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Pyrazolopyrimidines as Potent Stimulators for Transient Receptor Potential Canonical 3/6/7 Channels

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13 **KEYWORDS:** Pyrazolopyrimidines, agonists, cation channels
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20 **ABSTRACT:** Transient receptor potential canonical 3/6/7 (TRPC3/6/7) are highly homologous recep-
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22 tor-operated non-selective cation channels. Despite their physiological significance, very few selective
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24 and potent agonists are available for functional examination of these channels. Using a cell-based high
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26 throughput screening approach, a lead compound with the pyrazolopyrimidine skeleton was identified
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28 as a TRPC6 agonist. Synthetic schemes for the lead and its analogs were established and structural-
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30 activity relationship studies were carried out. A series of potent and direct agonists of TRPC3/6/7 chan-
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32 nels were identified and among them, **4m-4p** have a potency order of TRPC3>C7>C6, with **4n** being
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34 the most potent with an EC₅₀ of <20 nM on TRPC3. Importantly, these compounds exhibited no stimu-
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36 latory activity on related TRP channels. The potent and selective compounds described here should be
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38 suitable for evaluation of the roles of TRPC channels in the physiology and pathogenesis of diseases,
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40 including glomerulosclerosis and cancer.
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■ INTRODUCTION

Transient receptor potential canonical (TRPC) channels constitute a group of receptor-operated calcium-permeable non-selective cation channels of the TRP superfamily. The 7 mammalian TRPC members can be further divided into four subgroups (TRPC1; TRPC2; TRPC4/5; and TRPC3/6/7) based on their amino acid sequences and functional similarities¹. Native TRPC channels may be formed of either homo- or hetero-tetramers from the same subgroup or from different subgroups; they also interact with a variety of other proteins, which exert modulatory roles on their assembly, trafficking and/or function². So far, the majority of studies on TRPC channels have focused on their roles in mediating Ca²⁺ and Na⁺ influx after the stimulation of phospholipase C (PLC) either by G protein-coupled receptors that signal through the G_{q/11} subgroup of heterotrimeric G proteins or by receptor tyrosine kinases³. These activities cause membrane depolarization and a sustained increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i), which are important for a plethora of cellular functions in many physiological systems⁴. On the other hand, a few studies also implicated TRPC channel expression and function in intracellular membranes⁵, suggesting that very diverse cellular activities may be regulated by these channels.

Intensive investigations have been instrumental in defining the physiological roles of TRPC channels in different tissues and organs, including excitation-contraction coupling in smooth muscles^{6,7}, protein filtration in the kidney^{8,9}, synaptic formation and transmission^{10,11}, myofibroblast transdifferentiation and wound healing¹², regulation of vascular tone, cell growth and proliferation¹³⁻¹⁵. Particularly, TRPC6 has been implicated in the physiological regulation of glomerular hemodynamics in kidney. Genetic mutations of TRPC6, including both gain-of-function and loss-of-function ones, have been linked to glomerular dysfunction in the kidney, where the channel is expressed in podocytes and involved in the regulation of slit diaphragm for protein filtration^{8,9,16}. Elevated expression of TRPC6 was found in glomeruli from patients with proteinuria, which is a common feature of kidney dysfunction of glomerular origin

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3 and is by itself a risk factor for both renal and extrarenal diseases¹⁷. Additionally, TRPC6 and its closely
4 related TRPC3, are both suggested to be involved in the development of cardiac hypertrophy and hyper-
5 tension^{14, 18- 20}. The roles for TRPC3/6 in cancer have also been suggested^{21,22}. However, the exact
6 roles played by TRPC channels in kidney and cardiovascular functions and the underlying mechanisms
7 concerning how they contribute to these functions and/or pathogenesis have proven difficult to elucidate
8 due to the lack of specific compounds that modulate these channels.
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10 TRPC3, TRPC6, and TRPC7 are closely related, sharing 65–78% sequence identity, and form channels
11 that are generally activated downstream from receptors that activate PLC. These channels are also di-
12 rectly activated by diacylglycerols (DAG), one of the products of phosphatidylinositol 4,5-bisphosphate
13 (PIP₂) hydrolysis by PLC²³⁻²⁴. Analogues of DAG, such as 1-oleoyl-2-acetyl-sn-glycerol **1** (OAG) and 1,
14 2-dioctanoyl-sn-glycerol **2** (DOG) have been widely used to activate TRPC3 and TRPC6 channels (Fig-
15 ure 1). However, not only these stimulators are non-specific for TRPC3/6/7 channels, but also they are
16 highly hydrophobic and unstable in the aqueous solution. Recently, small molecular probes, including
17 both natural products and synthetic compounds, have been reported for TRPC channels. For example, a
18 novel synthetic agonist for TRPC3/6 named GSK1702934A (**3**)²⁵ was used at 1 μM to study cardiac
19 contractility and arrhythmogenesis in heart²⁶. A few small-molecule compounds including Pyr3 and
20 analogs²⁷, cationic, N⁷-substituted 1-benzylpiperidines⁷, anilino-thiazole²⁸, norgestimate²⁹, 4-((1R,2R)-
21 2-[(3R)-3-aminopiperidin-1-yl]-2,3-dihydro-1H-inden-1-yl}oxy)-3- chlorobenzonitrile³⁰ and larixyl ace-
22 tate³¹ have been identified as inhibitors of TRPC3/6/7 channels. Englerin A³², riluzole³³, clemizole hy-
23 drochloride³⁴, 4-methyl-2-(1-piperidinyl)-quinoline (ML204)³⁵, and 2-aminobenzimidazole derivatives³⁶
24 have been described as TRPC4 and/or TRPC5 agonists or antagonists. Often, these probes cross-react
25 within the subgroup of TRPC3/6/7 or TRPC4/5, but not between the two subgroups.
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3 While selectivity remains lacking for most compounds, identification of their pharmacophores through
4 systematic structure-activity approaches can be expected to lead to development of subtype-selective
5 TRPC agonists or antagonists, and may perhaps lead to development of novel drug therapies, such as for
6 the treatment of kidney and cardiovascular diseases or cancer. For such a reason, we have carried out a
7 high-throughput screening (HTS) effort to identify small-molecule probes of TRPC6 and related chan-
8 nels as a starting point for lead optimization and target validation. Here we report the identification of a
9 novel pyrazolopyrimidine-based TRPC6 lead agonist, its resynthesis, and the structure-activity relation
10 (SAR) analysis of the initial set of its analogs on TRPC3, TRPC6, and TRPC7. With minor modifica-
11 tions, we obtained an agonist with relatively strong selectivity for TRPC3, exhibiting apparent affinity at
12 the low nanomolar range.

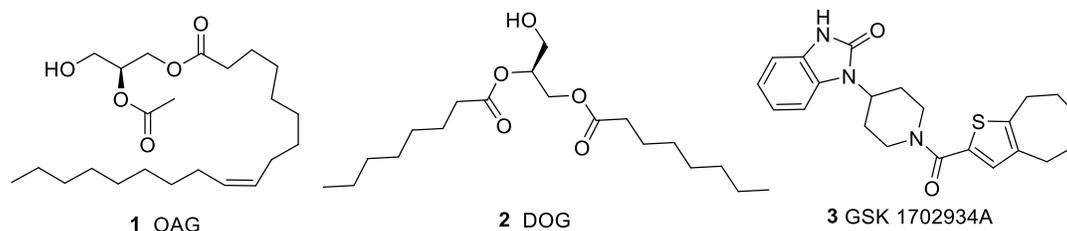


Figure 1. Examples of reported TRPC3/6/7 agonists.

■ RESULTS AND DISCUSSION

Chemistry. The scarcity of TRPC3/6/7 probes has severely hampered the characterization of these channels in their assembly, function and pathophysiological roles. To functionally screen for TRPC3/6/7 small molecular probes, we created a stable HEK293 cell line that co-expressed mouse TRPC6 and the $G_{q/11}$ -PLC β coupled M5 muscarinic receptor (TRPC6 cells). These cells responded to the muscarinic agonist, carbachol (CCh), with a strong sustained membrane depolarization, which can be detected by the FLIPR membrane potential (FMP) dye as an increase in fluorescence (Fig. 2A, *gray dashed line*). Using this cell-based assay, we screened 305,000 compounds from the Molecular Libraries Small Mole-

cule Repository (MLSMR) supported by the Molecular Libraries Probe Production Centers Network (MLPCN) and identified an activator with the pyrazolopyrimidine skeleton (PubChem CID: 5308428, Table 1, compound **4o**) as the primary hit. As shown in Fig. 2A, the original lead compound elicited membrane potential depolarization in TRPC6 cells in a concentration-dependent manner, but not in the parental HEK293 cells (Fig. 2B). It also evoked $[Ca^{2+}]_i$ rise in the TRPC6 cells (Fig. 2C), but not parental HEK293 cells (Fig. 2D). We then confirmed that the compound concentration-dependently elicited maximal stimulation (EC_{50}) were estimated to be $1.4 \pm 0.1 \mu\text{M}$ by the FMP assay ($n = 6$), $2.5 \pm 0.2 \mu\text{M}$ (at $+80 \text{ mV}$) by whole-cell recordings ($n = 5-7$), and $7.2 \pm 0.5 \mu\text{M}$ by the Ca^{2+} assay ($n = 12$) (Fig. 2F).

