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Identification and characterization of novel TRPV4 modulators ☆

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ABSTRACT

TRPV4, a close relative of the vanilloid receptor TRPV1, is activated by diverse modalities such as endogenous lipid ligands, hypotonicity, protein kinases and, possibly, mechanical inputs. While its multiple roles *in vivo* are being explored with KO mice and selective agonists, there is a dearth of selective antagonists available to examine TRPV4 function. Herein we detail the use of a focused library of commercial compounds in order to identify RN-1747 and RN-1734, a pair of structurally related small molecules endowed with TRPV4 agonist and antagonist properties, respectively. Their activities against human, rat and mouse TRPV4 were characterized using electrophysiology and intracellular calcium influx. Significantly, antagonist RN-1734 was observed to completely inhibit both ligand- and hypotonicity-activated TRPV4. In addition, RN-1734 was found to be selective for TRPV4 in a TRP selectivity panel including TRPV1, TRPV3 and TRPM8, and could thus be a valuable pharmacological probe for TRPV4 studies.

Introduction

The Transient Receptor Potential (TRP) family of ion channels is composed of 28 mammalian members divided among TRPA, TRPC, TRPM, TRPN, TRPML, TRPP and TRPV subfamilies [1]. As a class, they participate in a diverse set of sensory processes and, from the molecular level, can effect physiology of the whole organism [1,2]. An indication of their importance to normal physiological functions lies in the fact that mutations in TRP channels have been identified as the causative event in several human diseases [1,3].

TRPV1, also known as the vanilloid receptor, is the prototypical member of the TRPV subfamily. Identified through its activation by capsaicin, the active constituent of chili peppers [4], it has become the subject of an intense research effort aimed at developing a novel class of analgesics [5]. Predictably, this interest has been extended to encompass the other members of this subfamily. Among these, TRPV4 is arguably the most similar to TRPV1, at least in terms of the variety of its activation modalities: warm temperatures, hypotonicity, kinases and lipophilic ligands [6].

Identified only a decade ago as an osmosensor by two research teams [7,8], TRPV4 was found to be widely expressed with its presence being documented in the epithelium, kidney, lungs, central nervous system (CNS), sensory neurons (DRG), vascular endothelium, bladder and bone-related cell types (chondrocytes, osteoblasts and osteoclasts) [6]. Characterization of its biological roles in these settings has been pursued mostly with small molecule agonists, antisense oligodeoxynucleotides, and the analysis of KO mice [9–12]. Notably, TRPV4 was found to be a nociceptor playing an important role in inflammatory and neuropathic mechanical hyperalgesia in rodent models [9,13,14]. Interestingly, it appeared to be specifically activated under pathological conditions while not contributing to baseline mechanical nociceptive thresholds [13]. Other findings pointed to additional roles for TRPV4 in bladder, lung and bone function [10,15–19]. These may also be of potential therapeutic interest.

A variety of molecules can activate TRPV4 (Fig. 1A). Sources of chemical agonists can be endogenous to mammalian systems, as with arachidonic acid metabolite 5,6-epoxyeicosatrienoic acid (5,6-EET) [20], obtained from botanical extracts, as with Chinese-medicine plant derived bisandrographolide [21], and semi-synthetic, as with phorbol ester 4αPDD [22], and, more recently, fully synthetic with GSK1016790A [23]. On the other hand, only one potent TRPV4 antagonist is currently known, metal-derived ruthenium red which is known to act as a pore blocker of this and 12 other ion channels along with binding to tubulin and being an inhibitor of myosin light chain phosphatase [24–26]. As a selective antagonist would help in the elucidation of TRPV4 function, we sought to identify and characterize such a molecule. Accordingly,

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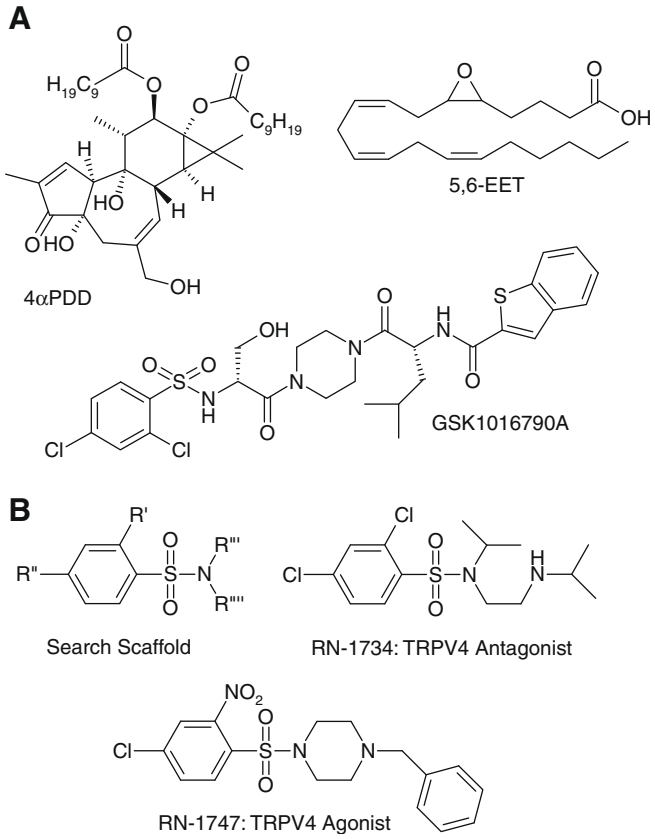

