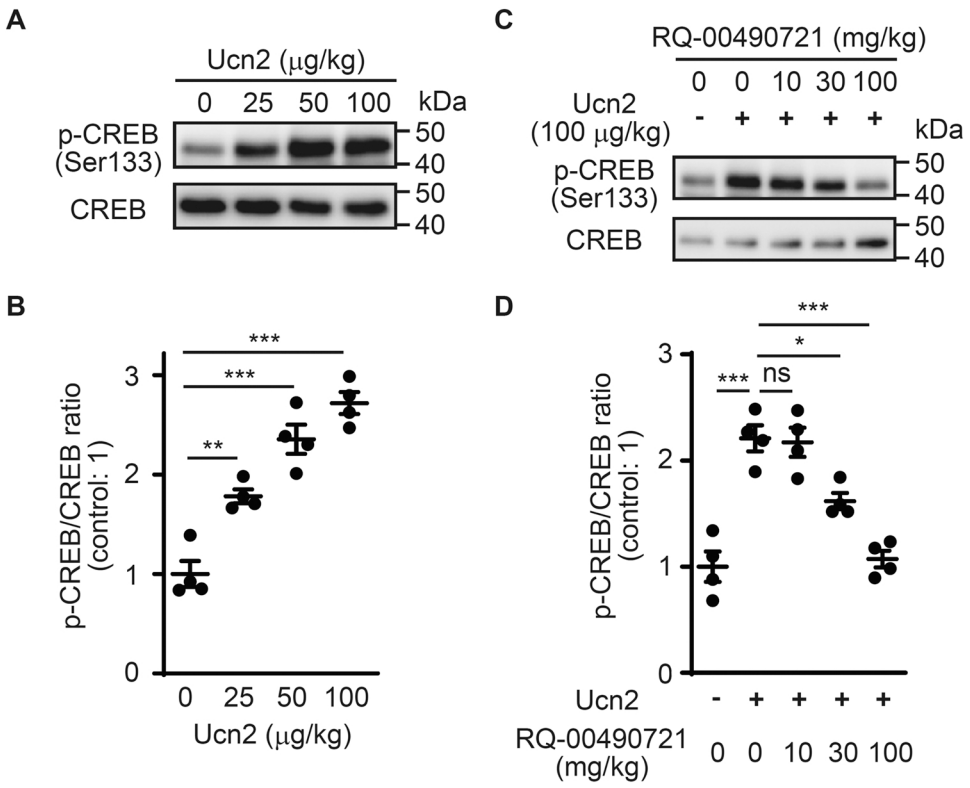


Fig. 3. Oral administration of RQ-00490721 suppressed the Unc2-induced phosphorylation of CREB in the heart. (A and B) Immunoblotting analysis showed the phosphorylation of CREB in the heart in response to Ucn2 ($n = 4$). (C and D) Ucn2-induced phosphorylation of CREB was suppressed by oral administration of RQ-00490721 ($n = 4$). Data are represented as mean ± SEM and analyzed with one-way analysis of variance with Tukey's *post hoc* test (B and D). ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1
Cardiac effects of RQ-00490721 after administration for 4 weeks in normal mice. Data are represented as mean ± SEM and analyzed using the Student's unpaired *t*-test. Abbreviations: ns, not significant.

	Control	RQ-00490721	
Body weight (g)	23.25 ± 0.22	22.90 ± 0.33	ns
Systolic blood pressure (mmHg)	96.00 ± 1.33	95.75 ± 0.56	ns
Heart rate (beat/min)	532.00 ± 1.07	523.50 ± 1.30	ns
PR interval (ms)	39.25 ± 0.56	39.50 ± 0.51	ns
QT interval (ms)	40.00 ± 0.17	41.25 ± 0.20	ns
Fractional shortening (%)	50.36 ± 0.16	50.19 ± 0.13	ns

clinical applications.