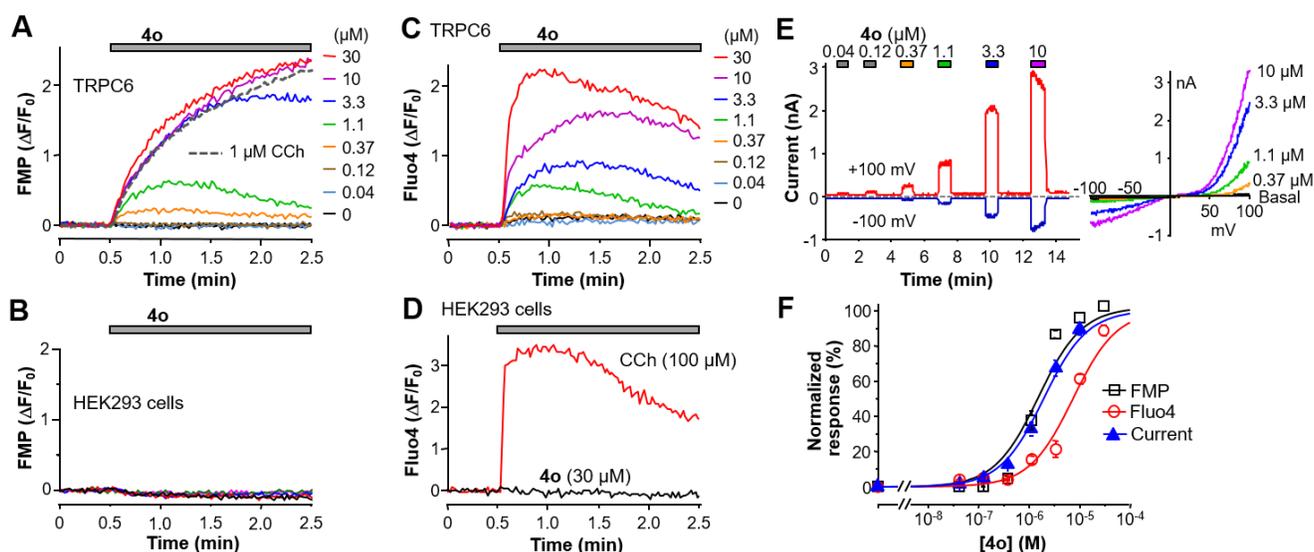


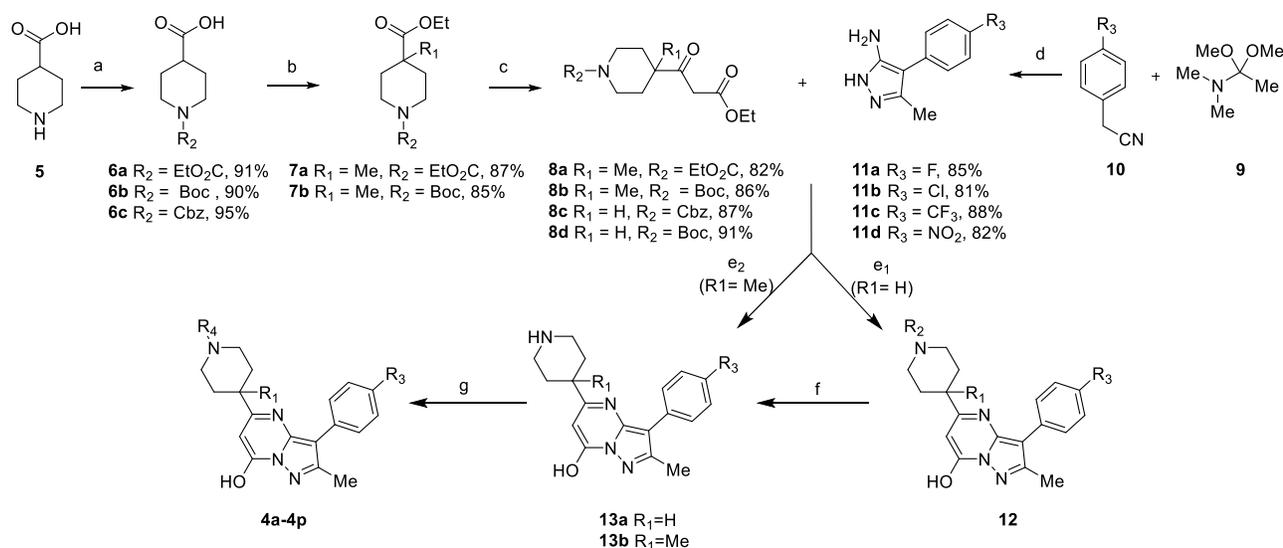
Figure 2. Lead compound **4o** Identified from HTS Activated TRPC6 Expressed in HEK293 Cells. (A) Representative traces of membrane potential changes evoked by carbachol (CCh, $1 \mu\text{M}$, gray dashed line) or varying concentrations of compound **4o** obtained from the compound library (colored lines) in the TRPC6 cells. The fluorescence increases indicate depolarization. (B) Compound **4o** did not induce membrane depolarization in parental HEK293 cells. (C) Representative traces of Fluo4 fluorescence changes, indicative of $[Ca^{2+}]_i$ increase, evoked by varying concentrations of compound **4o** in the TRPC6 cells. (D) CCh, but not compound **4o**, evoked $[Ca^{2+}]_i$ increase in the parental HEK293 cells. (E) Representative traces of whole-cell currents at $+100 \text{ mV}$ (red trace) and -100 mV (blue trace) elicited by varying concentrations of compound **4o** in a TRPC6 cell. The bath contained $2 \text{ mM } Ca^{2+}$ and the pipette

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3 solution had 400 nM free Ca²⁺ buffered by BAPTA. Voltage ramps from +100 mV to -100 mV were
4 applied every second. Current-voltage (I-V) relationships obtained from the voltage ramps for peak cur-
5 rents elicited by selected compound concentrations are shown at right. These I-V curves are typical to
6 TRPC6 currents evoked through receptor stimulation³⁷. (F) Concentration response curves for com-
7 pound **4o** activation of TRPC6, as determined by the membrane potential assay (FMP), Ca²⁺ assay
8 (Fluo4), and electrophysiology recording shown in (A), (C) and (E), respectively. For fluorescence as-
9 says, area under the curve, and for whole-cell recordings, peak current changes at +80 mV were used for
10 quantification. Solid lines represent fits by the Hill equation, which yielded the EC₅₀ values described in
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23 To further evaluate this compound and the SAR on TRPC6 activation, we first synthesized compounds
24 **4a-4p** (Scheme 1, Table 1). Starting from the commercially available piperidine-4-carboxylic acid **5**, our
25 initial attempt to introduce a methyl group or alkyl group into the R₂ position did not work until a pub-
26 lished protocol³⁸ was adapted to protect the carboxyl group before adding methyl at position R₂. Using
27 this strategy, compounds **6a-6c** were prepared with high yields of 90~95% by *N*-acylation with ethyl
28 chloroformate, di-*tert*-butyl dicarbonate or benzyl chloroformate groups at position R₂, respectively
29 (Scheme 1). Deprotonation of piperidine of **6a** and **6b** with 1.2 equiv of *n*-butyllithium followed by re-
30 action with methyl iodide afforded amides **7a** and **7b** in 87% and 85% yields, respectively. The ethyl
31 ester portion of **7a** and **7b** was converted to 3-oxopropanoate group using a modified Masamune proce-
32 dure³⁹, which furnished β-keto esters **8a** and **8b** in 82% and 86% yields, respectively. Compounds **8c**
33 and **8d** were obtained with high yields from **6c** and **6b**, respectively, using a similar procedure. Conden-
34 sation of nitrile intermediate **10** with 1, 1-dimethoxy-*N,N*-dimethylethan-1-amine **9** using dichloro-
35 methane as the solvent under microwave irradiation (MWI), followed by the cyclization reaction with
36 hydrazine hydrochloride in EtOH generated the 5-aminopyrazoles **11a-d** in excellent yields (81–88%)
37 over two steps. We then carried out a cyclization study of compounds **8** and **11** using a variety of acids,
38 such as HCl, trifluoroacetic acid (TFA), and H₂SO₄, in order to test the viability of our design. Unfortu-
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nately, only a trace amount of product **12** was formed in TFA at reflux for 18 h. However, mixing **8a-8c** with **11a-11d** in acetic acid in a microwave reactor at 120 °C overnight produced the desired products **12** in good yields. Deprotection of the Boc group of compounds **12** ($R_2 = \text{Boc}$) with TFA/dichloromethane (DCM) at 0 °C resulted in the formation of compounds **13** in high yield. Finally, amidation of compounds **13** gave rise to compounds **4** (**4a-4p**, Table 1) successfully (Scheme 1).

Scheme 1. The Synthetic Route for Compound **4a** and its Derivatives^a.

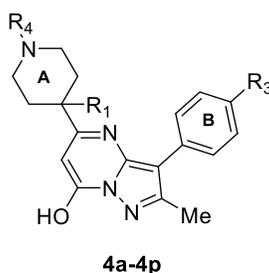


^aReagents and conditions: (a) $R_1\text{O}_2\text{CCl}$, NaOH, H_2O , rt, 5h, or $(\text{Boc})_2\text{O}$, K_2CO_3 , THF/ H_2O , rt, 7h. (b) i. ClCO_2Et , NEt_3 , rt, 12h; ii. *n*-BuLi, *i*-Pr₂NH, THF, -78 °C, 1h, MeI, THF, 5h. (c) i. 2M NaOH; ii. CDI, THF, 4h; $\text{KO}_2\text{CCH}_2\text{CO}_2\text{Et}$, MgCl_2 , DMAP, THF/ CH_3CN , overnight. (d) i. DCM, microwave, 100 °C; ii. $\text{N}_2\text{H}_4 \cdot \text{HCl}$, H_2O , EtOH, 80 °C, 8h. (e₁) AcOH, reflux, 120 °C, overnight. (e₂) EtOH, TFA, reflux, 85 °C, 48h. (f) TFA, DCM, 0 °C, 2h. (g) DMF, HOBt, HBTU, DIPEA, rt, overnight.

Biological Evaluation. The effect of the newly synthesized compounds (Table 1) on TRPC6 channel function was evaluated using the fluorescence Ca^{2+} assay. As the original lead, the re-synthesized compound **4o** ($R_1 = \text{H}$, $R_3 = \text{F}$, $R_4 = \text{CO}_2\text{Et}$) evoked $[\text{Ca}^{2+}]_i$ rise in the TRPC6 cells in a concentration de-

pendent manner, with a mean EC₅₀ value of 4.66 ± 0.03 μM (Fig. 2C and 2E, Table 1, entry 15), demonstrating a comparable activity of the re-synthesized compound on TRPC6 as the original lead. Compound **4o** was also tested on HEK293 cells that co-expressed μ opioid receptor (MOR) and TRPC4β^{35,36}, which belongs to a different subgroup of TRPC channels than TRPC3/6/7. Upon stimulation by a μ agonist, (D-Ala, N-MePhe, Gly-ol]-enkephalin (DAMGO, 1 μM), these cells show a robust increase in [Ca²⁺]_i³⁶. However, compound **4o** did not elicit a Ca²⁺ response in the MOR/TRPC4β cells; nor did it alter the DMAGO-evoked response, demonstrating its selectivity for TRPC6 over TRPC4.

Table 1. Structures of Compounds 4a-4p and Their Effects on TRPC6 and TRPC4.



Compound	R ₁	R ₃	R ₄	TRPC6 EC ₅₀ (μM) ^a	TRPC4β+MOR IC ₅₀ (μM) ^b
4a	H	F		12.3 ± 1.2	~10
4b	Me	F		NA	NA
4c	Me	F		NA	NA
4d	Me	F		NA	NA
4e	Me	NO ₂		NA	NA
4f	Me	NO ₂		NA	NA
4g	Me	NO ₂	CO ₂ Et	~8 ^c	NA
4h	Me	F	Boc	NA	NA
4i	H	NO ₂	CO ₂ Et	~2 ^c	NA

4j	H	F		28.8±8.3	~35
4k	H	F		NA	NA
4l	H	Cl	Boc	NA	NA
4m	H	Cl	Cbz	7.79±0.05	NA
4n	H	CF ₃	CO ₂ Et	1.39±0.01	NA
4o	H	F	CO ₂ Et	4.66±0.03	NA
4p	H	F	Cbz	3.91±0.02	NA

^aEC₅₀ determined based on Ca²⁺ assay.

^bIC₅₀ against that activated by 1 μM DAMGO, estimated based on one experiment with triplicates; ^cestimated value because of the self fluorescence of the compound; NA: No activity.