Fig. 1. Compound structures. (A) Chemical structures of selected TRPV4 agonists. (B) Scaffold used for the compound search and chemical structures of TRPV4 agonist RN-1747 and TRPV4 antagonist RN-1734.

we report herein the discovery and characterization of commercially available RN-1747 and RN-1734, two related small molecules that function, respectively, as selective agonist and antagonist of the TRPV4 channel.

Materials and methods

Reagents

Tissue culture reagents were purchased from Mediatech. RN-1747 and RN-1734 were purchased from Menai Organics Ltd. under the catalog numbers H4689 and NC1207, respectively. These compounds are also available from ABCR (AB164403 and AB159908, respectively).

Constructs and cell culture

Human, rat and mouse TRPV4 were cloned into a pcDNA5/TO vector from Invitrogen and stably transformed into the T-REx HEK 293 cell line from Invitrogen. HEK 293 cells expressing TRPV4 were grown in DMEM medium containing 5% PenStrep, 5% glutamax, 200 μg/mL hygromycin, 5 μg/mL blasticidin and 10% heat inactivated FBS.

Experimental protocols

Electrophysiology. Injected oocytes expressing hTRPV4 are stored at 16 °C in standard oocyte incubation solution containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 50 μg/mL gentamicin and 1 μM ruthenium red. The addition of ruthenium red is required to prevent premature TRPV4 channel activation under these hypotonic conditions (the osmolarity of the above solution is 200 (195–205) mOsm).

The low osmolarity recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 5 mM HEPES/NaOH at pH 7.6 while the high osmolarity solution for inhibition of TRPV4 in the absence of antagonists contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 100 mM mannitol, 5 mM HEPES/NaOH at pH 7.6 with an osmolarity of 300 mOsm.

^{45}Calcium uptake assay. The functional activity of RN-1747 and RN-1734 against the TRPV4 receptor was determined by measuring changes in intracellular calcium in HEK 293 cells expressing TRPV4. Cells expressing TRPV4 were cultured in DMEM medium containing 5% PenStrep, 5% glutamax, 200 μg/mL hygromycin, 5 μg/mL blasticidin and 10% heat inactivated FBS. Twenty-four hours prior to the assay, cells were transferred to PDL-coated, plastic 96-well black-walled plates and grown using culture media supplemented with 1 μg/mL doxycycline and 1 μM ruthenium red. The assay medium itself comprises DMEM + 0.25 mg/mL BSA. Plates are cooled for 1 min by being placed on a metal block kept at −20 °C, the medium aspirated and ice-cold wash buffer I (PBS without Ca^{2+}/Mg^{2+}) is added to the cells. The wash buffer is aspirated and $^{45}Ca^{2+}$ in medium is added to each well. The agonist EC_{50} curves of 4αPDD and RN-1747 are determined using concentrations ranging from 13 nM to 30 μM. For IC_{50} determination, the agonists used are 4αPDD (2 μM, except for Fig. 3A: 1 μM), RN-1747 (1 μM) and 30% hypotonicity (addition of 30% water to the cell media). Compounds are tested at concentrations ranging from 13 nM to 30 μM. The cells are then incubated on a shaker for 10 min before the medium is aspirated and the cells are washed in wash buffer II (PBS with Ca^{2+}/Mg^{2+}). Scintillation fluid is added and cells incubated in it for at least 15 min before counting using a MicroBeta liquid scintillation counter (Perkin-Elmer).

Data analysis

IC_{50} and EC_{50} curves were generated by fitting the data with a 4-parameter sigmoidal curve (variable slope) equation using the Graphpad Prism software: y = Bottom + (Top − Bottom)/(1 − 10^((log EC_{50} − x) ∗ Hillslope), where x = log(concentration). Error bars in figures indicate standard error of the mean (SEM).

Results and discussion

Compound identification

A study of the SAR data present in two patents applications from GlaxoSmithKline [27,28], and of screening results obtained in-house, pointed to a *para*- and/or *ortho*-substituted aryl sulfonamide motif as being primarily responsible for the affinity of small molecules to the TRPV4 channel. To test this hypothesis, we conducted a search of commercial vendor databases based on this scaffold (Fig. 1B), selected a diverse set of molecules and assembled a focused library of approximately 70 compounds. Screening this library at 10 μM against rat TRPV4-expressing HEK293 cells revealed three agonists and five antagonists of the channel (data not shown). Basing our choice on a combination of potency, efficacy and selectivity versus other TRP channels, we selected agonist RN-1747 and antagonist RN-1734 for further characterization (Fig. 1B).

Characterization of the potency and efficacy of RN-1747 and RN-1734

Small molecule probes that display minimal species difference may be of greater usefulness in elucidating the function of a biolog-