cAMP signaling in response to CRHR2-mediated Gαs activation modulates adenylyl cyclase and protein kinase A (PKA). CREB is a transcription factor regulated by phosphorylation in response to cAMP [33]; CREB-regulated transcription coactivators have functions in cancer, Parkinson's disease, and Alzheimer's disease [34,35]. Several growth factors and stress signals promote the phosphorylation of CREB at Ser133 in response to increased cAMP levels, caused by the phosphorylation of PKA or other kinases [36]. CREB phosphorylation at Ser133 enhances the expression of genes related to heart failure [37,38]. We showed that RQ-00490721 decreased the Ucn2-induced phosphorylation of CREB at Ser133 in a dose-dependent manner in the heart (Fig. 3C and D), and pressure overload-induced phosphorylation of CREB at Ser133 in the heart (Supplemental Fig. 2A). In addition to Gαs signaling, Ucn2 stimulation activates Gβγ-mediated phosphatidylinositol 3-kinase (PI3K) - AKT signaling [13], which is regulated by a growth factor receptor tyrosine kinase and by GPCR-mediated Gβγ subunit binding [39]. AKT initially promotes physiological cardiac hypertrophy; however, the chronic overactivation of AKT signaling induces pathological cardiac hypertrophy and dysfunction [40]. RQ-00490721 decreased the pressure overload-induced phosphorylation of AKT at Ser473 (Supplemental Fig. 2A). These findings suggest that orally administered RQ-00490721 inhibits CRHR2-mediated Gαs-PKA-CREB and Gβγ-PI3K-AKT signaling in the heart (Supplemental Fig. 2B).

Gαs-mediated signaling controls the cardiac activation *via* the positive chronotropic, lusitropic, and inotropic effects [41]; however, chronic stimulation resulting from transgenic overexpression of Gαs in mice caused them to develop cardiac hypertrophy and dilation as they aged [42]. A paradoxical effect is observed with catecholamines. While β-receptor stimulation in the acute phase of heart failure improves heart failure, the continuous suppression of β-receptors in chronic heart failure contributes to the improvement of cardiac function [43,44]. Animal studies have shown that injection of urocortin in mice, rats, and sheep results in inotropic and lusitropic effects [45]; however, it remains unclear whether treatment with a CRHR2 antagonist improves cardiac function in chronic heart failure. Our data demonstrated that the oral administration of CRHR2 antagonists for 4 weeks suppressed pressure overload-induced cardiac dysfunction in cases of pre-existing hypertrophy. Cardiomyocyte-specific CRHR2-deficiency protected mice from further deterioration of cardiac output after TAC surgery [13], supporting our hypothesis that RQ-00490721 treatment prevents cardiac dysfunction. Further studies are required to determine the mortality after long-term RQ-00490721 treatment and the physiological conditions of chronic heart failure for which RQ-00490721 treatment is suitable.

In conclusion, we developed a novel orally-available CRHR2 antagonist, RQ-00490721, and demonstrated its ability to prevent cardiac dysfunction in mouse models. The *in vitro* potency of RQ-00490721 against human CRHR2 was higher than that against murine CRHR2. Cardiac toxicity following oral administration was not observed *in vivo*. Therefore, RQ-00490721 may act as a starting point to facilitate the development of novel therapies for the treatment of chronic heart failure.