We next investigated SAR between substituents in R₁ and R₄ of ring A and R₃ of ring B using the Ca²⁺ assay on the TRPC6 cells (Table 1). For most compounds, the MOR/TRPC4β cells were used as a control for subtype selectivity. First, the hydrogen is required in R₁, as the substitution by a methyl group rendered all compounds (**4b-4h**) inactive at stimulating TRPC6 (Table 1, entries 2-8), except for **4g**, which however, showed high fluorescence by the compound itself (not shown). This could be due in part to the ability of methyl at position R₁ to bias a particular A ring conformer. Second, the substitution of F at R₃ by Cl moderately increased the EC₅₀ (**4m**, Fig. 3A and 3E, Table 1, entry 13) whereas that by NO₂ or CF₃ decreased the EC₅₀ by ~60-70% to ≤ 2 μM (**4i** and **4n**, Fig. 3B and 3E, Table 1, entries 9 and 14), suggesting a possible position for further modifications to improve the agonist potency. However, like **4g**, the introduction of NO₂ at R₃ rendered the compound (**4i**) to become self-fluorescent (not shown). Third, substitutions at R₄ of the A ring revealed that the carbamate group, such as ethyl carbamate, is optimal. To identify suitable carbamate group replacements for R₄, we used 6-bromo-nicotine (**4a**), ethyl carbamoyl (**4j**), *tert*-butyloxycarbonyl (Boc, **4l**) and carboxybenzyl (Cbz, **4m**, **4p**). Intriguingly, compound **4l** did not exhibit stimulatory activity on TRPC6, likely because of the steric hin-

drance blockade by the Boc group introduced at the R₄ position. The benzyl carbamate or Cbz group in R₄ (**4p**) displayed comparable stimulatory activity on TRPC6 as the corresponding ethyl carbamate (**4o**), giving an EC₅₀ value of $3.91 \pm 0.02 \mu\text{M}$ (Fig. 3D and 3E, Table 1, entry 16).

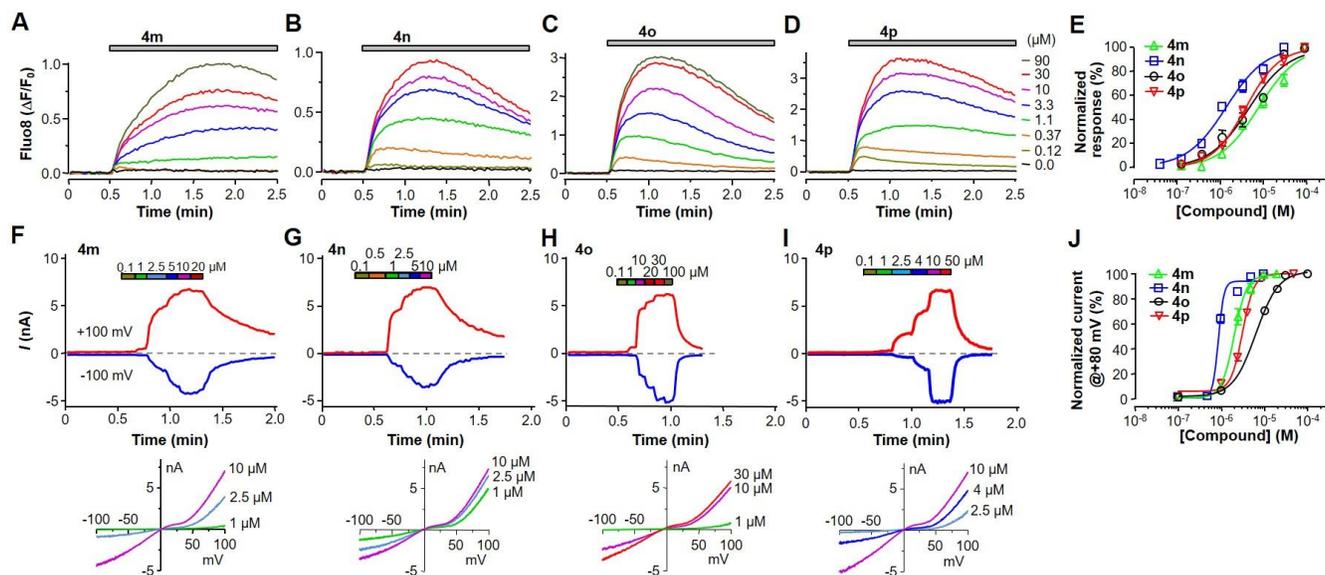


Figure 3. Compounds **4m-4p** Activated TRPC6 Expressed in HEK293 Cells. (A)-(E) Compounds **4m-4p** were synthesized as described in the text and tested in the Ca²⁺ assay using Fluo8-loaded TRPC6 cells. (A)-(D) Representative traces of [Ca²⁺]_i rise, as indicated by the Fluo8 fluorescence increase, evoked by varying concentrations of **4m** (A), **4n** (B), **4o** (C), or **4p** (D). (E) Summary data for (A)-(D) and the fits by the Hill equation. (F)-(J) Compounds **4m-4p** were tested on TRPC6 cells in whole-cell recordings. The bath contained 0.1 mM Ca²⁺ and the pipette solution had 400 nM free Ca²⁺ buffered by BAPTA. (F)-(I) Representative current traces at +100 mV (*red traces*) and -100 mV (*blue traces*) in response to consecutive applications of increasing concentrations of **4m** (F), **4n** (G), **4o** (H), or **4p** (I) (*upper panels*) and I-V curves for selected compound concentrations (*lower panels*). (J) Summary data for (F)-(I) and the fits by the Hill equation.

Functional characterization of 4m-4p. To validate the agonistic action of pyrazolopyrimidine compounds on TRPC6 channels, we focused on the last four compounds listed in Table 1 (**4m-4p**), which evoked robust [Ca²⁺]_i increases in TRPC6-expressing cells in the Ca²⁺ assay with EC₅₀ values < 8 μM (Fig. 3A-3E) and no self-fluorescence. In whole-cell voltage clamp recordings, we used a low Ca²⁺ bath

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3 solution (0.1 mM Ca²⁺) in order to minimize current rundown. Under these conditions, increasing con-
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5 concentrations (0.1 – 100 μM) of the test compound were applied consecutively and in each case the com-
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7 pound evoked stepwise current increases in individual cells at both negative and positive potentials (Fig.
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9 3F-3I). Noticeably, the outward currents at positive potentials developed earlier at low compound con-
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11 centrations than the inward currents at negative potentials, indicative of a shift of voltage dependence as
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13 the compound concentration increased. Typically, the currents did not desensitize even at highest con-
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15 centrations (20-100 μM) used. Based on the currents at +80 mV, the EC₅₀ values for monovalent cation
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17 currents of TRPC6 evoked by **4m-4p** were determined to be 2.05 ± 0.02 μM, 0.89 ± 0.01 μM, 6.28 ±
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19 0.02 μM and 3.24 ± 0.01 μM (n = 6 - 8), respectively (Fig. 3J). These values are comparable to that ob-
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21 tained from the Ca²⁺ assay (Table 1).
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28 We then examined whether the compounds directly activate closely related TRPC3 and TRPC7 channels.
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30 Although our initial attempts using the FMP and Ca²⁺ assays did not reveal obvious activation of human
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32 TRPC3 stably expressed in HEK293 cells by compound **4o** (Fig. 4A₁, 4A₂), lowering the Ca²⁺ concen-
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34 tration in the extracellular solution from the standard 2 mM to 0.5 mM allowed **4o** to evoke membrane
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36 depolarization in these cells in a concentration dependent manner (Fig. 4B). These results suggest that
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38 the activation of TRPC3 by **4o** is sensitive to inhibition by extracellular Ca²⁺, consistent with the previ-
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40 ous finding that physiological concentrations of extracellular Ca²⁺ suppressed receptor-operated TRPC3
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42 currents⁴⁰. Supporting the FMP assay data, **4o** also evoked whole-cell currents in HEK293 cells that ex-
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44 pressed human TRPC3 (Fig. 4E). Using the FMP assay with 0.5 mM extracellular Ca²⁺, we determined
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46 the EC₅₀ values of compounds **4m-4p** on stimulating TRPC3 to be 138 ± 23 nM, 19.2 ± 2.3 nM, 450 ±
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48 87 nM, and 236 ± 47 nM (Fig. 4B-4D, n = 6 for each), respectively. Among the four analogs, **4n** really
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50 stands out, exhibiting >7-fold increase in the potency on TRPC3 as compared to other compounds
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52 and >70-fold increase as compared to the same compound on TRPC6.
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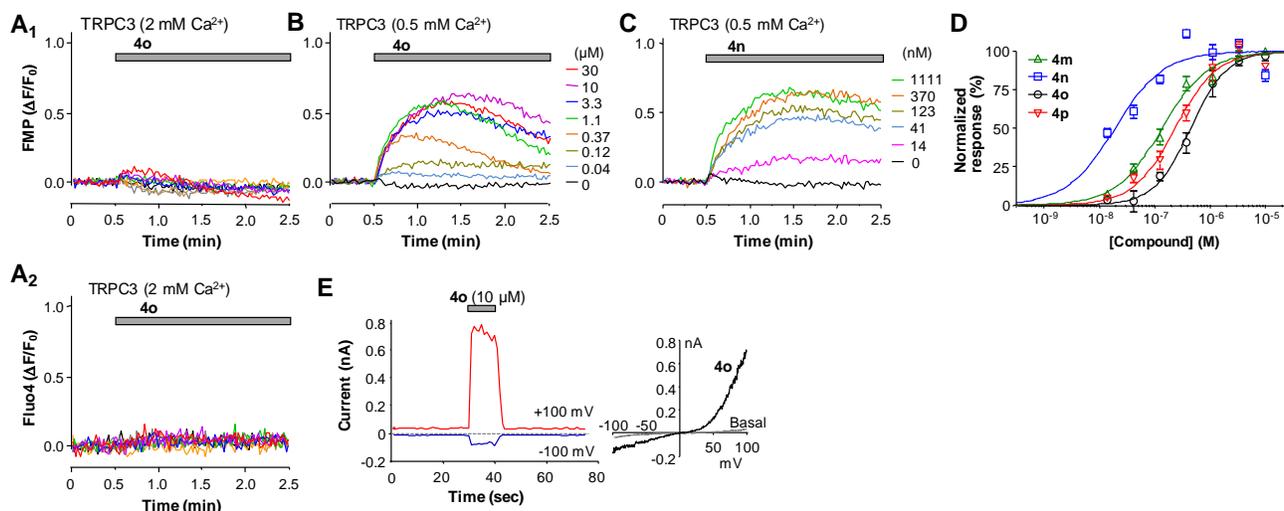


Figure 4. Compounds **4m-4p** Activated TRPC3 Expressed in HEK293 Cells when Extracellular Solution Contained 0.5 mM Ca^{2+} . **(A)** Compound **4o** failed to elicit membrane depolarization (**A₁**) or $[\text{Ca}^{2+}]_i$ rise (**A₂**) in TRPC3 cells in normal extracellular solution that contained 2 mM Ca^{2+} . **(B)** and **(C)** Representative traces of membrane potential depolarization induced by varying concentrations of compounds **4o** (**B**) and **4n** (**C**) in TRPC3 cells in an extracellular solution that contained 0.5 mM Ca^{2+} . **(D)** Summary data for membrane depolarization induced by compounds **4m-4p** in TRPC3 cells bathed in the 0.5 mM Ca^{2+} solution. The data points were fitted by the Hill equation. **(E)** Compound **4o** (10 μM) elicited TRPC3 currents in the bath that contained 0.5 mM Ca^{2+} . The I-V curves under basal and **4o**-stimulated conditions are shown at *right*.

Among the three members of the TRPC3/6/7 subgroup, the sequence of TRPC7 is more similar to TRPC3 than to TRPC6. Interestingly, compound **4o** was able to elicit membrane depolarization and $[\text{Ca}^{2+}]_i$ elevation in HEK293 cells that stably expressed human TRPC7 in the normal extracellular solution that contained 2 mM Ca^{2+} (Fig. 5A, 5B), although the extents of the response, even when they reached the maximum appeared to be markedly lower than that seen in the TRPC6 cells. Previously, it was shown that extracellular Ca^{2+} potentiated TRPC6 but inhibited TRPC7 currents⁴¹. Thus, the lower response of TRPC7 to **4o** than TRPC6 reflected at least in part a block by the 2 mM Ca^{2+} included in the extracellular solution, although such a block may be much weaker than TRPC3, which was almost com-

pletely inhibited by the same concentration of Ca^{2+} (see above and Fig. 4A). In whole-cell voltage clamp recordings, **4o** elicited concentration dependent current increases in the TRPC7 cells (Fig. 5D). Different from TRPC6, the currents showed some desensitization in the high **4o** concentrations (10 and 30 μM) at both negative and positive potentials even though the low Ca^{2+} (0.1 mM) bath solution was used. Nonetheless, a stepwise increase in the TRPC7 currents was still detected at $<10 \mu\text{M}$ **4o**. Based on the whole-cell recordings performed in the low Ca^{2+} bath solution, **4o** had an estimated EC_{50} of $1.13 \pm 0.03 \mu\text{M}$ ($n = 6$) on TRPC7. This value is only slightly higher than that obtained from the Ca^{2+} assay. Based on the Ca^{2+} assay, the EC_{50} values of **4m-4p** were estimated to be $213 \pm 36 \text{ nM}$, $90 \pm 12 \text{ nM}$, $497 \pm 91 \text{ nM}$, and $314 \pm 59 \text{ nM}$ (Fig. 5C, $n = 6$ for each), respectively. For each of the compounds, the EC_{50} value for TRPC7 is in between those for TRPC3 and TRPC6 (Table 2).

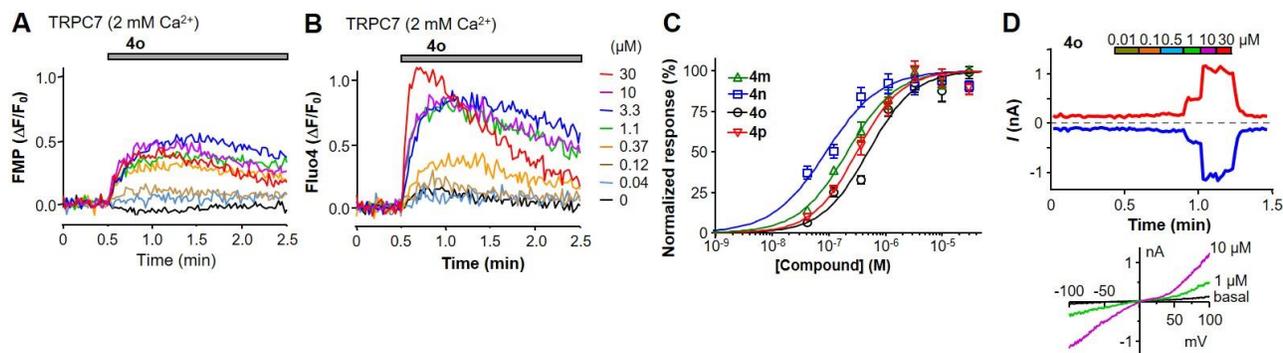


Figure 5. Compounds **4m-4p** Activated TRPC7 Expressed in HEK293 Cells. **(A)** and **(B)** Representative traces of membrane potential depolarization **(A)** and $[\text{Ca}^{2+}]_i$ increase **(B)** induced by varying concentrations of compounds **4o** in TRPC7 cells in normal extracellular solution that contained 2 mM Ca^{2+} . **(C)** Summary data for $[\text{Ca}^{2+}]_i$ rise induced by compounds **4m-4p** in TRPC7 cells bathed in the 2 mM Ca^{2+} solution, as exemplified in **(B)**. The data points were fitted by the Hill equation. **(D)** Activation of TRPC7 currents by increasing concentrations of compound **4o** in the bath solution that contained 0.1 mM Ca^{2+} . The I-V curves under basal and selected concentrations of **4o** are shown below.

The above data clearly demonstrate the agonistic activity of the pyrazolopyrimidine compounds **4m-4p** on the TRPC3/6/7 subgroup of TRPC channels. To verify the selectivity, we tested the effects of **4m-4p** on a number of related TRP channels, including TRPC4, TRPC5, TRPA1, TRPV1, TRPV3, TRPV4, TRPM5 and TRPM8, using either the FMP or Ca²⁺ assay. All compounds were applied at 33 μM when tested for stimulatory activity, which revealed no obvious effect (data not shown). Then the channels were stimulated with their corresponding agonists in the absence or presence of 22 μM **4m-4p**. For most channels, the compounds did not significantly alter the agonist-evoked responses, except for TRPC4, where **4n** and **4p** suppressed the MOR agonist DAMGO-evoked membrane depolarization by ~33% and 13%, respectively, and for TRPM8, where **4n**, **4o** and **4p** suppressed the menthol-evoked [Ca²⁺]_i increase by ~17%, 22% and 35%, respectively (Fig. 6). A small (<20%) inhibition by **4n** was also detected for TRPA1, TRPV1 and TRPM5 (Fig. 6).

Table 2. The EC₅₀ Values (μM) for Compounds **4m-4p** to TRPC3/6/7 Channels.

Compound	TRPC3 ^a	TRPC7 ^b	TRPC6 ^b	TRPC6 ^c
4m	0.138 ± 0.023	0.213 ± 0.036	7.79 ± 0.05	2.05 ± 0.02
4n	0.019 ± 0.002	0.090 ± 0.012	1.39 ± 0.01	0.89 ± 0.01
4o	0.450 ± 0.087	0.497 ± 0.091	4.66 ± 0.03	6.28 ± 0.02
4p	0.236 ± 0.047	0.314 ± 0.059	3.91 ± 0.02	3.24 ± 0.01

^a Cells were loaded with the FLIPR membrane potential (FMP) blue dye; the [Ca²⁺] in the bath was 0.5 mM.

^b Cells were loaded with Fluo8-AM; the [Ca²⁺] in the bath was 2 mM.

^c Determined based on currents at +80 mV by whole-cell patch clamp recordings using a bath solution that contained 0.1 mM Ca²⁺ and a peptide solution that contained 400 nM free Ca²⁺ buffered with BAPTA.

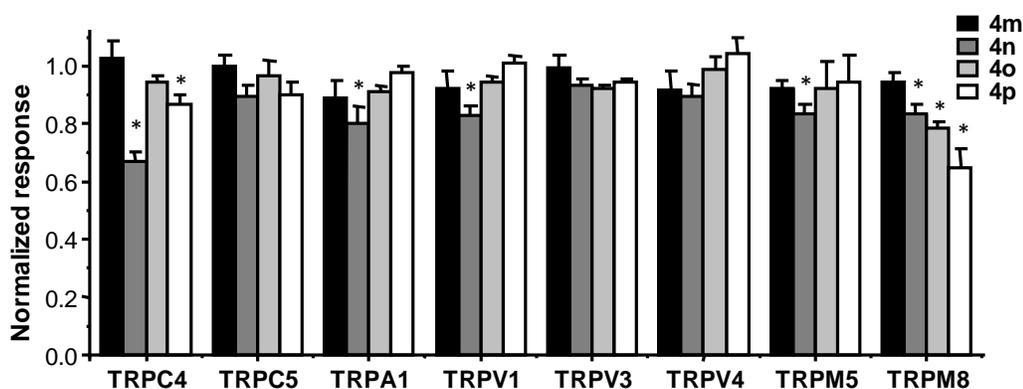


Figure 6. Compounds **4m-4p** had Little or no Effect on Related TRP Channels. Stable HEK293 cell lines expressing MOR/TRPC4 β , TRPC5, TRPA1, TRPV1, TRPV3, TRPV4, TRPM5 and TRPM8 were seeded in wells of 96-well plates and loaded with either FMP dye (TRPC4, C5, and M5) or Fluo8-AM (TRPA1, V1, V3, V4, and M8). Changes in membrane potential or $[Ca^{2+}]_i$ were read in a microplate reader while compound **4m**, **4n**, **4o**, or **4p** (33 μ M) was applied for 2 min before the corresponding agonist known to activate the channel (C4, 1 μ M DAMGO; C5, 100 μ M CCh; A1, 100 μ M flufenamic acid; V1, 1 μ M capsaicin; V3, 200 μ M 2-APB; V4, 30 μ M RN-1747; M5, 100 μ M CCh; M8, 100 μ M menthol) was added and measurement continued for 2.5 min. After the agonist addition, the test compound was diluted to 22 μ M. Shown are summary data for the agonist-evoked response (area under the curve) in the presence of the test compound normalized to that in the absence of the test compound. $n = 4-10$ measurements. * $p < 0.05$ compared to no test compound by Student's t test.

Finally, we evaluated the ability of compound **4p** to elicit $[Ca^{2+}]_i$ rise in rat primary glomerular mesangial cells, which endogenously express functional TRPC3/6 channels^{42,43}. **4p** (1 μ M) evoked marked increases in $[Ca^{2+}]_i$, as revealed by the elevation in Fluo-4 fluorescence (Fig. 7A). These activities were abolished by 2-aminoethoxydiphenyl borate (2-APB, 30 μ M), a non-specific TRP modulator that inhibits TRPC channels^{7,37}.

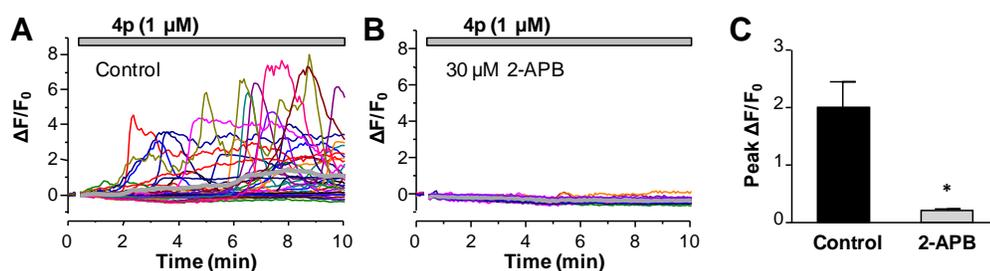


Figure 7. Compound **4p** Induced Ca^{2+} Transients in Rat Glomerular Mesangial Cells. **(A)** Representative traces of Fluo4-loaded mesangial cells exposed to 1 μM compound **4p**. Each trace represents a single cell and the thick gray line is the average of all 36 cells in the same coverslip. **(B)** Similar to **(A)** but the cells were pretreated for 2 min with 30 μM 2-APB, which was also present throughout the period when compound **4p** was applied. Each trace represents a single cell and the thick gray line is the average of all 30 cells in the same coverslip. **(C)** Summary of four experiments quantified by the mean peak fluorescence changes induced by compound **4p** for the individual experiments. * $p < 0.05$ compared to no 2-APB control by Student's t test.