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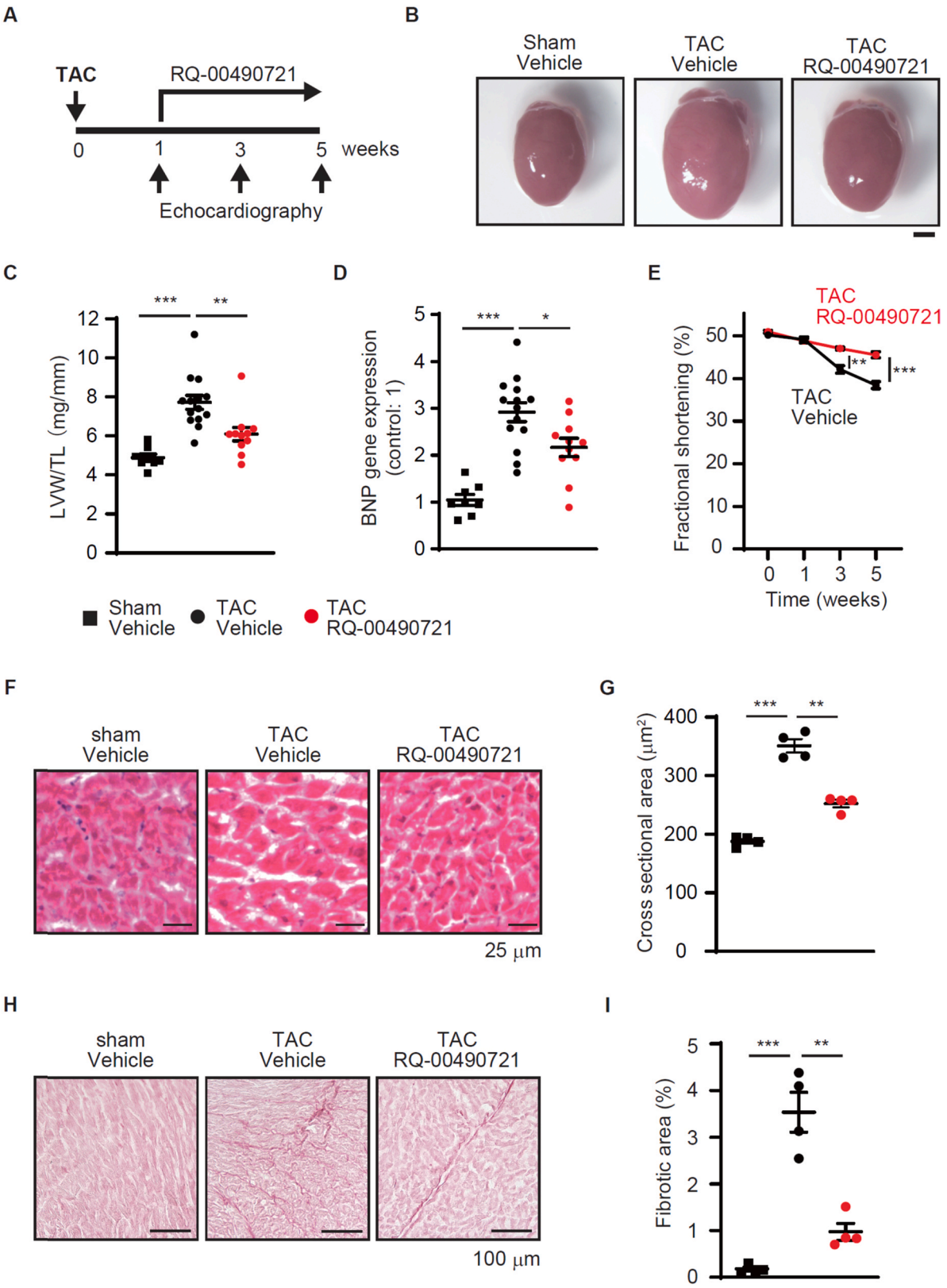

Fig. 4. Oral administration of RQ-00490721 prevented pressure overload-induced cardiac dysfunction in mice. (A) Experimental design. (B) Cardiac morphology at 5 weeks after TAC surgery, with or without RQ-00490721 treatment (scale bar, 1 mm). (C and D) Left ventricular weight/tibia length ratio (C) and BNP gene expression in the left ventricular were measured at 5 weeks after TAC surgery (D; $n = 8$–14). (E) Echocardiography was performed before and at 1, 3, and 5 weeks after TAC surgery to assess left ventricular fractional shortening ($n = 11$–14). (F and G) Cross-sectional area of left ventricles 5 weeks after TAC was assessed by hematoxylin and eosin staining ($n = 4$). (H and I) Fibrotic changes in left ventricles at 5 weeks after TAC surgery were assessed using Picrosirius red staining ($n = 4$). Data are presented as mean ± SEM and analyzed using one-way analysis of variance with Tukey's *post hoc* test (C, D, E, G, and I). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Abbreviations: TAC, transverse aortic constriction; LVW/TL, left ventricular weight/tibia length ratio; BNP, brain natriuretic peptide.

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Contributions

A.T., A.F., M.O., Y.I., H.M.P., and M.T. designed the experiments. M. O. generated the compound. A.T. performed *in vitro* experiments. Y.I., Y. M., T.S., T.Y., S.Y., S.I., S.E., T.T., and K.K. performed *in vivo* experiments. Y.M., A.T., A.F., and H.M.P. wrote the manuscript. K.O., N.O., and T.M. supervised the study and commented on the manuscript. M.T. initiated the study, performed experiments, analyzed and discussed data, and wrote the manuscript.

Conflict of interest statement

A.T., A.F., M.O., Y.I., and H.M.P. are employees of RaQualia Pharma Inc.

Data availability

All data are available in this manuscript and supplementary files.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112566.

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