CONCLUSIONS

Pharmacological tool compounds play a vital role in drug development and functional characterization of TRPC channels. Using HTS and SAR studies, we have identified a series of potent and direct agonists of TRPC3/6/7 channels. Four of them, compounds **4m-4p** were further characterized and shown a potency order of TRPC3 > C7 > C6, with **4n** being the most potent with an EC_{50} value of <20 nM on TRPC3. Importantly, these compounds exhibited no stimulatory activity on related TRPC channels, including the subfamily members TRPC4 and C5. Although not directly tested, it is unlikely that compounds **4m-4p** would activate other Ca^{2+} permeable channels endogenously expressed in HEK293 cells, such as inositol 1,4,5-trisphosphate receptors and Orai1 or inhibit the sarco/endoplasmic reticulum Ca^{2+} -ATPase as they did not elicit detectable $[\text{Ca}^{2+}]_i$ in parental HKE293 cells and a number of stable cell

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3 lines. A few compounds exhibited weak inhibition on TRPC4 and TRPM8 at high concentrations.
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5 Therefore, they are quite selective for the TRPC3/6/7 subgroup of TRPCs. It remains to be determined if
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7 the compounds affect ryanodine receptors or voltage-gated Ca²⁺ channels, which are mainly present in
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9 excitable cells. Nevertheless, given the current general lack of good pharmacological agonists for the
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11 TRPC channels and our demonstration that they activated native TRPC3/6 channels in rat glomerular
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13 mesangial cells, the compounds described here should find excellent use in TRPC channel research. Our
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15 results may lead to more potent and specific agonists or antagonists of targeting TRPC channels and fa-
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17 cilitate the development of related disease therapeutics.
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26 ■ EXPERIMENTAL SECTION

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28 **General Methods.** Unless noted otherwise, all reagents and solvents were purchased from commercial
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30 suppliers and used without further purification. Melting points were determined on a YUHUA X-5 melt-
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32 ing point apparatus. All reactions were performed under an argon atmosphere unless otherwise specified.
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34 Reaction progress was monitored using analytical thin-layer chromatography (TLC). The purification of
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36 all compounds was processed by silica gel column chromatography. ¹H and ¹³C NMR spectra were rec-
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38 orded on a Bruker AV400 spectrometer (400 MHz, ¹H NMR; 101 MHz, ¹³C NMR) at room temperature
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40 (rt). NMR spectra were calibrated to the solvent signals of CDCl₃ (δ 7.26 and 77.16 ppm), CD₃OD (δ
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42 3.31 and 49.00ppm), or *d*₆-DMSO (δ 2.50 and 39.52 ppm). The chemical shifts are provided in ppm,
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44 and the coupling constants, in Hz. The following abbreviations for multiplicities are used: s, singlet; d,
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46 doublet; dd, double doublet; ddd, triple doublet; t, triplet; dt, double triplet; q, quadruplet; m, multiplet;
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48 and br, broad. High-resolution MS was carried out with a Thermo LTQ XL Orbitrap instrument. The
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50 yield of all compounds for biological testing was determined by HPLC analysis, confirming >95% puri-
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3 **General procedure for the synthesis of 4a, 4c, 4d, 4f, 4i, 4j, 4k.** To a solution of **13** (1eq) in DMF was
4 added appropriate carboxylic acid (1.2 eq), HOBT (3eq), HBTU (3eq). Diisopropyl ethyl amine (9eq)
5 was added 15 min later. The reaction was stirred for 5-24 h, quenched with water, diluted with EtOAc,
6 and then washed with water. The organic layer was dried over anhydrous MgSO₄ and the solvent evapo-
7 rated under reduced pressure. The residue was purified by silica gel column chromatography (CH₂Cl₂:
8 MeOH= 100: 1- 40: 1) to obtain the title compound.
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18 **General procedure⁴⁴ for the synthesis of 4b, 4e, 4g, 4h.** To a solution of **13** (1eq) in CH₂Cl₂ was add-
19 ed appropriate acyl chloride or anhydride. Et₃N (1.2eq) was added and the reaction was stirred at rt. for
20 4-8 h. After removing the solvent, the title compound was purified by silica gel column chromatography
21 (CH₂Cl₂: MeOH = 100: 1- 40: 1).
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28 **General procedure for the synthesis of 4l-4p.** Compound **4l-4p** were synthesized according to the
29 general procedure for the synthesis of **12** (see later).
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33 **(6-Bromopyridin-3-yl)(4-(3-(4-fluorophenyl)-7-hydroxy-2-methyl-pyrazolo[1,5-a]pyrimidin-5-**
34 **yl)piperidin-1-yl) methanone (4a)⁴⁴.** The title compound was isolated as a white solid in 83% yield. ¹H
35 NMR (400 MHz, DMSO-*d*₆) δ 11.70 (s, 1H), 8.48 (d, *J* = 2.3 Hz, 1H), 7.84 (dd, *J* = 8.2, 2.2 Hz, 1H),
36 7.75 (d, *J* = 8.1 Hz, 1H), 7.47-7.44 (m, 2H), 7.30 (t, *J* = 8.8 Hz, 2H), 5.67 (s, 1H), 4.61 (d, *J* = 10.8 Hz,
37 1H), 3.62 (d, *J* = 11.1 Hz, 1H), 3.13 (s, 1H), 2.91 (t, *J* = 11.4 Hz, 1H), 2.78 (s, 1H), 2.25 (s, 3H), 1.98 (d,
38 *J* = 5.2 Hz, 1H), 1.79 (s, 1H), 1.69 (d, *J* = 9.0 Hz, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.7, 162.4,
39 160.0, 156.2, 150.0, 148.4, 142.1, 138.0, 131.7, 131.6, 131.5, 128.1, 115.6, 115.4, 102.2, 92.8, 47.3,
40 41.7, 30.6, 13.0. HRMS (ESI) calcd for C₂₄H₂₂BrFN₅O₂ [M + H]⁺ 510.0941, found 510.0960.
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52 **3-(4-Fluorophenyl)-5-(1-((4-methoxyphenyl)sulfonyl)-4-methyl-piperidin-4-yl)-2-**
53 **methylpyrazolo[1,5-a]pyrimidin-7-ol (4b)⁴⁴.** The title compound was isolated as a white solid in 60%
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3 yield. mp decomposition; ^1H NMR (400 MHz, DMSO- d_6) δ 10.99 (s, 1H), 7.68 (d, J = 8.6 Hz, 2H), 7.38
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5 (m, 2H), 7.27 (dd, J = 8.7, 6.7 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 5.62 (s, 1H), 3.82 (s, 3H), 2.97 (d, J =
6
7 10.8 Hz, 4H), 2.19 (m, 5H), 1.77 (d, J = 4.5 Hz, 2H), 1.17 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ
8
9 162.6, 158.1, 155.8, 142.8, 131.7, 131.7, 129.6, 127.4, 127.4, 127.3, 115.4, 115.2, 114.5, 93.7, 55.7,
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11 42.2, 36.5, 33.6, 24.7, 13.0. HRMS (ESI) calcd for $\text{C}_{26}\text{H}_{28}\text{FN}_4\text{O}_4\text{S}$ [$\text{M} + \text{H}$] $^+$ 511.1815, found 511.1803
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15 **1-(4-(3-(4-Fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a] pyrimidin-5-yl)-4-methylpiperidin-1-**
16 **yl)propan-1-one (4c)**⁴⁴. The title compound was isolated as a grey solid in 73% yield. mp decomposi-
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18 tion. ^1H NMR (400 MHz, CD_3OD) δ 7.45 (s, 2H), 7.21 (s, 2H), 5.36-5.33 (m, 1H), 4.65 (s, 2H), 3.60 (s,
19
20 2H), 2.43-2.39 (m, 2H), 2.32 (s, 3H), 2.04 (s, 2H), 1.76 (s, 2H), 1.39 (s, 3H), 1.11 (t, J = 7.5 Hz, 3H).
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22 ^{13}C NMR (101 MHz, DMSO- d_6) δ 171.2, 162.5, 160.1, 158.9, 156.1, 150.7, 138.9, 131.8, 127.5, 115.5,
23
24 115.3, 102.6, 93.6, 41.1, 37.4, 34.8, 34.1, 25.6, 24.3, 13.1, 9.5. HRMS (ESI) calcd for $\text{C}_{22}\text{H}_{26}\text{FN}_4\text{O}_2$ [M
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26 + H] $^+$ 397.2040, found 397.2053.
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33 **1-(4-(3-(4-Fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a] pyrimidin-5-yl)-4-methylpiperidin-1-**
34 **yl)-2,2-dimethyl-propan-1-one (4d)**⁴⁴. The title compound was isolated as a yellow solid in 55% yield.
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36 mp 203-206 °C. ^1H NMR (400 MHz, CD_3OD) δ 7.42 (s, 2H), 7.20 (s, 2H), 5.87 (s, 0.5H), 5.34 (s, 0.5H),
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38 4.65 (s, 1H), 3.71 (s, 3H), 2.30 (s, 3H), 2.10 (s, 2H), 1.78 (d, J = 12.7 Hz, 2H), 1.40 (s, 3H), 1.27 (s, 9H).
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40 HRMS (ESI) calcd for $\text{C}_{24}\text{H}_{30}\text{FN}_4\text{O}_2$ [$\text{M} + \text{H}$] $^+$ 425.2353, found 425.2351.
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46 **5-(1-((2,6-Difluorophenyl)sulfonyl)-4-methylpiperidin-4-yl)-2-methyl-3-(4-**
47 **nitrophenyl)pyrazolo[1,5-a]pyrimidin-7-ol (4e)**⁴⁴. The title compound was isolated as an orange solid
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49 in 67% yield. mp 224-227 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.74 (s, 1H), 8.23 (d, J = 8.9 Hz, 3H),
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51 7.65-7.57 (m, 2H), 7.22 (t, J = 9.1 Hz, 2H), 5.74 (d, J = 28.9 Hz, 1H), 2.96 (t, J = 9.7 Hz, 2H), 2.56 (s,
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53 2H), 2.41-2.34 (m, 3H), 1.70 (s, 2H), 1.23 (s, 2H), 1.18 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ
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55 169.6, 160.0, 157.5, 145.1, 143.7, 140.7, 137.8, 135.9, 128.4, 123.7, 113.8, 113.8, 113.5, 113.5, 112.1,
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3 112.1, 93.2, 45.7, 42.9, 35.2, 22.7, 10.8, 9.0. HRMS (ESI) calcd for C₂₅H₂₄F₂N₅O₅S [M + H]⁺ 544.1466,
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5 found 544.1461.
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8 **2-(3,4-Dimethoxyphenyl)-1-(4-(7-hydroxy-2-methyl-3-(4-nitro-phenyl)pyrazolo[1,5-a]pyrimidin-5-**
9 **yl)-4-methyl-piperidin-1-yl)ethan-1-one (4f)**⁴⁴. The title compound was isolated as an orange solid in
10 58% yield. mp 113-116 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.95 (s, 1H), 8.20 (s, 4H), 6.82-6.80 (m,
11 2H), 6.71 (d, *J* = 8.1 Hz, 1H), 5.85 (d, *J* = 8.1 Hz, 1H), 3.78 (d, *J* = 6.5 Hz, 1H), 3.66 (d, *J* = 5.7 Hz, 6H),
12 3.60 (s, 2H), 3.30 (d, *J* = 10.5 Hz, 2H), 3.17 (d, *J* = 9.5 Hz, 1H), 2.54 (s, 2H), 2.16 (s, 2H), 1.45 (d, *J* =
13 39.3 Hz, 2H), 1.21 (s, 1H), 1.18 (s, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 168.8, 158.0, 149.3, 148.5,
14 147.3, 129.7, 128.4, 123.7, 120.7, 112.7, 111.8, 55.5, 55.4, 43.0, 40.1, 38.6, 36.3, 35.7, 22.1, 14.0.
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25 HRMS (ESI) calcd for C₂₉H₃₂N₅O₆ [M + H]⁺ 546.2353, found 546.2322.
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28 **Ethyl 4-(7-hydroxy-2-methyl-3-(4-nitrophenyl) pyrazolo[1,5-a] pyrimidin-5-yl)-4-**
29 **methylpiperidine-1-carboxylate (4g)**⁴⁴. The title compound was isolated as an orange solid in 76%
30 yield. mp 174-178 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.20 (s, 4H), 5.73 (s, 1H), 3.97 (q, *J* = 6.9 Hz,
31 2H), 3.57 (d, *J* = 12.4 Hz, 2H), 3.16 (s, 2H), 2.54 (s, 3H), 2.19 (s, 2H), 1.51 (s, 2H), 1.18 (s, 3H), 1.13 (t,
32 *J* = 7.0 Hz, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 154.7, 123.7, 60.5, 40.8, 35.7, 28.8, 14.7. HRMS
33 (ESI) calcd for C₂₂H₂₆N₅O₅ [M + H]⁺ 440.1934, found 440.1931.
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42 **Tert-butyl 4-(3-(4-fluorophenyl)-7-hydroxy-2-methylpyrazolo [1, 5-a]pyrimidin-5-yl)-4-**
43 **methylpiperidine-1-carbo-xylate (4h)**⁴⁴. The title compound was isolated as a white solid in 62%
44 yield. mp decomposition. ¹H NMR (400 MHz, CDCl₃) δ 8.92 (s, 1H), 7.21 (dd, *J* = 8.0, 5.5 Hz, 2H),
45 7.04 (t, *J* = 8.5 Hz, 2H), 5.75 (s, 1H), 3.60-3.55 (m, 2H), 3.42-3.37 (m, 2H), 2.26 (s, 3H), 2.02-1.98 (m,
46 2H), 1.73 (s, 2H), 1.44 (s, 9H), 1.41 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 163.1, 160.7, 157.7, 157.0,
47 154.8, 151.7, 138.2, 130.7, 130.6, 127.1, 116.3, 116.1, 105.3, 103.1, 95.1, 80.2, 37.4, 28.5, 25.4, 13.1,
48 1.2, 0.13. HRMS (ESI) calcd for C₂₄H₃₀FN₄O₃ [M + H]⁺ 441.2302, found 441.2310.
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3 **Ethyl 4-(7-hydroxy-2-methyl-3-(4-nitrophenyl)pyrazolo[1,5-a] pyrimidin-5-yl)piperidine-1-**
4 **carboxylate (4i)**⁴⁴. The title compound was isolated as a yellow solid in 61% yield. mp decomposition.
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7 ¹H NMR (400 MHz, CDCl₃) δ 11.37 (s, 1H), 8.05 (d, *J* = 8.7 Hz, 2H), 7.45 (d, *J* = 8.6 Hz, 2H), 5.65 (s,
8 1H), 4.30 (d, *J* = 10.9 Hz, 2H), 4.12 (q, *J* = 7.1 Hz, 2H), 2.95 (t, *J* = 12.1 Hz, 1H), 2.80 (s, 2H), 2.31 (s,
9 3H), 2.06-2.04 (m, 2H), 1.67 (ddd, *J* = 25.3, 12.7, 4.3 Hz, 2H), 1.27 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (101
10 MHz, CDCl₃) δ 157.9, 157.3, 155.4, 151.0, 145.9, 139.3, 138.4, 130.1, 123.3, 102.6, 94.1, 61.7, 43.9,
11 40.3, 30.7, 14.8, 13.2. HRMS (ESI) calcd for C₂₁H₂₄N₅O₅ [M + H]⁺ 426.1777, found 426.1771.
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20 **1-(4-(3-(4-Fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a] pyrimidin-5-yl)piperidin-1-**
21 **yl)propan-1-one (4j)**⁴⁴. The title compound was isolated as a white solid in 85% yield. mp 164-166 °C.
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23 ¹H NMR (400 MHz, CD₃OD) δ 7.51 (s, 2H), 7.17 (s, 2H), 5.80 (s, 1H), 4.67 (d, *J* = 15.8 Hz, 2H), 4.08
24 (d, *J* = 11.4 Hz, 1H), 3.16 (t, *J* = 12.2 Hz, 1H), 2.87 (s, 1H), 2.67 (d, *J* = 11.0 Hz, 1H), 2.45 (d, *J* = 7.0
25 Hz, 2H), 2.32 (s, 3H), 1.99 (t, *J* = 15.4 Hz, 2H), 1.75-1.63 (m, 2H), 1.14 (t, *J* = 7.2 Hz, 3H). ¹³C NMR
26 (101 MHz, CD₃OD) δ 174.7, 164.8, 162.3, 159.2, 153.0, 140.3, 133.0, 132.9, 127.9, 116.6, 116.4, 104.7,
27 93.9, 46.6, 42.8, 41.1, 32.3, 31.9, 27.3, 12.9, 10.0. HRMS (ESI) calcd for C₂₁H₂₄FN₄O₂ [M + H]⁺
28 383.1883, found 383.1876.
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39 **1-(4-(3-(4-Fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a] pyrimidin-5-yl)piperidin-1-yl)-2,2-**
40 **dimethylpropan-1-one (4k)**⁴⁴. The title compound was isolated as a white solid in 85% yield. mp 267-
41 270 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.71 (s, 1H), 7.43 (dd, *J* = 8.5, 5.6 Hz, 2H), 7.30 (t, *J* = 8.8
42 Hz, 2H), 5.59 (s, 1H), 4.41 (d, *J* = 13.3 Hz, 2H), 2.90-2.84 (m, 1H), 2.75 (dd, *J* = 20.8, 8.0 Hz, 2H), 2.23
43 (s, 3H), 1.89 (d, *J* = 12.2 Hz, 2H), 1.50 (qd, *J* = 12.5, 3.3 Hz, 2H), 1.19 (s, 9H). ¹³C NMR (101 MHz,
44 DMSO-*d*₆) δ 175.1, 162.6, 160.2, 157.3, 156.3, 150.3, 138.8, 131.8, 131.7, 127.2, 127.1, 115.8, 115.5,
45 102.3, 92.8, 44.8, 38.3, 30.9, 30.8, 28.3, 13.1. HRMS (ESI) calcd for C₂₃H₂₈FN₄O₂ [M + H]⁺ 411.2196,
46 found 411.2170.
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Tert-butyl 4-(3-(4-chlorophenyl)-7-hydroxy-2-methylpyrazolo [1, 5-a]pyrimidin-5-yl)pi -peridine-1-carboxylate (4l)⁴⁴. The title compound was isolated as a white solid in 82% yield. mp 119-123 °C. ¹H NMR (400 MHz, CDCl₃) δ 11.35 (s, 1H), 7.17 (d, *J* = 8.2 Hz, 2H), 7.09 (d, *J* = 8.1 Hz, 2H), 5.51 (s, 1H), 4.22 (s, 2H), 3.07 (t, *J* = 11.7 Hz, 1H), 2.74 (s, 2H), 2.16 (s, 3H), 2.09 (d, *J* = 12.0 Hz, 2H), 1.62 (dt, *J* = 12.2, 8.7 Hz, 2H), 1.45 (s, 9H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 157.6, 156.5, 154.2, 150.4, 139.1, 132.0, 131.8, 130.1, 129.1, 102.4, 93.3, 79.2, 30.7, 28.5, 13.4. HRMS (ESI) calcd for C₂₃H₂₈ClN₄O₃ [M + H]⁺ 443.1850, found 443.1832.

Benzyl 4-(3-(4-chlorophenyl)-7-hydroxy-2-methylpyrazolo[1,5 -a]pyrimidin-5-yl)piper -idine-1-carboxylate (4m)⁴⁴. The title compound was isolated as a white solid in 85% yield. mp 236-239 °C. ¹H NMR (400 MHz, CD₃OD) δ 7.69 – 7.66 (m, 2H), 7.39 – 7.31 (m, 7H), 5.68 (s, 1H), 5.15 (s, 2H), 4.27 (d, *J* = 13.3 Hz, 2H), 2.94 (s, 2H), 2.70 – 2.67 (m, 1H), 2.48 (s, 3H), 1.93 (d, *J* = 9.3 Hz, 2H), 1.71 (tt, *J* = 12.5, 6.3 Hz, 2H). ¹³C NMR (101 MHz, CD₃OD) δ 168.0, 161.4, 156.9, 150.5, 150.2, 138.2, 134.6, 131.4, 131.1, 129.5, 129.1, 129.0, 128.8, 105.0, 91.2, 68.2, 45.5, 45.2, 32.7, 14.5. HRMS (ESI) calcd for C₂₆H₂₆ClN₄O₃ [M + H]⁺ 477.1693, found 477.1691.

Ethyl 4-(7-hydroxy-2-methyl-3-(4-(trifluoromethyl) phenyl) pyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (4n)⁴⁴. The title compound was isolated as a white solid in 78% yield. mp 258-261 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.74 (s, 1H), 7.85-7.81 (m, 2H), 7.68-7.66 (m, 2H), 5.64 (s, 1H), 4.06 (dt, *J* = 14.1, 9.2 Hz, 4H), 2.78 (t, *J* = 11.8 Hz, 3H), 2.30 (s, 3H), 1.85 (d, *J* = 12.4 Hz, 2H), 1.56 (tt, *J* = 12.4, 6.3 Hz, 2H), 1.18 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 157.2, 156.0, 154.5, 150.0, 139.0, 135.3, 130.2, 125.5, 125.4, 101.9, 93.3, 60.7, 43.4, 30.2, 14.6, 13.1. HRMS (ESI) calcd for C₂₂H₂₄F₃N₄O₃ [M + H]⁺ 449.1801, found 449.1792.

Ethyl 4-(3-(4-fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a] pyrimidin-5-yl) piperi- dine-1-carboxylate (4o)⁴⁴. The title compound was isolated as a white solid in 87% yield. mp 241-244 °C. ¹H

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3 NMR (400 MHz, CDCl₃) δ 12.12 (s, 1H), 7.14 (dd, *J* = 8.5, 5.4 Hz, 2H), 6.74 (t, *J* = 8.7 Hz, 2H), 5.48 (s,
4 1H), 4.26 (s, 2H), 4.09 (q, *J* = 7.1 Hz, 3H), 3.20 (t, *J* = 11.9 Hz, 1H), 2.78 (s, 2H), 2.12 (d, *J* = 9.7 Hz,
5 6H), 1.64 (dt, *J* = 12.1, 8.7 Hz, 3H), 1.24 (t, *J* = 7.1 Hz, 4H). ¹³C NMR (101 MHz, CDCl₃) δ 160.3,
6 158.0, 157.7, 155.6, 150.9, 139.0, 131.2, 126.8, 115.0, 114.8, 103.17, 92.9, 61.6, 43.9, 39.8, 31.0, 14.8,
7 12.8. HRMS (ESI) calcd for C₂₁H₂₄FN₄O₃ [M + H]⁺ 399.1832, found 399.1813.
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15 **Benzyl 4-(3-(4-fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a] pyrimidin-5-yl)piperi -dine-1-**
16 **carboxylate (4p)**⁴⁴. The title compound was isolated as a white solid in 82% yield. mp 206-209 °C. ¹H
17 NMR (400 MHz, DMSO-*d*₆) δ 7.79 (dd, *J* = 8.5, 5.8 Hz, 2H), 7.28 (d, *J* = 4.3 Hz, 4H), 7.23 (dd, *J* = 8.4,
18 4.0 Hz, 1H), 7.09 (t, *J* = 8.9 Hz, 2H), 5.34 (s, 1H), 5.01 (s, 2H), 4.04 (t, *J* = 12.0 Hz, 2H), 3.09 (d, *J* =
19 4.9 Hz, 1H), 2.82 (s, 2H), 2.37 (s, 3H), 1.74 (d, *J* = 11.9 Hz, 2H), 1.52 (qd, *J* = 12.6, 3.8 Hz, 2H). ¹³C
20 NMR (101 MHz, DMSO-*d*₆) δ 165.5, 158.6, 158.2, 154.6, 149.0, 147.5, 137.2, 131.7, 128.6, 128.6,
21 128.5, 127.9, 127.6, 114.8, 114.6, 102.1, 90.3, 66.2, 48.7, 44.0, 43.5, 15.2. HRMS (ESI) calcd for
22 C₂₆H₂₆FN₄O₃ [M + H]⁺ 461.1989, found 461.1984.
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35 **General procedure for the synthesis of 6a-6c.** To a solution of **5** (1eq) in water was added NaOH (**6a**,
36 **6c**) (1.2eq) or K₂CO₃ (**6b**) (1.2eq), appropriate acyl chloride (**6a**, **6c**) (1.2eq) or (Boc)₂O (**6b**) (1.2eq)
37 dissolved in THF. The mixture was stirred at rt. for 5-7 h. The solvent was removed under reduced pres-
38 sure and the aqueous phase was adjusted to pH 2 with 1M HCl. The sample was then extracted with
39 CH₂Cl₂ and dried over anhydrous MgSO₄. The solvent was evaporated under reduced pressure. The res-
40 idue was used in the next step without further purification.
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49 **1-(Tert-butoxycarbonyl)piperidine-4-carboxylic acid (6a)**⁴⁵. The title compound was isolated as a
50 white solid in 90% yield. ¹H NMR (400 MHz, CDCl₃) δ 4.01 (s, 2H), 2.85 (t, *J* = 11.7 Hz, 2H), 2.48 (tt,
51 *J* = 10.9, 3.9 Hz, 1H), 1.90 (d, *J* = 10.9 Hz, 2H), 1.68-1.58 (m, 2H), 1.45 (s, 9H).
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3 **1-(Ethoxycarbonyl)piperidine-4-carboxylic acid (6b)**⁴⁶. The title compound was isolated as a white
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5 solid in 91% yield. ¹H NMR (400 MHz, CDCl₃) δ 11.01 (s, 1H), 4.14-4.05 (m, 4H), 2.90 (t, *J* = 10.8 Hz,
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7 2H), 2.49 (tt, *J* = 10.9, 3.8 Hz, 1H), 1.91 (d, *J* = 11.9 Hz, 2H), 1.64 (qd, *J* = 11.5, 4.1 Hz, 2H), 1.24 (t, *J*
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9 = 7.1 Hz, 3H).

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13 **1-((Benzyloxy)carbonyl)piperidine-4-carboxylic acid (6c)**⁴⁷. The title compound was isolated as a
14
15 white solid in 95% yield. ¹H NMR (400 MHz, CDCl₃) δ 10.72 (s, 1H), 7.39-7.29 (m, 5H), 5.13 (s, 2H),
16
17 4.10 (s, 2H), 2.95 (s, 2H), 2.51 (tt, *J* = 10.7, 3.8 Hz, 1H), 1.93 (s, 2H), 1.67 (d, *J* = 10.3 Hz, 2H).

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20 **General procedure for the synthesis of 7a-7b.** To a solution of diisopropyl amine (6.73 mL, 48 mmol)
21
22 in THF (145 mL) was added *n*-BuLi (2M in hexane, 24mL, 48 mmol) at 0 °C. The reaction was stopped
23
24 to -78 °C 15 min later. Compound **6** (40mmol) in THF (60 mL) was added to the above solution and
25
26 stirred for 1h. Iodomethane (3.75 mL, 60 mmol) in THF (60 mL) was then added. The reaction was
27
28 warmed to rt. and stirred overnight, quenched with saturated NH₄Cl aqueous solution at 0 °C and the
29
30 solvent removed. The sample was extracted with EtOAc (3 × 100 mL). The combined organic layer was
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32 washed with brine (100 mL), dried over anhydrous MgSO₄ and solvent evaporated under reduced pres-
33
34 sure. The residue was purified by silica gel column chromatography (petroleum ether / EtOAc, 150: 1-
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36 50: 1) to obtain the title compound.
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43 **Diethyl 4-methylpiperidine-1, 4-dicarboxylate (7a)**⁴⁶. The title compound was isolated as a light yel-
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45 low oil in 87% yield. ¹H NMR (400 MHz, CDCl₃) δ 4.13-4.04 (m, 4H), 3.79 (s, 2H), 2.98 (t, *J* = 11.4
46
47 Hz, 2H), 2.03 (d, *J* = 13.5 Hz, 2H), 1.35-1.28 (m, 2H), 1.21 (td, *J* = 7.1, 5.5 Hz, 6H), 1.16 (s, 3H).

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50 **1-(Tert-butyl) 4-ethyl 4-methylpiperidine-1,4-dicarboxylate (7b)**⁴⁸ The title compound was isolated
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52 as a light yellow oil in 85% yield. ¹H NMR (400 MHz, CDCl₃) δ 4.17-4.09 (m, 2H), 3.76-3.71 (m, 2H),
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54 2.96 (t, *J* = 11.5 Hz, 2H), 2.04 (d, *J* = 13.1 Hz, 2H), 1.43 (s, 9H), 1.37-1.30 (m, 2H), 1.24 (t, *J* = 7.1 Hz,
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56 3H), 1.18 (s, 3H).

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3 **General procedure for the synthesis of 8a-8d.** To a solution of compound **7** (40 mmol) in THF (10
4 mL) was added 5M NaOH (40 mL, 200 mmol) at 0 °C, the resulting solution was then reacted at 70 °C
5 overnight. After removing the solvent, the sample was diluted with water and extracted with EtOAc (3
6 ×15 mL). The resulting aqueous phase was adjusted to pH 3 with 1M HCl and extracted with CH₂Cl₂
7 for three times, which was then combined with the organic phase and dried over anhydrous MgSO₄. The
8 solvent was removed under reduced pressure. The corresponding carboxylic acid was obtained and used
9 directly in the next step without further purification. Then, to a round bottom flask with the above car-
10 boxylic acid (1eq) dissolved in THF was added N, N'-carbonyldiimidazole (1.3 eq) at 0 °C. The resulting
11 solution was stirred at rt. for 4 h. To another solution of potassium 3-ethoxy-3-oxopropanoate (1 eq) in
12 MeCN: THF (1: 2) was added MgCl₂ (2 eq) and 4-dimethylaminopyridine (DMAP, 0.1 eq) at 0 °C. The
13 resulting solution was stirred at rt for 5 h. The former solution was then added to the latter solution at
14 0 °C. The resulting mixture was stirred at rt. overnight. After removing the solvent and adjusting pH to
15 4 with 1 M HCl at 0 °C, the product was extracted with EtOAc for three times. The combined organic
16 layer was washed with 1M HCl, 1M NaOH and brine separately, dried over anhydrous MgSO₄ and the
17 solvent removed under reduced pressure to obtain **8** as a yellow oil, which was used directly in the next
18 step without further purification.
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41 **Ethyl 4-(3-ethoxy-3-oxo)propanoyl)-4-methylpiperidine-1-carboxylate (8a)**⁴⁶. Yield of 82%. ¹H
42 NMR (400 MHz, CDCl₃) δ 4.21-4.15 (m, 2H), 4.10 (q, 2H), 3.62 (s, 2H), 3.51 (s, 2H), 3.35-3.20 (m,
43 2H), 1.97 (m, 2H), 1.44 (d, *J* = 3.7 Hz, 2H), 1.26 (tt, *J* = 10.5, 7.1 Hz, 6H), 1.18 (s, 3H).
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48 **Tert-butyl 4-(3-ethoxy-3-oxopropanoyl)-4-methylpiperidine-1-carboxylate (8b)**⁴⁶. Yield of 86%. ¹H
49 NMR (400 MHz, CDCl₃) δ 4.17 (qd, *J* = 7.1, 1.3 Hz, 2H), 3.53-3.46 (m, 4H), 3.29-3.16 (m, 2H), 1.96-
50 1.91 (m, 2H), 1.42 (s, 11H), 1.29-1.23 (m, 3H), 1.16 (s, 3H).
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Benzyl 4-(3-ethoxy-3-oxopropanoyl)piperidine-1-carboxylate (8c)⁴⁶. Yield of 87%. ¹H NMR (400 MHz, CDCl₃) δ 7.39-7.36 (m, 5H), 5.14 (s, 2H), 4.21 (q, *J* = 7.1 Hz, 4H), 3.51 (s, 2H), 2.89 (d, *J* = 10.8 Hz, 2H), 2.67 (tt, *J* = 11.2, 3.7 Hz, 1H), 1.88 (s, 2H), 1.59 (m, 2H), 1.30 (td, *J* = 7.1, 5.6 Hz, 3H).

Tert-butyl 4-(3-ethoxy-3-oxopropanoyl) piperidine-1-carboxylate (8d)⁴⁴. Yield of 91%. ¹H NMR (400 MHz, CDCl₃) δ 4.12 (dd, *J* = 14.1, 7.0 Hz, 2H), 4.04 (s, 2H), 3.43 (s, 2H), 2.72 (t, *J* = 11.4 Hz, 2H), 2.59-2.54 (m, 1H), 1.77 (t, *J* = 13.6 Hz, 2H), 1.52-1.43 (m, 2H), 1.38 (s, 9H), 1.22-1.19 (m, 3H).

General procedure for the synthesis of 11a-11d. To a sealing tube was added **9** (14.4 mL, 100 mmol), **10** (14.6 mL, 120 mmol), dry CH₂Cl₂ (10 mL) under N₂ atmosphere. The mixture was heated at 100 °C for 24 h. After cooling to rt., the resulting solution was added to a solution of hydrazine hydrochloride (10.3 g, 150 mmol) in EtOH (20 mL) and H₂O (20 mL) in a 250 mL round-bottom flask. The mixture was then heated at 80 °C for 12 h. The resulting solution was concentrated and neutralized with saturated solution of NaHCO₃ to pH 9. The mixture was extracted with EtOAc (3×100 mL). The combined organic layer was washed with brine (100mL), dried over anhydrous MgSO₄ and the solvent removed under reduced pressure. The product was recrystallized with EtOAc to obtain the title compound.

4-(4-Fluorophenyl)-3-methyl-1H-pyrazol-5-amine (11a)⁴⁴. The title compound was isolated as a white solid in 85% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.28-7.24 (m, 2H), 7.07 (t, *J* = 8.7 Hz, 2H), 2.22 (s, 3H).

4-(4-Chlorophenyl)-3-methyl-1H-pyrazol-5-amine (11b)⁴⁴. The title compound was isolated as a white solid in 81% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.35 (d, *J* = 8.4 Hz, 2H), 7.25 (d, *J* = 8.2 Hz, 2H), 2.24 (s, 3H).

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3 **3-Methyl-4-(4-(trifluoromethyl) phenyl)-1H-pyrazol-5-amine (11c)**⁴⁴. The title compound was iso-
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5 lated as a yellow solid in 88% yield. ¹H NMR (400 MHz, CDCl₃) δ 7.65 (d, *J* = 8.1 Hz, 2H), 7.47 (d, *J* =
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7 8.0 Hz, 2H), 2.31 (d, *J* = 5.5 Hz, 3H).

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10 **3-Methyl-4-(4-nitrophenyl)-1H-pyrazol-5-amine (11d)**⁴⁴. The title compound was isolated as a yel-
11
12 low solid in 82% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.76 (s, 1H), 8.21-8.19 (m, 2H), 7.63 (d, *J* =
13
14 8.7 Hz, 2H), 4.95 (s, 2H), 2.26 (s, 3H).

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17 **General procedure for the synthesis of 12.** To a solution of **8** (1 eq) in AcOH was added **11** (1.1 eq),
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19 the resulting solution was refluxed overnight. After removing the solvent and adjusting pH of the aque-
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21 ous phase to 8 with a saturated NaHCO₃ solution, the product was extracted with CH₂Cl₂ and dried over
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23 anhydrous MgSO₄. The solvent was evaporated under reduced pressure and the residue was purified by
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25 silica gel column chromatography (CH₂Cl₂ / MeOH, 100 : 1- 40: 1) to obtain compound **12**.
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30 **General procedure for the synthesis of 13a.** To a solution of **12** (R₂ = Boc) in CH₂Cl₂ (3V/V) was
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32 added TFA (V/V) and the mixture stirred at 0 °C for 2 h, After removing the solvent and adjusting pH
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34 of the aqueous phase to 8 with a saturated NaHCO₃ solution, the resulting precipitation was filtrated,
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36 washed with water, and dried over vacuum. **13a** was obtained and used in the next step without further
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38 purification.
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43 **General procedure for the synthesis of 13b.** To a solution of **8** (1 eq) in EtOH: TFA = (50 mL : 10 mL)
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45 was added **11a** (1.2 eq). The resulting solution was stirred at 85 °C when another 6.5 mL, 8.5 mL, and 25
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47 mL of TFA was added after 10 h, 20 h, and 30 h separately. The reaction was quenched with water at 48
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49 h. After removing the solvent and adjusting pH of the aqueous phase to 8 with a saturated NaHCO₃ so-
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51 lution, the product was filtered, the residue washed with water, CH₃OH, and CH₂Cl₂ separately, and
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53 then dried over vacuum to obtain **13b** as a white solid, which was used directly in the next step without
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55 further purification.
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3 **Cell Lines and Cell Culture.** HEK293 cells were grown in DMEM (high glucose) supplemented with
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5 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin at
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7 37 °C, 5% CO₂. Stable cell lines that express human TRPC3, mouse TRPC4, mouse TRPC6, mouse
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9 TRPV3, or mouse TRPM8 were established as described previously and maintained in the medium sup-
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11 plemented with G418 (400 µg/mL; Invitrogen)⁴⁹. For those that co-expressed MOR or M5 muscarinic
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13 receptor, 100 µg/ml hygromycin B (Calbiochem) was also included in the culture medium. Stable cell
14
15 lines that inducibly express human TRPC5, TRPC7, TRPA1, TRPV1, TRPV4, or TRPM5, were estab-
16
17 lished as described previously and maintained in the medium supplemented with 100 µg/mL hygromy-
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19 cin B and 5 µg/mL blasticidin⁵⁰. The expression of TRP channels was induced by the addition of 0.1 µM
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21 doxycycline to the culture medium 24 hrs prior to the assay.
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27 The use of animals was approved by the Animal Experimentation Ethics Committee of Anhui Medical
28
29 University and in accordance with the guidelines for ethical conduct in the care and use of animals.
30

31
32 Glomerular mesangial cells were prepared according to previous report^{43, 51}. Briefly, glomeruli were iso-
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34 lated from male Sprague-Dawley rats (260–280 g) using the graded sieving technique. Glomeruli were
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36 collected by centrifugation and digested by collagenase (2 mg/mL, Sigma-Aldrich) for 45 min at 37 °C.
37
38 Following the wash-off of the enzymes, mesangial cells were cultured in RPMI 1640 medium supple-
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40 mented with 17% FBS, 100 units/mL penicillin G, and 100 µg/mL streptomycin at 37 °C in a 5% CO₂
41
42 humidified incubator.
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46 **Fluorescence Ca²⁺ and Membrane Potential Assays.** Cells were seeded in wells of 96-well plates pre-
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48 coated with polyornithine at ~100,000 cells/well. Cells were loaded with Fluo4-AM (Invitrogen) or
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50 Fluo8-AM (TEFLabs) to monitor intracellular Ca²⁺ changes or loaded with the FLIPR Membrane Po-
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52 tential dye (FMP, Molecular Devices) to monitor membrane potential changes with the use of a FlexSta-
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54 tion microplate reader (Molecular Devices). The detailed protocols for the FlexStation FMP and Ca²⁺
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3 assays have been described previously^{37, 49, 52}. Briefly, the extracellular solution for all FlexStation as-
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5 says contained (in mM): 140 NaCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH adjusted to 7.4
6
7 with NaOH, except for TRPC3 when the 2 CaCl₂ was replaced with 0.5 CaCl₂. Probenecid (2 mM) was
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9 included in all Ca²⁺ assays except for TRPV1. Assays were run at 32 °C.

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12 [Ca²⁺]_i measurement in rat mesangial cells was carried out as described previously⁵³. Briefly, glomerular
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14 mesangial cells were seeded on glass coverslips and allowed to grow overnight. In the next day, the cells
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16 were loaded with 10 μM Fluo4-AM in the presence of 0.02% pluronic acid F-127 at 37 °C for 1 h in the
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18 dark in 1 mM Ca²⁺ saline containing (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10
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20 HEPES, pH adjusted to 7.4 with NaOH. After washing, the fluorescent signal was measured using a
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22 Leica TCS SP5 laser scanning confocal imaging system. The excitation and emission wavelength were
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24 488 nm and 515 nm, respectively.
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30 For all fluorescence measurement, changes in the fluorescence intensity were displayed as a ratio of flu-
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32 orescence change to the fluorescence before the application of stimulating compounds ($\Delta F/F_0$).
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35 **Electrophysiology recordings.** HEK293 cells stably expressing TRPC channels were seeded in 35-mm
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37 dishes at least 5 hrs before whole-cell patch clamp recordings were performed. Recording pipettes were
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39 pulled from micropipette glass (Sutter Instrument) to 2-5 MΩ when filled with a pipette solution con-
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41 taining (in mM): 110 CsCl, 10 HEPES, 10 BAPTA, 1 MgCl₂, 4.77 CaCl₂, pH adjusted to 7.2 with HCl
42
43 (400 nM free Ca²⁺) and placed in the bath solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1
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45 MgCl₂, 10 glucose, and 10 HEPES, pH 7.4. Individual cells were voltage-clamped in the whole-cell
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47 mode using either an EPC9 or EPC10 (HEKA Instruments Inc., Bellmore, NY) amplifier. Voltage com-
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49 mands were made from the PatchMaster program (version 2.60; HEKA) and currents were recorded at 5
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51 kHz. Voltage ramps of 100 ms to -100 mV after a brief (20-ms) step to +100 mV from holding potential
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53 of 0 mV were applied every 1 s. Cells were continuously perfused with the bath solution through a grav-
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ity-driven multi-channel system with the desired channel opening placed about 50 μm away from the cell being recorded. For some recordings, CaCl_2 in the bath solution was reduced to 0.5 or 0.1 mM as indicated in the figure legends. Drugs were diluted in the bath solution with the desired Ca^{2+} concentration and applied to the cell through perfusion.

Data analysis. All data were analyzed and plotted using Origin 7.5 (OriginLab) and Graphpad prism (V 5.01). Summary data are presented as the mean \pm S.E.M. Statistical comparisons were made using Student's t test. A p value of < 0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX

^1H NMR spectral information for compounds **6a-6c**, **7a-7b**, **8a-8d** **11a-11c**; ^1H NMR and ^{13}C NMR spectral information for compounds **4a-4p**; HPLC results and HPLC traces information for compounds **4a-4p** (PDF)

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Author Contributions

※Chunrong Qu, Mingmin Ding, Yingmin Zhu contributed equally to this work.

X. Hong, M.X. Zhu, M. Li, B. Shen, Z. Cao, H. Wang, H.R. Luo designed the research; C. Qu, M. Ding, Y. Zhu, Y. Lu, J. Zhu, J. Du, M. Miller, J. Tian, J. Xu, M. Wen, M. Wu, J. Wu performed experiments; X. Hong., M.X. Zhu., H. Wang, C. Qu, M. Ding, Y. Lu, Y. Xiao, O.B. McManus, J. Wang performed data analyses; M. Ding, AGA Er-Bu performed HPLC analyses; X. Hong., M.X. Zhu., H. Wang, C. Qu, M. Ding, Y. Lu, H.R. Luo wrote the paper.

Notes

The authors declare no competing financial interest.

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10 ■ ABBREVIATIONS USED

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12 $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Cbz, carboxybenzyl; CCh, carbachol; DAG, diacylglycerols;
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14 DAMGO, [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin; DCM, dichloromethane; EC₅₀, half maximal
15
16 stimulation; FBS, fetal bovine serum; FMP, FLIPR Membrane Potential; HTS, high-throughput
17
18 screening; MOR, μ opioid receptor, PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate;
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20 SAR, structure-activity relationship; TFA, trifluoroacetic acid; TRPA, Transient receptor potential
21
22 ankyrin; TRPC, Transient receptor potential canonical; TRPM, Transient receptor potential melatatin;
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24 TRPV, Transient receptor potential vanilloid.
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Table of Contents Graphic:

