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# Improved PIEZO1 agonism through 4-benzoic acid modification of Yoda1

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## DATA AND MATERIALS AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions. All original data are available in an Excel file. Unique laboratory materials created in the project are available on request (D.J.B. for biological materials and R.F. for chemicals).

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## AUTHOR CONTRIBUTIONS

G.P., A.J.H. and J.A.K. designed and performed calcium measurement assays. K.C. and C.H.R. designed, synthesized and analysed chemicals. N.E. designed and performed myography assays. O.V.P. designed and performed PIEZO1 patch-clamp experiments and J.A.K. PIEZO2 patch-clamp experiments. N.M., M.G.R., N.B. and A.B. designed automated patch-clamp experiments. N.M. and M.G.R. performed automated patch-clamp assays. T.S.F. and L.L. bred and maintained genetically engineered mice. M.J.L. generated cell lines. G.P., N.E., N.M. and O.V.P. made the figures. G.P. orchestrated the figure designs, data analysis and data transparency. CHR generated the supplementary chemistry information. E.C.-B. made intellectual contribution and performed PIEZO2 and HeLa cell experiments with M.D. and F.B.. G.P. and D.J.B. interpreted data and wrote most of the manuscript with input from

54 M.G.R., R.F., O.V.P., N.M., E.C.-B. and M.D.. D.J.B. and R.F. conceptualised the study,  
55 supervised the project team and generated funding.

56  
57

## 58 **CONFLICT OF INTEREST DISCLOSURE**

59

60 Automated patch-clamp studies were at Nanion Technologies GmbH, which has interest in  
61 the commercial success of the SyncroPatch 384. Authors at Leeds and Homburg have interest  
62 in successful outcomes from research grants and studentships as indicated in the Funding  
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64  
65

## 66 **ETHICS APPROVAL STATEMENT**

67

68 All animal experiments were authorised by the University of Leeds Animal Welfare and Ethics  
69 Committee and the UK Home Office under the authority of the Project Licences P606320FB  
70 and PP8169223.

71  
72

## 73 **PERMISSION TO REPRODUCE INFORMATION FROM OTHER SOURCES**

74

75 Not applicable.

76  
77

## 78 **DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR**

79

80 This Declaration acknowledges that this paper adheres to the principles for transparent  
81 reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design  
82 and Analysis, and Animal Experimentation, and as recommended by funding agencies,  
83 publishers and other organisations engaged with supporting research.

84  
85

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87

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91

## KEYWORDS

92 Calcium channel, Non-selective cation channel, Mechanical force, Pharmacology, Medicinal  
93 chemistry, Vascular biology, Endothelial cell.

94

95

## 96 **WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT**

97

98 PIEZO1 channel is an important mechanical force sensor

99

100 Options for pharmacological modulation of PIEZO1 are limited

101

## 102 **WHAT THIS STUDY ADDS**

103

104 Novel PIEZO1 agonists and new agonism structure-activity relationships

105

106 Improved PIEZO1 agonism

107

## 108 **CLINICAL SIGNIFICANCE**

109

110 Potential foundations for treating diseases linked genetically to PIEZO1, such as lymphatic  
111 dysplasia

112

113

## 114 **ABSTRACT**

115

116 **Background and Purpose:** PIEZO1 forms mechanically activated calcium-permeable non-  
117 selective cation channels in numerous species and cell types. Options for pharmacological  
118 modulation are limited and so we modified a PIEZO1 small-molecule agonist (Yoda1) to  
119 advance capability for modulation.

120 **Experimental Approach:** Medicinal chemistry generated Yoda1 analogues that were tested  
121 in intracellular calcium and patch-clamp assays on cultured cells exogenously expressing  
122 human or mouse PIEZO1 or mouse PIEZO2, physico-chemical assays and wire myography  
123 assays on vein from mice in which there was genetic disruption of PIEZO1.

124 **Key Results:** A Yoda1 analogue (KC159) containing 4-benzoic acid instead of the pyrazine  
125 of Yoda1 and its potassium salt (KC289) have equivalent or improved reliability, efficacy and  
126 potency compared to Yoda1 in functional assays. Tested against over-expressed mouse  
127 PIEZO1 in calcium assays, potency order is KC289 (150)>KC159 (280)>Yoda1 (600) ( $EC_{50}$ s  
128 in parentheses, nM). There is selectivity for PIEZO1 over other membrane proteins and the  
129 physico-chemical properties are more suited to physiological conditions than those of Yoda1.  
130 Vasorelaxant effects occur that are consistent with PIEZO1 agonism. 2-benzoic acid  
131 substitution, by contrast, fails to generate a modulator.

132 **Conclusion and Implications:** 4-Benzoic acid modification of Yoda1 improves PIEZO1  
133 agonism. We suggest naming this new modulator Yoda2. It should be a useful tool compound  
134 in physiological assays and facilitate efforts to identify a binding site. Such compounds may  
135 have therapeutic potential, for example in diseases linked genetically to PIEZO1 such as  
136 lymphatic dysplasia.

137

## 138 **NON-STANDARD ABBREVIATIONS**

139  
140 EC<sub>50</sub>, concentration for 50% effect; HEK, human embryonic kidney; GFP, green fluorescent  
141 protein; TRPC, transient receptor potential canonical; DMEM, Dulbecco's minimum essential  
142 medium; HUVEC, Human Umbilical Vein Endothelial Cells; ECGM-2, Endothelial Cell Basal  
143 Medium 2; EDTA, ethylenediamine tetraacetic acid; HeLa cells, immortal cells named after  
144 Henrietta Lacks; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEPES, 4-(2-  
145 hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulphoxide; PTFE,  
146 polytetrafluoroethylene; RED, rapid equilibrium dialysis; TAM, tamoxifen; L-NAME, L-N<sup>G</sup>-nitro  
147 arginine methyl ester; PE, phenylephrine; EC, endothelial cell; Cdh5, Cadherin 5; NOS3,  
148 endothelial nitric oxide synthase; mPIEZO1, mouse PIEZO1; hPIEZO1, human PIEZO1;  
149 mPIEZO2, mouse PIEZO2; siPIEZO1, short-interfering RNA targeted to PIEZO1 expression;  
150 siPIEZO2, short-interfering RNA targeted to PIEZO2 expression; siCtrl, control short-  
151 interfering RNA; M-Stim, mechanical stimulation.

152

## 153 INTRODUCTION

154

155 [PIEZO1](#) is a mechanically activated calcium ion (Ca<sup>2+</sup>)-permeable non-selective cation  
156 channel subunit (Coste et al., 2010; Murthy, Dubin & Patapoutian, 2017). It assembles as  
157 trimers to form a central ion pore (Guo & MacKinnon, 2017; Jiang, Yang, Jiang & Xiao, 2021).  
158 Its N-terminal propeller blade-like structures curve and indent the membrane, most likely to  
159 enable response to increased membrane tension (De Vecchis, Beech & Kalli, 2021; Guo &  
160 MacKinnon, 2017; Yang, Lin, Chen, Li, Li & Xiao, 2022; Young, Lewis & Grandl, 2022).  
161 Channel activation leads to Ca<sup>2+</sup> influx and intracellular Ca<sup>2+</sup> elevation and thus cellular effects  
162 due to the second messenger roles of Ca<sup>2+</sup>. The channels are widely expressed and have  
163 diverse functions, including in blood flow sensing by endothelium (Li et al., 2014; Rode et al.,  
164 2017; Wang, Chennupati, Kaur, Iring, Wettschureck & Offermanns, 2016) and detection of  
165 cyclical force in innate immunity (Solis et al., 2019). Mutations in human *PIEZO1* associate  
166 with anaemia (Zarychanski et al., 2012), malarial resistance (Ma et al., 2018), lymphatic  
167 dysplasia (Fotiou et al., 2015) and varicose vein disease (Fukaya et al., 2018), suggesting  
168 important roles in red blood cell, vascular wall and other human biology (Beech & Kalli, 2019;  
169 Jiang, Yang, Jiang & Xiao, 2021).

170

171 The pharmacology available for PIEZO1 is limited and so the opportunities for PIEZO1  
172 manipulation and therapeutic drug discovery are restricted. Most importantly so far, screening  
173 of a library of small-molecules revealed a promising agent called [Yoda1](#), named in reference  
174 to the "may the force be with you" phrase of the Star Wars films (Syeda et al., 2015). This  
175 substance (2-[5-[[2,6-dichlorophenyl)methyl]thio]-1,3,4-thiadiazol-2-yl]pyrazine) is an agonist  
176 of mouse and human PIEZO1 channels (Syeda et al., 2015). It does not activate the only  
177 related channel, [PIEZO2](#), in studies of mouse PIEZO2 overexpressed in HEK 293 cells (Syeda  
178 et al., 2015). Yoda1 is used extensively in the field and, while some concern has been raised  
179 about its suitability, it appears to be a valuable tool compound (Beech & Kalli, 2019). It  
180 activates PIEZO1 channels reconstituted in lipid bilayers (Syeda et al., 2015) and may act  
181 somewhere in the propeller blade region proximal to the ion pore domain, putatively serving  
182 as a "molecular wedge" to lower the threshold for mechanical activation (Botello-Smith et al.,  
183 2019). Despite the synergy with mechanical force (Syeda et al., 2015), direct application of  
184 Yoda1 in the absence of exogenous force is often sufficient to cause strong PIEZO1 channel  
185 activation (Evans et al., 2018), perhaps because there is already endogenous force acting on  
186 the channel (e.g., from the substrate or cell-cell contact). Therefore, Yoda1 is a test agent to  
187 determine the presence of functional PIEZO1 channels and indicate physiological roles of  
188 PIEZO1. Nevertheless, although Yoda1 is a useful tool compound, it has limitations such as  
189 its relatively low potency and aqueous solubility (Syeda et al., 2015).

190

191 A potential route to improving PIEZO1 pharmacology is through better understanding of the  
192 structure-activity relationships of Yoda1. Integrity of the 2,6-dichlorophenyl moiety is important  
193 (Evans et al., 2018; Syeda et al., 2015) with only limited possibilities for modification with

194 retained activity (Li, Xiong, Yan, O'Brien & Schuller de Almeida, 2021). The pyrazine moiety  
 195 at the other end of the molecule, however, appears to be less critical while still important for  
 196 agonism (Evans et al., 2018). Therefore, we hypothesized that modification of the pyrazine  
 197 could be a route to improved agonism and physico-chemical properties.

198  
 199

## 200 METHODS

201  
 202

### 202 Stable cell lines

203

204 HEK T-REx™ 293 cells that overexpress human PIEZO1 upon induction with tetracycline were  
 205 generated as previously described (Rode et al., 2017). PIEZO1-GFP7 was used as a PCR  
 206 template to clone human PIEZO1 coding sequence into pcDNA™4/TO between HindIII and  
 207 EcoRI restriction sites. PIEZO1 was amplified as two fragments using the following primers:  
 208 (HindIII-PIEZO1-Fw: AATAAGCTTATGGAGCCGCACGTG and BamHI-Int.PIEZO1-Rv:  
 209 AATGGATCCCCCTGGACTGTCTG) and (BamHI-Int. PIEZO1-Fw:  
 210 AATGGATCCTCCCCGCCACGGA and EcoRI-PIEZO1-Rv:  
 211 AATGAATTCTTACTCCTTCTCACGAGT). The two fragments were fused using BamHI  
 212 restriction site, resulting in the full-length PIEZO1 coding sequence with the c4182a silent  
 213 mutation. HEK T-REx™ 293 cells (Invitrogen) were transfected with pcDNA4/TO-PIEZO1  
 214 using Lipofectamine 2000 (Thermo Fisher Scientific). Subsequently cells were treated with 10  
 215 µg mL<sup>-1</sup> blasticidin and 200 µg mL<sup>-1</sup> zeocin (InvivoGen) to select stably transfected cells.  
 216 Single cell clones were isolated and analysed individually. Expression was induced by treating  
 217 the cells for 24 hours with 10 ng mL<sup>-1</sup> tetracycline and analysed by quantitative RT-PCR and  
 218 western blot. These cells are referred to as hPIEZO1-TREx.

219 HEK T-REx™ cells that constitutively overexpress murine PIEZO1 were generated as  
 220 previously described (Blythe et al., 2019). pcDNA3 mouse PIEZO1 IRES GFP, a gift from  
 221 Ardem Patapoutian (Coste et al., 2010), was used as a template to clone the mouse PIEZO1  
 222 coding sequence into pcDNA4/TO. Overlapping mouse PIEZO1 (forward primer 5'-  
 223 GTAACAACCTCCGCCCCATTG-3' and reverse primer 5'-GCTTCTACTCCCTCTCACGTGTC-  
 224 3') and pcDNA4/TO (forward primer 5'-  
 225 GACACGTGAGAGGGAGTAGAAGCCGCTGATCAGCCTCGACTG-3' and reverse primer 5'-  
 226 CAATGGGGCGGAGTTGTTAC-3') PCR products were assembled using Gibson Assembly  
 227 (New England Biolabs). This construct does not contain tetracycline operator sequences. HEK  
 228 T-REx™ 293 cells were transfected with pcDNA4/TO-mPIEZO1 using Lipofectamine 2000  
 229 (Invitrogen) and treated with 200 µg mL<sup>-1</sup> zeocin to select stably transfected cells. Individual  
 230 clones were isolated and analyzed for expression using Yoda1 and intracellular Ca<sup>2+</sup>  
 231 measurements. These cells are referred to as mPIEZO1-TREx.

232

233 HEK 293 cells stably expressing tetracycline-regulated human [TRPC5](#) have been described  
 234 previously (Zeng et al., 2004). For the TRPC5 expressing cells, selection was achieved by  
 235 including 5 µg mL<sup>-1</sup> blasticidin and 400 µg mL<sup>-1</sup> zeocin in the cell medium. To induce  
 236 expression, cells were incubated with 1 µg mL<sup>-1</sup> tetracycline for 24 h prior to experiments.  
 237 These cells are referred to as hTRPC5-TREx.

238

### 239 PIEZO2 and cell transfection

240

241 Mouse PIEZO2 (mPIEZO2) pCMV-Sport6 was a gift from Ardem Patapoutian (Coste et al.,  
 242 2015), obtained via Addgene (Addgene plasmid # 81073; <http://n2t.net/addgene:81073>;  
 243 RRID:Addgene\_81073). It was sequenced for validation. Transient transfection of the plasmid  
 244 was achieved using Lipofectamine 3000 in HEK293 cells, 48 hr prior to measurements being  
 245 made.

246

### 247 Cell culture

248

249 HEK T-REx™ 293 cells were maintained in DMEM supplemented with 10% heat-inactivated  
 250 fetal calf serum, 100 units mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin (Sigma-Aldrich). Stably  
 251 transfected HEK T-REx™ 293 cells continued to receive 10 µg mL<sup>-1</sup> blasticidin, and 200 µg  
 252 mL<sup>-1</sup> zeocin in maintenance cultivation. Non-transfected HEK T-REx™ 293 cells were used  
 253 as control cells.

254

255 Human Umbilical Vein Endothelial Cells (HUVEC) purchased from PromoCell (# C-12203)  
 256 were maintained in Endothelial Cell Basal Medium (ECGM-2, PromoCell # C-22111)  
 257 supplemented with: 500 pg mL<sup>-1</sup> recombinant human vascular endothelial growth factor 165,  
 258 10 ng mL<sup>-1</sup> recombinant human basic fibroblast growth factor, 200 ng mL<sup>-1</sup> hydrocortisone,  
 259 22.5 µg mL<sup>-1</sup> heparin, and 2% (vol./vol.) foetal calf serum. Antibiotic-antimycotic (Gibco  
 260 #15240062) was also added and resulted in final concentrations of 10 U mL<sup>-1</sup> penicillin, 10 U  
 261 mL<sup>-1</sup> streptomycin, and 250 ng mL<sup>-1</sup> amphotericin B. HUVEC were passaged at a maximum  
 262 seeding ratio of 1:4 (parent:daughter flask) after 3 to 6 days' growth, when they had reached  
 263 densities of between 3.4 to 6.6 x 10<sup>4</sup> cells cm<sup>-2</sup>. After washing the cell monolayer with D-PBS  
 264 [Sigma # D8537], 2 mL pre-warmed 0.05% Trypsin-EDTA (Gibco # 25300054) was added to  
 265 75 cm<sup>2</sup> flasks until the cells had detached (between 2-5 minutes at 37°C). 8 mL complete  
 266 ECGM2 medium was added to inhibit the trypsin, the cells were counted, their density adjusted  
 267 before seeding into fresh vessels for maintenance cultivation or experimental purposes. The  
 268 medium for maintenance of cultures was completely refreshed every 48 hours. HUVEC were  
 269 used for experiments after a minimum of 3 and a maximum of 5 passages. All cells were grown  
 270 at 37°C and in 5% CO<sub>2</sub> humidified incubator.

271

272 HeLa and HEK 293 cells were purchased from ATCC and maintained in DMEM supplemented  
 273 with 10% heat-inactivated fetal calf serum, 100 units.mL<sup>-1</sup> penicillin and 100 µg.mL<sup>-1</sup>  
 274 streptomycin (Sigma-Aldrich).

275

## 276 RNA interference

277

278 Cells were transfected using using Opti-MEM™ I Reduced Serum Medium (ThermoFisher  
 279 Scientific) and Lipofectamine 2000 (ThermoFisher Scientific). For transfection of cells in 6-well  
 280 plates, 20 nmol siRNA (siCtrl : Dharmacon ON-TARGETplus Non-targeting Control Pool, D-  
 281 001810-10-05 ; siPIEZO2 : Dharmacon ON-TARGETplus SmartPool PIEZO2, L-013925-02-  
 282 0005 and PIEZO1 : Sigma-Aldrich, GCAAGUUCGUGCGCGGAUU[DT][DT]) and 3 µL of  
 283 Lipofectamin 2000 complexed in 200 µL of Opti-MEM were added to 0.8 mL cell culture  
 284 medium per well. The culture medium was changed 4 hours post transfection and cells were  
 285 used for experiments 48 hr later. RNA isolation and RT-qPCR: RNA was isolated using Trizol  
 286 according to manufacturer's protocol. 1 µg of total RNA was reverse transcribed using the  
 287 iScript™ cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. qPCR  
 288 was performed using SyBR Green (Bio-Rad). qPCR reactions were performed on a  
 289 LightCycler® 480 Real Time PCR System (Roche). Samples were analysed using the  
 290 comparative CT method, and expressed as percentage of GAPDH housekeeping gene. The  
 291 sequences of PCR primers (synthesized by Integrated DNA Technologies) were:

292

Species	Gene	Forward (5'-3')	Reverse (5'-3')
Homo sapiens	<i>PIEZO1</i>	CGTCTTCGTGGAGCAGATG	GCCCTTGACGGTGCATAC
	<i>PIEZO2</i>	GACAGACGAAGCAGCACAGA	GTGCTTTCTTCCAACCTCGCC
	<i>GAPDH</i>	GCCTCAAGATCATCAGCAAT	GGACTGTGGTCATGAGTCCT
Mus musculus	<i>Piezo1</i>	TGAGCCCTTCCCCAACAATAC	CTGCAGGTGGTTCTGGATATAG
	<i>Piezo2</i>	AGAGTCGGAAAAGAGATACCCTC	CCAGACGATACAGATGAGAAGGA

293

## 294 Ca<sup>2+</sup> measurement

295



296 HEK 293-TREx cells and their stably-transfected derivatives or transiently transfected HEK  
297 293 or HeLa cells were seeded at a density of  $7.5 \times 10^4$  cells per well into poly-D-Lysine-  
298 coated 96-well plates. The plates were either commercially pre-coated with poly-D-Lysine  
299 (Greiner Bio-One # 655946) or (Greiner Bio-One # 655090) were coated in-house with 35  $\mu\text{L}$   
300 of a  $10 \mu\text{g mL}^{-1}$  poly-D-Lysine (Bio-Techne, Cultrex # 3439-100-01) in sterile deionised water  
301 per well for at least 24 hours and rinsed 3 times with 100  $\mu\text{L}$  per well sterile D-PBS before  
302 addition of cells. HUVEC were seeded into tissue culture-treated 96-well plates (Greiner Bio-  
303 One #655090) at a density of either  $2.5 \times 10^4$  cells 24 h before experimentation, or  $1.25 \times 10^4$   
304 cells 48 h before experimentation. Cells were incubated with 2  $\mu\text{M}$  Fura-2-AM (Molecular  
305 Probes™) in the presence of 0.01% (weight/vol.) pluronic F127 (Sigma-Aldrich #P2443) in  
306 standard bath solution (SBS) for 1 h at 37°C. Cells were washed with SBS for 30 min at room  
307 temperature. Alternatively, if inhibitors were being tested, these were added at this time,  
308 immediately following a wash in SBS. (-)-Englerin A (Akbulut et al., 2015) was used in SBS  
309 containing 0.01% pluronic acid as a dispersing agent to minimise aggregation of compound.  
310 Unless otherwise stated, the final concentration of DMSO was 0.2% (vol./vol.). Working 2000X  
311 stock solutions of test compounds were freshly made in DMSO and diluted 1000-fold in SBS  
312 during the 30-minute SBS wash/inhibitor preincubation period to obtain 2X solutions for  
313 injection. When testing the highest concentration of Yoda1, KC159 and KC289 (30  $\mu\text{M}$ ), the  
314 final DMSO concentration was 0.6% (vol./vol.). Measurements were made at room  
315 temperature on a 96-well fluorescence plate reader (FlexStationIII, Molecular Devices,  
316 Sunnyvale, CA, USA) controlled by Softmax Pro software v7.0.3. Recipient wells containing  
317 80  $\mu\text{L}$  SBS/ 0.2% (vol./vol.) DMSO vehicle or inhibitor compound received 80  $\mu\text{L}$  2X compound  
318 at an injection rate of  $1 \mu\text{L s}^{-1}$ , and the pipette introduced liquids from a height setting of 70  
319  $\mu\text{L}$ . No trituration was performed. For recordings using Fura-2, the change in intracellular  
320 calcium was indicated as the ratio of Fura-2 emission (510 nm) intensities at 340 and 380 nm  
321 excitation. Photomultipliers were set to medium sensitivity. SBS contained: 130 mM NaCl, 5  
322 mM KCl, 8 mM D-glucose, 10 mM HEPES, 1.2 mM  $\text{MgCl}_2$ , 1.5 mM  $\text{CaCl}_2$  and the pH was  
323 adjusted to 7.4 with NaOH. The solution was filtered through a 0.2  $\mu\text{m}$  bottle-top filter prior to  
324 use, and handled aseptically thereafter.

325  
326 We did not randomize compounds in the multiwell plate  $\text{Ca}^{2+}$  assays because of the risk of it  
327 introducing errors due to the complexity of constructing and analysing randomized plates. We  
328 did, however, take steps to minimize the risk of systematic errors by altering the position of  
329 specific compounds in the plate to test for the possibility of position-related effects, for which  
330 we found no evidence.

331

### 332 **Patch-clamp recording**

333

334 For manual patch-clamp, macroscopic transmembrane ionic currents of mPIEZO1 HEK-TREx  
335 cells (or control HEK-TREx cells) were recorded using the standard whole-cell configuration  
336 for patch-clamp in voltage-clamp mode. For studies of HEK 293 cells overexpressing PIEZO2  
337 (or control HEK 293 cells), the outside-out patch configuration was used in voltage-clamp  
338 mode. All recordings were made using an AXOpatch 200B amplifier (Axon Instruments, Inc.,  
339 USA) equipped with Digidata 1550B hosted by a PC running pClamp 10.7 software (Molecular  
340 Devices, USA) at room temperature. The cells were maintained during the experiment in an  
341 external salt buffer solution (SBS) of the following composition: 135 mM NaCl, 5 mM KCl, 1.5  
342 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 8 mM D-glucose, 10 mM HEPES (titrated to pH 7.4 with NaOH).  
343 Patch pipettes were fire-polished and had a resistance of 4–7 M $\Omega$  when filled with the pipette  
344 solution of the following composition: 145 mM CsCl, 2 mM  $\text{MgCl}_2$ , 10 mM HEPES, 1 mM  
345 EGTA, 5 mM  $\text{Na}_2\text{ATP}$ , 0.1 mM  $\text{Na}_2\text{GTP}$  and the pH was titrated to 7.2 with CsOH (whole-cell  
346 experiments). For outside-out experiments,  $\text{Na}_2\text{ATP}$  and  $\text{Na}_2\text{GTP}$  were omitted. For whole-  
347 cell experiments, cells were held at 0 mV and current amplitude was monitored by application  
348 of ramp voltage protocol from -100 mV to +100 mV at 10 s intervals. Currents were measured  
349 at -100 mV and +100 mV. Test compounds were applied in the external solution using a bath  
350 perfusion system. SBS flow was applied to the cells for 1 min before the addition of

351 compounds. For outside-out experiments the patches were held at -80 mV and currents were  
352 activated by application of 100-ms pressure pulse from 0 to 75 mmHg applied directly to the  
353 patch pipette with an interval of 12.387 s using High Speed Pressure Clamp HSPC-1 System  
354 (ALA Scientific Instruments, USA). Current records were analogue-filtered at 1 kHz and  
355 digitally acquired at 10 kHz. Data were analysed and plotted using pClamp 10.7 and MicroCal  
356 Origin 2018 (MicroCal software, USA).

357  
358 For automated patch-clamp, cells were cultured and harvested according to Nanion's standard  
359 cell culture protocol (Obergrussberger et al., 2014). hPIEZO1-TREx cells were induced to  
360 express PIEZO1 by incubation with 0.5  $\mu\text{g ml}^{-1}$  tetracycline 24 hours prior to experiments.  
361 Whole cell patch-clamp recordings were conducted on HEK-TREx, hPIEZO1-TREx and  
362 mPIEZO1-TREx cells according to Nanion's standard procedure for the SyncroPatch 384,  
363 using medium resistance (4-5 M $\Omega$ ) chips (Obergrussberger et al., 2018). Cells were held at -  
364 80 mV for the duration of the experiment. The channel was activated by mechanical forces in  
365 the absence and presence of the agonists. To stimulate PIEZO1 channels mechanically  
366 (mechanical stimulation, M-Stim), we dispensed 20  $\mu\text{L}$  of solution (either reference or agonist)  
367 locally to the cell at a pipetting speed of 110  $\mu\text{L.s}^{-1}$  in synchrony with a triggered recording of  
368 the current response at the holding potential. The internal solution contained: 110 mM KF, 10  
369 mM KCl, 10 mM NaCl, 10 mM HEPES, 10 mM EGTA pH adjusted to 7.2 with KOH, and the  
370 external solution contained: 140 mM NaCl, 4 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM  
371 Glucose, 10 mM HEPES, pH adjusted to 7.4 with NaOH, and 0.1% DMSO to match the DMSO  
372 concentration of the compound solutions. For PIEZO1 stimulation, KC159, KC157 or Yoda1  
373 was applied directly onto the cells via a pipette application. One chip of the SyncroPatch 384  
374 is equipped with 384 wells served by 384 robotic pipettes and connected to 384 individual  
375 amplifiers, allowing us to test 384 distinct cells in one experiment. We tested the effect of each  
376 specified analogue on mPIEZO1-, hPIEZO1- and untransfected HEK-TREx cells in parallel  
377 and in comparison to mechanical and Yoda1 stimulation. To estimate the EC<sub>50</sub> values, 4  
378 concentrations of each agonist were tested per cell line in one chip, with each cell receiving  
379 single concentrations, and the concentration response curve was calculated across the whole  
380 chip (384 cells). Only cells with seal resistance >0.3 G $\Omega$ , series resistance <20 M $\Omega$  and cell  
381 capacitance >5 pF and <40 pF were used for analysis. We considered responding cells as the  
382 cells treated with the agonist that displayed current with peak amplitude >-100 pA and area  
383 under the curve (AUC) >-10 pA.

384

### 385 **Chemical synthesis**

386

387 Analogues of Yoda1 were synthesised using a 4-step synthetic route utilising a Suzuki-  
388 coupling to introduce diversity (Scheme X) whilst compound **KC289** is the potassium salt of  
389 **KC159**, synthesised by treating **KC159** with KOH. All synthesised chemicals were purified by  
390 column chromatography or trituration and determined as >97% pure by <sup>1</sup>H NMR (proton  
391 nuclear magnetic resonance) and <sup>13</sup>C NMR (carbon-13 nuclear magnetic resonance).  
392 Synthetic and analytical details are reported in the Supporting Information (SI).

393

### 394 **Eurofins Hit Profiling Screen PP70**

395

396 These experiments were performed by Eurofins Scientific and the methods below are from  
397 this company's information (<https://www.eurofins.co.uk/>). †Membrane protein/ membrane  
398 amounts may have varied and the concentrations used were adjusted as necessary. KC289  
399 was tested at 5  $\mu\text{M}$ .

400

401 Acetylcholine M2: Human recombinant muscarinic M2 receptors expressed in CHO-K1 cells  
402 were used in modified Tris-HCl buffer pH 7.4. An 8  $\mu\text{g}^\ddagger$  aliquot was incubated with 0.8 nM  
403 [<sup>3</sup>H]N-Methylscopolamine for 120 minutes at 25°C. Non-specific binding was estimated in the  
404 presence of 1  $\mu\text{M}$  atropine. Receptors were filtered and washed, the filters were counted to  
405 determine [<sup>3</sup>H]N-Methylscopolamine specifically bound.

406  
407 Acetylcholine M3: Human recombinant muscarinic M3 receptors expressed in CHO-K1 cells  
408 were used in modified Tris-HCl buffer pH 7.4. A 12  $\mu\text{g}^\pm$  aliquot was incubated with 0.8 nM  
409 [ $^3\text{H}$ ]N-Methylscopolamine for 120 minutes at 25°C. Non-specific binding was estimated in the  
410 presence of 1  $\mu\text{M}$  atropine. Receptors were filtered and washed, the filters were then counted  
411 to determine [ $^3\text{H}$ ]N-Methylscopolamine specifically bound.

412  
413 Adenosine A1: This assay measured binding of [ $^3\text{H}$ ]DPCPX to adenosine A1 receptors. CHO-  
414 K1 cells stably transfected with a plasmid encoding the human adenosine A1 receptor were  
415 used to prepare membranes in modified HEPES pH 7.4 using standard techniques. A 10  $\mu\text{g}^\pm$   
416 aliquot of membrane was incubated with 1 nM [ $^3\text{H}$ ]DPCPX for 90 minutes at 25°C. Non-specific  
417 binding was estimated in the presence of 100  $\mu\text{M}$  R(-)-PIA. Membranes were filtered and  
418 washed 3 times and the filters were counted to determine [ $^3\text{H}$ ]DPCPX specifically bound.

419  
420 Adenosine A2A: This assay measured binding of [ $^3\text{H}$ ]CGS-21680 to human adenosine A2A  
421 receptors. HEK 293 (human embryonic kidney) cells stably transfected with a plasmid  
422 encoding the human adenosine A2A receptor were used to prepare membranes in modified  
423 Tris-HCl pH 7.4 buffer using standard techniques. A 15  $\mu\text{g}^\pm$  aliquot of membrane was  
424 incubated with 50 nM [ $^3\text{H}$ ]CGS-21680 for 90 minutes at 25°C. Non-specific binding was  
425 estimated in the presence of 50  $\mu\text{M}$  NECA. Membranes were filtered and washed 3 times and  
426 the filters were counted to determine [ $^3\text{H}$ ]CGS-21680 specifically bound.

427  
428 Adrenoceptor alpha1A: Submaxillary glands of male Wistar derived rats weighing  $175 \pm 25$  g  
429 were used to prepare adrenergic  $\alpha$ 1A receptors in modified Tris-HCl buffer pH 7.4. A 5  $\text{mg}^\pm$   
430 aliquot was incubated with 0.25 nM [ $^3\text{H}$ ]Prazosin for 60 minutes at 25°C. Non-specific binding  
431 was estimated in the presence of 10  $\mu\text{M}$  phentolamine. Membranes were filtered and washed,  
432 the filters were then counted to determine [ $^3\text{H}$ ]Prazosin specifically bound.

433  
434 Adrenoceptor alpha1B: Livers of male Wistar derived rats weighing  $175 \pm 25$  g were used to  
435 prepare adrenergic  $\alpha$ 1B receptors in modified Tris-HCl buffer pH 7.4. A 5  $\text{mg}^\pm$  aliquot was  
436 incubated with 0.25 nM [ $^3\text{H}$ ]Prazosin for 60 minutes at 25°C. Non-specific binding was  
437 estimated in the presence of 10  $\mu\text{M}$  phentolamine. Membranes were filtered and washed, the  
438 filters were then counted to determine [ $^3\text{H}$ ]Prazosin specifically bound.

439  
440 Adrenoceptor alpha2A: Human recombinant adrenergic  $\alpha$ 2A receptors expressed in CHO-K1  
441 cells were used in modified Tris-HCl buffer pH 7.4. A 2  $\mu\text{g}^\pm$  aliquot was incubated with 1.5 nM  
442 [ $^3\text{H}$ ]Rauwolscine for 60 minutes at 25°C. Non-specific binding was estimated in the presence  
443 of 10  $\mu\text{M}$  WB-4101. Receptors were filtered and washed, the filters were then counted to  
444 determine [ $^3\text{H}$ ]Rauwolscine specifically bound. KC289 was screened at 5  $\mu\text{M}$ .

445  
446 Adrenoceptor beta1: Human recombinant adrenergic  $\beta$ 1 receptors expressed in CHO-K1 cells  
447 were used in modified Tris-HCl buffer pH 7.4. A 25  $\mu\text{g}^\pm$  aliquot of membrane was incubated  
448 with 0.03 nM [ $^{125}\text{I}$ ]Cyanopindolol for 120 minutes at 25°C. Non-specific binding was estimated  
449 in the presence of 100  $\mu\text{M}$  S(-)-propranolol. Membranes were filtered and washed 3 times and  
450 the filters were counted to determine [ $^{125}\text{I}$ ]Cyanopindolol specifically bound.

451  
452 Adrenoceptor beta2: This assay measured binding of [ $^3\text{H}$ ]CGP-12177 to human adrenergic b2  
453 receptors. Mammalian CHO-hNBR1 cells stably transfected with a plasmid encoding the  
454 human adrenergic b2 receptor were used to prepare membranes in modified Tris-HCl pH 7.4  
455 buffer using standard techniques. A 50  $\mu\text{g}^\pm$  aliquot of membrane was incubated with 0.2 nM  
456 [ $^3\text{H}$ ]CGP-12177 for 60 minutes at 25°C. Non-specific binding was estimated in the presence  
457 of 10  $\mu\text{M}$  ICI-118551. Membranes were filtered and washed 3 times and the filters were  
458 counted to determine [ $^3\text{H}$ ]CGP-12177 specifically bound.

459

460 Cav1.2 (L-type): Cerebral cortices of Wistar derived rats weighing  $175 \pm 25$  g were used to  
461 prepare membranes in Tris-HCl buffer pH 7.4. A  $2.5 \text{ mg}^\dagger$  aliquot was incubated with  $0.1 \text{ nM}$   
462 [ $^3\text{H}$ ]Nitrendipine for 90 minutes at  $25^\circ\text{C}$ . Non-specific binding was estimated in the presence  
463 of  $1 \text{ }\mu\text{M}$  nitrendipine. Membranes were filtered and washed, the filters were then counted to  
464 determine [ $^3\text{H}$ ]Nitrendipine specifically bound.

465  
466 CB1 Human Cannabinoid: Human recombinant cannabinoid CB1 receptors expressed in rat  
467 hematopoietic Chem-1 cells were used in modified HEPES buffer pH 7.4. A  $5 \text{ }\mu\text{g}^\dagger$  aliquot of  
468 membrane was incubated with  $2 \text{ nM}$  [ $^3\text{H}$ ]SR141716A for 60 minutes at  $37^\circ\text{C}$ . Non-specific  
469 binding was estimated in the presence of  $10 \text{ }\mu\text{M}$  CP 55,940. Membranes were filtered and  
470 washed 4 times and the filters were counted to determine [ $^3\text{H}$ ]SR141716A specifically bound.

471  
472 Dopamine D1: This assay measured binding of [ $^3\text{H}$ ]SCH-23390 to human dopamine D1  
473 receptors. CHO cells stably transfected with a plasmid encoding the human dopamine D1  
474 receptor were used to prepare membranes in modified Tris-HCl pH 7.4 buffer using standard  
475 techniques. A  $20 \text{ }\mu\text{g}^\dagger$  aliquot of membrane was incubated with  $1.4 \text{ nM}$  [ $^3\text{H}$ ]SCH-23390 for 120  
476 minutes at  $37^\circ\text{C}$ . Non-specific binding was estimated in the presence of  $10 \text{ }\mu\text{M}$  (+)-butaclamol.  
477 Membranes were filtered and washed 3 times and the filters were counted to determine  
478 [ $^3\text{H}$ ]SCH-23390 specifically bound.

479  
480 Dopamine D25: This assay measured binding of [ $^3\text{H}$ ]Spiperone to human dopamine D25  
481 (D2B) receptors. CHO cells stably transfected with a plasmid encoding the human dopamine  
482 D25 receptor were used to prepare membranes in modified Tris-HCl pH 7.4 buffer using  
483 standard techniques. A  $15 \text{ }\mu\text{g}^\dagger$  aliquot of membrane was incubated with  $0.16 \text{ nM}$  [ $^3\text{H}$ ]Spiperone  
484 for 120 minutes at  $25^\circ\text{C}$ . Non-specific binding was estimated in the presence of  $10 \text{ }\mu\text{M}$   
485 haloperidol. Membranes were filtered and washed 3 times and the filters were counted to  
486 determine [ $^3\text{H}$ ]Spiperone specifically bound.

487  
488 GABA A (1): Whole brains (except cerebellum) of male Wistar derived rats weighing  $175 \pm 25$   
489 g were used to prepare GABAA agonist site receptors in Tris-HCl buffer pH 7.4. A  $10 \text{ mg}^\dagger$   
490 aliquot was incubated with  $1 \text{ nM}$  [ $^3\text{H}$ ]Muscimol for 10 minutes at  $4^\circ\text{C}$ . Non-specific binding was  
491 estimated in the presence of  $0.1 \text{ }\mu\text{M}$  muscimol. Membranes were filtered and washed, the  
492 filters were then counted to determine [ $^3\text{H}$ ]Muscimol specifically bound. GABA A (2): Whole  
493 brain (except cerebellum) of male Wistar derived rats weighing  $175 \pm 25$  g were used to  
494 prepare GABAA central benzodiazepine membrane receptor in Na-K phosphate buffer pH 7.4.  
495 A  $5 \text{ mg}^\dagger$  aliquot was incubated with  $1 \text{ nM}$  [ $^3\text{H}$ ]Flunitrazepam for 60 minutes at  $25^\circ\text{C}$ . Non-  
496 specific binding was estimated in the presence of  $10 \text{ }\mu\text{M}$  diazepam. Membranes were filtered  
497 and washed, the filters were then counted to determine [ $^3\text{H}$ ]Flunitrazepam specifically bound.

498  
499 Glutamate: Cerebral cortices of Wistar derived rats weighing  $175 \pm 25$  g were used to prepare  
500 glutamate NMDA phencyclidine receptors in Tris-HCl buffer pH 7.4. A  $6.3 \text{ mg}^\dagger$  aliquot was  
501 incubated with  $4 \text{ nM}$  [ $^3\text{H}$ ]TCP for 45 minutes at  $25^\circ\text{C}$ . Non-specific binding was estimated in  
502 the presence of  $1 \text{ }\mu\text{M}$  dizocilpine ((+)-MK-801). Membranes were filtered and washed, the  
503 filters were then counted to determine [ $^3\text{H}$ ]TCP specifically bound.

504  
505 Histamine H1: Human recombinant histamine H1 receptor expressed in CHO-K1 cells were  
506 used in modified Tris-HCl buffer pH 7.4. A  $10 \text{ }\mu\text{g}^\dagger$  aliquot was incubated with  $1.2 \text{ nM}$   
507 [ $^3\text{H}$ ]Pyrilamine for 180 minutes at  $25^\circ\text{C}$ . Non-specific binding was estimated in the presence  
508 of  $1 \text{ }\mu\text{M}$  pyrilamine. Receptor proteins were filtered and washed, the filters were then counted  
509 to determine [ $^3\text{H}$ ]Pyrilamine specifically bound.

510  
511 Imidazoline I2: Brains (except cerebella) of male Wistar derived rats weighing  $175 \pm 25$  g were  
512 used to prepare imidazoline I2 receptors in modified Tris-HCl buffer pH 7.4. A  $15 \text{ mg}^\dagger$  aliquot  
513 was incubated with  $2 \text{ nM}$  [ $^3\text{H}$ ]Idazoxan for 30 minutes at  $25^\circ\text{C}$ . Non-specific binding was

514 estimated in the presence of 1  $\mu\text{M}$  idazoxan. Membranes were filtered and washed, the filters  
515 were then counted to determine [ $^3\text{H}$ ]Idazoxan specifically bound.

516

517  $\mu$  Opioid: Human opiate  $\mu$  receptors expressed in CHO-K1 cells were used in modified Tris-  
518 HCl buffer pH 7.4. An 11  $\mu\text{g}^\dagger$  aliquot was incubated with 0.6 nM [ $^3\text{H}$ ]Diprenorphine for 60  
519 minutes at 25°C. Nonspecific binding was estimated in the presence of 10  $\mu\text{M}$  naloxone.  
520 Membranes were filtered and washed, the filters were then counted to determine  
521 [ $^3\text{H}$ ]Diprenorphine specifically bound.

522

523 nAChR ( $\alpha 1$ ): Human Nicotinic Acetylcholine  $\alpha 1$  receptors expressed in RD cells were  
524 used in 150 mM NaCl, 4 mM KCl, 2.3 mM  $\text{CaCl}_2$ . An aliquot was incubated with 0.6 nM [ $^{125}\text{I}$ ] $\alpha$ -  
525 Bungarotoxin for 120 minutes at 25°C. Nonspecific binding was estimated in the presence of  
526 1  $\mu\text{M}$   $\alpha$ -Bungarotoxin. Membranes were filtered and washed, the filters were then counted to  
527 determine [ $^{125}\text{I}$ ] $\alpha$ -Bungarotoxin specifically bound.

528

529 nAChR Nicotinic Acetylcholine: Human Nicotinic Acetylcholine receptors expressed in IMR-  
530 32 cells were used in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  
531  $\text{MgSO}_4$ . An aliquot was incubated with 0.10 nM [ $^{125}\text{I}$ ] Epibatidine for 60 minutes at 25°C.  
532 Nonspecific binding was estimated in the presence of 300  $\mu\text{M}$  (-)-Nicotine. Membranes were  
533 filtered and washed, the filters were then counted to determine [ $^{125}\text{I}$ ] Epibatidine specifically  
534 bound.

535

536 Norepinephrine Transporter: Human norepinephrine transporters expressed in dog kidney  
537 MDCK cells were used in modified Tris-HCl buffer pH 7.4. A 40  $\mu\text{g}^\dagger$  aliquot was incubated with  
538 0.2 nM [ $^{125}\text{I}$ ]RTI-55 for 3 hours at 4°C. Non-specific binding was estimated in the presence of  
539 10  $\mu\text{M}$  desipramine. Membranes were filtered and washed, the filters were then counted to  
540 determine [ $^{125}\text{I}$ ]RTI-55 specifically bound.

541

542 PDE: This assay measured binding of [ $^3\text{H}$ ]Rolipram to rolipram binding sites. Whole brains  
543 (except cerebellum) of male Wistar derived rats weighing  $175 \pm 25$  g were prepared in modified  
544 Tris-HCl pH 7.4 buffer using standard techniques. A 180  $\mu\text{g}^\dagger$  aliquot of membrane was  
545 incubated with 1.8 nM [ $^3\text{H}$ ]Rolipram for 60 minutes at 4°C. Non-specific binding was estimated  
546 in the presence of 10  $\mu\text{M}$  rolipram. Membranes were filtered and washed 3 times and the filters  
547 were counted to determine [ $^3\text{H}$ ]Rolipram specifically bound.

548

549 Phorbol Ester: This assay measures binding of [ $^3\text{H}$ ]PDBu to phorbol ester receptors. Brains  
550 (except cerebella) membranes of male ICR derived mice weighing  $20 \pm 2$  g were prepared in  
551 modified Tris-HCl pH 7.4 buffer using standard techniques. A 20  $\mu\text{g}^\dagger$  aliquot of membrane was  
552 incubated with 3 nM [ $^3\text{H}$ ]PDBu for 60 minutes at 25°C. Non-specific binding was estimated in  
553 the presence of 1  $\mu\text{M}$  PDBu. Membranes were filtered and washed 3 times and the filters were  
554 counted to determine [ $^3\text{H}$ ]PDBu specifically bound.

555

556 Potassium Channel (hERG): Human recombinant potassium channel HERG expressed in  
557 human HEK 293 cells were used in modified HEPES buffer pH 7.4. A 10  $\mu\text{g}^\dagger$  aliquot was  
558 incubated with 1.5 nM [ $^3\text{H}$ ]Astemizole for 60 minutes at 25°C. Non-specific binding was  
559 estimated in the presence of 10  $\mu\text{M}$  Astemizole. Channel proteins were filtered and washed,  
560 the filters were then counted to determine [ $^3\text{H}$ ]Astemizole specifically bound.

561

562 Potassium Channel (KATP): This assay measured binding of [ $^3\text{H}$ ]Glyburide to voltage  
563 insensitive ATP-sensitive potassium channel sites [KATP]. HIT-T15 Syrian hamster pancreatic  
564 b cells were used to prepare membranes in modified MOPS pH 7.4 buffer using standard  
565 techniques. A 100  $\mu\text{g}^\dagger$  aliquot of membrane was incubated with 5 nM [ $^3\text{H}$ ]Glyburide for 120  
566 minutes at 25°C. Non-specific binding was estimated in the presence of 1  $\mu\text{M}$  glyburide.  
567 Membranes were filtered and washed 3 times and the filters were counted to determine  
568 [ $^3\text{H}$ ]Glyburide specifically bound.

569  
570 Prostanoid EP4: Human recombinant prostanoid EP4 receptors expressed in Chem-1 cells  
571 were used in modified MES buffer pH 6.0. A 3  $\mu\text{g}/\text{ml}^\dagger$  aliquot was incubated with 1 nM  
572 [ $^3\text{H}$ ]PGE<sub>2</sub> for 120 minutes at 25°C. Non-specific binding was estimated in the presence of 10  
573  $\mu\text{M}$  PGE<sub>2</sub>. Receptors were filtered and washed, the filters were then counted to determine  
574 [ $^3\text{H}$ ]PGE<sub>2</sub> specifically bound.

575  
576 Serotonin (5-HT<sub>2B</sub>): This assay measured binding of [ $^3\text{H}$ ]Lysergic acid diethylamide (LSD) to  
577 human serotonin 5-HT<sub>2B</sub> receptors. CHO-K1 cells stably transfected with a plasmid encoding  
578 the human serotonin 5-HT<sub>2B</sub> receptor were used to prepare membranes in modified Tris-HCl  
579 pH 7.4 buffer using standard techniques. A 30  $\mu\text{g}^\dagger$  aliquot of membrane protein was incubated  
580 with 1.2 nM [ $^3\text{H}$ ]LSD for 60 minutes at 37°C. Non-specific binding was estimated in the  
581 presence of 10  $\mu\text{M}$  serotonin. Membranes were filtered and washed 3 times and the filters  
582 were counted to determine [ $^3\text{H}$ ]LSD specifically bound.

583  
584 Sigma 1: This assay measured binding of [ $^3\text{H}$ ]Haloperidol to sigma  $\delta$ 1 receptors. Human  
585 Jurkat cells were used to prepare membranes in potassium phosphate buffer pH 7.5 using  
586 standard techniques. A 140  $\mu\text{g}^\dagger$  aliquot of membrane was incubated with 8 nM [ $^3\text{H}$ ]Haloperidol  
587 for 4 hours at 25°C. Non-specific binding was estimated in the presence of 10  $\mu\text{M}$  Haloperidol.  
588 Membranes were filtered and washed 3 times and the filters were counted to determine  
589 [ $^3\text{H}$ ]Haloperidol specifically bound.

590  
591 Sodium Ion Channel: This assay measured binding of [ $^3\text{H}$ ]Batrachotoxinin to the site 2 of the  
592 sodium channel-batrachotoxin. Whole brain (except cerebellum) membranes of male Wistar  
593 derived rats weighing  $175 \pm 25$  g were prepared in modified HEPES/Tris-HCl containing 40  
594 mg/ml LqTx (alpha scorpion toxin from *Leiurus quinquestriatus*) to block site 2 at pH 7.4 buffer  
595 using standard techniques. A 7.5  $\text{mg}^\dagger$  aliquot of membrane was incubated with 5 nM  
596 [ $^3\text{H}$ ]Batrachotoxinin for 60 minutes at 37°C. Non-specific binding was estimated in the  
597 presence of 100  $\mu\text{M}$  veratridine. Membranes were filtered and washed 3 times and the filters  
598 were counted to determine [ $^3\text{H}$ ]Batrachotoxinin specifically bound.

## 599 600 **Aqueous solubility**

601  
602 Standard curves for each test compound were constructed. DMSO-dissolved test compounds  
603 were added to universal aqueous buffer (45 mM ethanolamine, 45 mM KH<sub>2</sub>PO<sub>4</sub>, 45 mM KOAc,  
604 pH 7.4): acetonitrile 80:20 (298.5  $\mu\text{L}$ ) at a final DMSO concentration of 0.5%. Samples were  
605 incubated at room temperature with 300 rpm shaking for 30 min. 200  $\mu\text{L}$  of each well was  
606 transferred to a 96-well polypropylene V-bottomed collection plate and absorbance readings  
607 were taken at 250 - 500 nm in 10 nm increments. Thus the  $\lambda_{\text{max}}$  was identified at which  
608 concentration versus absorbance was plotted to provide standard curves.

609  
610 For estimating aqueous solubility 2  $\mu\text{L}$  of 50 mM test compound in DMSO was added to 398  
611  $\mu\text{L}$  of universal aqueous buffer and the samples were incubated for 1.5 hours with 300 rpm  
612 shaking at room temperature. After filtration through hydrophilic PTFE 0.2  $\mu\text{m}$  filters, 160  $\mu\text{L}$   
613 sample was transferred to wells of a 96-well polypropylene V-bottomed collection plate and  
614 40  $\mu\text{L}$  acetonitrile was added to each well. Absorbance readings were taken at 250-500 nm in  
615 10 nm increments. Solubility was calculated at the appropriate  $\lambda_{\text{max}}$  with reference to the  
616 standard curve.

617  
618 Kinetic and thermodynamic solubility assays were also performed by Malvern PanAnalytical  
619 (UK). Briefly, 1000  $\mu\text{L}$  of 0.1 M phosphate buffer (pH 7.4) received either; 25  $\mu\text{L}$  of a 10 mM  
620 DMSO stock of test compound (for kinetic solubility assays) or; 1 mg of test compound (for  
621 thermodynamic solubility assays). The mixtures were shaken on an orbital mixer to reach  
622 equilibrium for 1 or 24 hours, respectively. The equilibrated solution was centrifuged, the  
623 supernatant removed to a fresh vial and then re-centrifuged. High and low dilution samples

624 were prepared from the secondary supernatant and quantified by LC-MSMS against a  
625 standard curve.

626

### 627 **Microsomal half-life**

628

629 Compound half-lives were measured by Malvern PanAnalytical (UK). Briefly, microsomal  
630 mouse liver microsomes (1 mg protein/ mL) were pre-incubated with NADPH cofactor solution  
631 at 37°C. Biotransformation was initiated by addition and mixing of 1 µM test compound in a  
632 final volume of 350 µL. 25 µL aliquots were removed at 5 minute intervals for 35 minutes and  
633 quenched in 300 µL ice cold methanol containing internal standard. The protein in the samples  
634 was then precipitated by centrifugation at 4°C, and the supernatant analysed by LC-MS/MS  
635 to quantify the test parent compound remaining at each time-point.

636

### 637 **Plasma protein binding**

638

639 Mouse plasma protein binding was measured by Rapid Equilibrium Dialysis (RED) performed  
640 by Malvern PanAnalytical (UK). Briefly, mouse plasma was warmed to 37°C for 10 minutes  
641 and test compound stock solution added to achieve a 5 µM solution. 500 µL of dialysis buffer  
642 was added to one side of the chamber of the device insert (Piercenet, Dialysis membrane  
643 MWCO 8000) housed within a heated Teflon block, and the incubation initiated by the addition  
644 of 300 µL of the test compound protein solution to the opposite chamber. Equilibrium was  
645 reached by shaking the device on an orbital mixer at 37°C for 4 hours. 50 µL aliquots from  
646 the buffer and the plasma chambers were transferred into separate wells of a deep well plate.  
647 50 µL of plasma were added to the buffer samples, and 50 µL of buffer were added to the  
648 plasma samples. 300 µL of ice cold acetonitrile containing internal standard were added to  
649 precipitate the protein. Samples were then centrifuged (2700 x g at 4°C for 20 minutes) to  
650 pellet the protein and the supernatant analysed by LC-MS/MS to quantify the % input  
651 compound unbound to plasma protein with reference to a standard curve.

652

### 653 **Stability in plasma**

654

655 Pooled heparinised mouse plasma was warmed to 37°C for 10 minutes, mixed and cleared of  
656 aggregated protein by centrifugation. Aliquots of the clear supernatant were transferred into  
657 the assay plate. Following equilibration to 37°C, biotransformation was initiated by adding and  
658 mixing of 1 µM compound solution in a final incubation volume of 300 µL. 25 µL aliquots were  
659 removed at 0, 5, 15, 30, 60, and 120 minutes and quenched in 300µL ice cold acetonitrile  
660 containing internal standard. After storing the samples at -20°C for a minimum of 4 hours, the  
661 protein in the samples was precipitated by centrifugation at 4°C and the supernatants analysed  
662 by LC-MS/MS to quantify the % input test compound remaining.

663

### 664 **Mice**

665

666 The mouse was selected for animal ex vivo studies because it is the smallest known  
667 mammalian species that can be used to provide blood vessels suitable for myography and  
668 enable suitable genetic manipulation, which in this study was used to test the role of PIEZO1.  
669 The portal vein was selected as the tissue to study because our pilot studies had shown that  
670 this blood vessel has a robust response to PIEZO1 agonists that is more reliable and larger  
671 than we have observed for other blood vessels. Animal studies are reported in compliance  
672 with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson & Altman, 2010; McGrath &  
673 Lilley, 2015). All animal experiments were authorised by the University of Leeds Animal Ethics  
674 Committee and the UK Home Office under the authority of the UK Home Office Project  
675 Licences P606320FB and PP8169223.

676

677 All mice were housed in GM500 individually ventilated cages (Animal Care Systems) at 21°C,  
678 50-70% humidity and with a 12 hour alternating light/dark cycle. They had *ad libitum* access

679 to water and RM1 diet (SpecialDiet Services, Witham, UK) with bedding from Pure'o Cell  
680 (Datesand, Manchester, UK). The number of cage companions was up to 5. Animals were  
681 visually inspected and weighed at a minimum of weekly intervals for welfare-related  
682 assessments. Local animal welfare advice and steps were taken in the rare cases of concern  
683 for an animal or animals. The genetically modified mice did not display any obvious adverse  
684 effects. Animals weighed 25-35 g.

685  
686 Genotypes were determined by a service using real-time PCR with specific probes designed  
687 for each gene (Transnetyx, Cordova, TN). C57BL/6 J mice from the University of Leeds with  
688 *PIEZO1* gene flanked with LoxP sites (*PIEZO1<sup>flox</sup>*) (Li et al., 2014) were used to generate  
689 tamoxifen (TAM)-inducible disruption of the *PIEZO1* gene in the endothelium. *PIEZO1<sup>flox/flox</sup>*  
690 mice were crossed with mice expressing cre recombinase under the Cadherin5 promoter  
691 (Tg(Cdh5-cre/ERT2)1Rha and inbred to obtain *PIEZO1<sup>flox/flox</sup>/Cdh5-cre* mice (Rode et al.,  
692 2017). TAM (Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich) at 20 mg mL<sup>-1</sup>. Mice  
693 were injected intra-peritoneally with 75 mg kg<sup>-1</sup> TAM for 5 consecutive days and studies were  
694 performed 10–14 days later. *PIEZO1<sup>flox/flox</sup>/Cdh5-cre* mice that received TAM injections are  
695 referred to as *PIEZO1<sup>ΔEC</sup>*. *PIEZO1<sup>flox/flox</sup>* littermates (lacking Cdh5-cre) that received TAM  
696 injections were the controls (control genotype). For experiments, mice were males aged 12–  
697 16 weeks. Only male mice were used in order to reduce variability that might arise due to sex  
698 differences and reproductive cycle. TAM injections and genotyping were performed by a  
699 researcher independently from the myographer, such that the genotypes were blind to the  
700 myographer. The different genotypes were studied at random as they became available,  
701 depending on the genotypic spread of each litter.

702  
703 The study sought to address the 3Rs by using in vitro cell-based (non-animal) technical  
704 approaches as much as possible (i.e., Replacement), only using animals once the aim and  
705 design of such studies was informed by the in vitro studies (i.e., Reduction).

## 706 707 **Myography**

708  
709 Mice were anaesthetized with isoflurane (5% induction, 1.5% maintenance) in 95% O<sub>2</sub>  
710 according to Schedule 1 procedure approved by the UK Home Office. The portal vein was  
711 quickly dissected and placed in Krebs solution. The Krebs PV solution consisted of (in mM):  
712 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>(7H<sub>2</sub>O), 25.2 mM  
713 NaHCO<sub>3</sub> and 11.1 mM glucose. By means of a dissecting microscope, adhering perivascular  
714 tissue was carefully removed and the vein was cut into 1-mm segments.

715  
716 Vein segments were mounted onto two thin stainless-steel wires in an isometric myograph  
717 (Multi Wire Myograph System 620 M from Danish Myograph Technology, DMT), for which the  
718 force transducer was calibrated once per month according to the manufacturer's instructions.  
719 The recording chamber was filled with gassed Krebs solution (95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4). The  
720 segments were then stretched to a normalized internal diameter according to the  
721 manufacturer's instructions. The mounted rings were kept in a 5 mL chamber containing Krebs  
722 solution at 37°C and continuously bubbled with a gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4).  
723 After an equilibration period of 60 minutes the contractile function of the vessel was tested by  
724 replacing the Krebs solution by 60 mM K<sup>+</sup> solution. Following washout, the vein was contracted  
725 once with 10 μM phenylephrine (PE) and then Yoda1, KC159 or KC289 was applied with the  
726 concentrations of 0.1 to 10 μM. The nitric oxide synthase inhibitor, N<sup>G</sup>-nitro-L-arginine methyl  
727 ester (L-NAME, 100 μM) was pre-incubated for 20 minutes before the *PIEZO1* modulator.

728  
729 Since the anticipated effect of test compounds was to cause vessel relaxation, phenylephrine  
730 (PE) was applied to induce tension and the maximum value of this tension was defined as 0%  
731 relaxation (no relaxation). The value of tension immediately prior to application of PE was  
732 defined as 100% relaxation. Data are normalised to these values because, otherwise, the



733 intrinsic variability in the absolute amount of tension induced by PE obscured exploration of  
734 the relaxant effect of compounds.

735

## 736 Reagents

737

738 Unless stated otherwise, all commercially available chemicals were purchased from Sigma-  
739 Aldrich. Stocks of chemicals were reconstituted in DMSO and stored at -20 °C unless stated  
740 otherwise. Fura-2-AM was dissolved at 1 mM. Pluronic acid F-127 was stored at 10% (w/v) in  
741 DMSO at room temperature in the dark. Yoda1 (Tocris) was stored at 10 mM. All Yoda1  
742 analogues were synthesised in-house and purified (for more information, see Supporting  
743 Information) and prepared as 10 mM stock solutions. [\(-\)-Englerin A](#) was prepared as a 10 mM  
744 stock solution and stored at -80°C. PE was dissolved in distilled water to make a stock solution  
745 of 100 mM. L-NAME was dissolved in distilled water to make a stock solution of 100 mM.

746

## 747 Data analysis

748

749 OriginPro 2020 (OriginLab) and Prism 9 (GraphPad) were used for all data analysis. For  
750 intracellular  $Ca^{2+}$  ( $Ca^{2+i}$ ) measurements, technical intra-experiment replicates were used to  
751 improve confidence in the data. Analysis of individual experiments was performed to obtain  
752 mean  $\pm$  SEM values. For collated analysis of independent experiments, where normal  
753 distributions were not evidenced by Shapiro-Wilk normality tests, non-parametric distributions  
754 were assumed and therefore median values were used. To compare the agonistic activity of  
755 Yoda1 analogues, background readings taken for the first 25 s prior to compound injection  
756 were subtracted and the resulting median peak values were compared ( $\Delta Ca^{2+i}$ ). Data  
757 subjected to statistical analysis arose from at least 5 independent experiments (n=5). For  
758 comparisons between two sets of paired data, Wilcoxon's signed rank tests were used. For  
759 comparisons of two sets of unpaired data, two-tailed Mann-Whitney signed rank tests were  
760 performed. For multiple comparisons (Figure 1D), Kruskal-Wallis ANOVA was used. Datasets  
761 in Figure 10 and 10 SI 1 satisfied the Shapiro-Wilk normality test, and therefore Repeated-  
762 Measure One-Way ANOVA was applied, followed by a Tukey's post-hoc test. Data for Figure  
763 10 SI 1Aii were not normally distributed, and so a Friedmann test for paired non-parametric  
764 data followed by a Dunn's post-hoc test was applied.  $p < 0.05$  was deemed significant  
765 throughout.  $EC_{50}$  estimates from appropriately saturating concentration-response curves were  
766 fitted with a standard Hill-equation (Hill1 function in OriginPro 2020 software).

767

768 DataControl 384 (Nanon Technologies GmbH), Prism 9 (GraphPad) and OriginPro 2020  
769 (OriginLab) were used for all analysis of automated patch clamp data. For manual and  
770 automated patch-clamp data, N represents the number of cells recorded from successfully in  
771 each experimental condition, sampled from 2 to 6 batches of cells or 384-well chips in  
772 automated patch-clamp. For manual patch-clamp experiments, each cell/patch recording (N)  
773 is considered as an independent experiment because each was made independently at a  
774 separate time, using a separate coverslip of cells and using a separate patch pipette. In some  
775 instances, more than one such independent recording was made from the same batch of  
776 cultured/ transfected cells. These N values are considered as independent. Each cell was  
777 studied alone and from a separate cover slip in the case for manual patch-clamp. To estimate  
778 the  $EC_{50}$  values of the agonists in automated patch-clamp studies, the peak current in the  
779 presence of the agonist was normalized to the peak current in the presence of the reference  
780 solution. Single-point concentration response curves were fitted with a standard Hill equation  
781 to estimate  $EC_{50}$  values.  $p < 0.05$  was deemed significant. For automated patch-clamp  
782 experiments we included some data for  $n < 5$  (Figure 7A, B). We did not apply a statistical test.  
783 We suggest that the results are, nevertheless, useful because of the high number of  
784 independent replicates per experiment (i.e., separate recordings from separate wells in each  
785 recording chip, which are referred to as N). Such replicates are a feature of, and a rationale  
786 for, this automated technology, which is an innovation for the type of research shown here but  
787 already commonly used in the pharmaceutical industry for other ion channel studies to

788 increase throughput and avoid waste from large volumes of independent cell cultures and  
789 minimise cost from expensive specially engineered plates. As a general strategy, our study  
790 used multiple independent technical approaches to address the same or similar questions.  
791 These multiple approaches include  $\text{Ca}^{2+}$  measurement, patch-clamp and vascular contraction  
792 assays. In this way, the overall conclusions do not rely on a single approach such as  
793 automated patch-clamp.

794

795 OriginPro 2020 was used for the analysis of myography data. Myography traces show  
796 readings taken every 0.5 or 1 s, smoothed with the Savitzky-Golay filter set to 70 points. To  
797 compare the agonistic activity of Yoda1 analogues, basal vessel tension prior to compound  
798 injection was subtracted. Maximal tension in the presence of phenylephrine (PE) alone was  
799 used to define 0% relaxation. The tension value prior to PE application was defined as 100%  
800 relaxation. Following the addition of test compounds, tension readings were taken at the last  
801 time point of the treatments and expressed as percentage relaxation.

802

803 Group sizes were not always equal because we did more experiments with analogues that  
804 were most active or newest. We have fully represented what we did rather than excluding  
805 data. While our target was 5, our observation of unexpected variability in effects of some  
806 compounds caused us to increase the number in some cases in an effort to increase the  
807 robustness of our conclusions.

808

809 Statistical analysis was undertaken only for studies where each group size was at least  $n=5$ .  
810 Group size is the number of independent values. Statistical analysis was done using these  
811 independent values (i.e., not treating technical replicates as independent values).

812

813 For multigroup studies with parametric variables, post hoc tests were conducted only if F in  
814 ANOVA (or equivalent) achieved the chosen necessary level of statistical significance and  
815 there was no significant variance inhomogeneity.

816

817 Potential outlier data points were retained in data analysis and presentation.

818

819 The identities of compounds and cell lines were not blinded to investigators. This is a limitation  
820 of the study that may have introduced bias.

821

822 The work complies with BJP's recommendations and requirements on experimental design  
823 and analysis (Curtis et al., 2018). A separate "Compliance with BJP Declaration of  
824 Transparency and Scientific Rigour" document is provided. A separate data transparency  
825 spreadsheet document is provided containing all data.

826

## 827 **Nomenclature of targets and ligands**

828

829 Key protein targets and ligands in this article are hyperlinked to corresponding entries in  
830 <http://www.guidetopharmacology.org>, and are permanently archived in the Concise Guide to  
831 PHARMACOLOGY 2021/22 (Alexander et al., 2021).

832

833

## 834 **RESULTS**

835

### 836 **4-benzoic acid substitution in Yoda1 improves PIEZO agonism**

837

838 We replaced the pyrazine ring of Yoda1 with a 2-, 3- or 4- benzoic acid group (**KC157**, 2-  
839 benzoic acid; **KC158**, 3-benzoic acid; **KC159**, 4-benzoic acid), a 3- or 4- benzamide group  
840 (**KC162**, 3-benzamide; **KC161**, 4-benzamide) or a potassium salt of the 4-benzoic acid  
841 (**KC289**) (Figure 1A). Each analogue was tested for its ability to evoke intracellular calcium  
842 ion ( $\text{Ca}^{2+}$ ) elevation in cells overexpressing human PIEZO1, compared directly with Yoda1 at

843 the same concentration of 10  $\mu\text{M}$  (Figure 1B and 1C). **KC159** and **KC289** evoke robust  
844 responses that are larger than those of Yoda1 (Figure 1B-D). The other analogues fail to evoke  
845 responses (Figure 1B and 1C). Yoda1, **KC159** and **KC289** fail to evoke responses in a similar  
846 cell line that expressed TRPC5  $\text{Ca}^{2+}$ -permeable cation channel in place of PIEZO1, suggesting  
847 that  $\text{Ca}^{2+}$  signals need PIEZO1 (Figure 1 SI 1). The selectivity of 5  $\mu\text{M}$  **KC289** was further  
848 investigated via Eurofins' Hit Profiling Screen PP70 to obtain binding data for 30 proteins  
849 including ion channels and receptors (SI Table 1). There is modest interaction with Adenosine  
850 A2A and Prostanoid EP4 receptors and little or no binding to others (SI Table 1). The data  
851 suggest that the 4-benzoic acid analogue (**KC159**) and its potassium salt (**KC289**) are PIEZO1  
852 agonists.

853

#### 854 **4-benzoic acid substitution improves physico-chemical properties**

855

856 Physico-chemical properties are important in physiological assays and therapeutics. Kinetic  
857 and thermodynamic solubility assays conducted by Malvern PanAnalytical were used to  
858 assess aqueous solubility and other properties. In both, **KC159** and **KC289** showed better  
859 solubility than Yoda1 in phosphate-buffered saline at physiological pH (SI Table 2). Our in-  
860 house solubility data suggested that **KC159** and **KC289** are at least 160 times more soluble  
861 in aqueous buffer than Yoda1 (Figure 1 SI 2). A microsomal stability assay performed by  
862 Malvern PanAnalytical yielded half-lives of 29.6 and 24.6 min, at least 20 times longer than for  
863 Yoda1 (SI Table 2). The fractions of **KC159** and **KC289** bound to plasma proteins are  
864 relatively high (99.4 and 99.35 %) but are lower than that of Yoda1 (SI Table 2). A plasma  
865 stability assay indicated good stability over 2 hours for Yoda1, **KC159** and **KC289** (SI Table  
866 2). The data suggest that key physico-chemical properties are better in **KC159** and **KC289**  
867 than Yoda1.

868

#### 869 **4-benzoic acid substitution improves concentration-response data for human PIEZO1**

870

871 Because of the potential advantages, we investigated **KC159** and **KC289** in more detail. We  
872 first attempted to construct concentration-response curves using cells overexpressing human  
873 PIEZO1, comparing the effects of Yoda1, **KC159** and **KC289** at concentrations up to 30  $\mu\text{M}$   
874 in  $\text{Ca}^{2+}$  assays. Yoda1 causes concentration-dependent increases in intracellular  $\text{Ca}^{2+}$  but a  
875 saturating effect is not observed at the highest concentration and its effects are highly variable  
876 (Figure 2A and 2B, Figure 2 SI 1). **KC159** and **KC289** also do not produce saturating effects  
877 but a saturating inflection point occurs at 10  $\mu\text{M}$  **KC289**, suggesting that a maximum is  
878 approached at approximately 30  $\mu\text{M}$ . The effects of **KC159** and **KC289** are less variable than  
879 those of Yoda1, particularly at high concentrations (Figure 2A and 2B, Figure 2 SI 1). We  
880 performed similar experiments with human umbilical vein endothelial cells (HUVECs), which  
881 are a physiologically relevant cell type that endogenously expresses PIEZO1. Again, the  
882 effects of Yoda1 are the most variable and in this cell system saturating inflection points occur  
883 at 3 - 10  $\mu\text{M}$  for both **KC159** and **KC289** (Figure 2C and 2D, Figure 2 SI 1). Estimated  
884 concentrations for 50% effect ( $\text{EC}_{50\text{S}}$ ) for **KC159** and **KC289** are 2.28  $\mu\text{M}$  and 1.14  $\mu\text{M}$   
885 respectively (Figure 2D). The data suggest that **KC159** and **KC289** provide more consistency  
886 than Yoda1 in concentration-response studies.

887

#### 888 **4-benzoic acid substitution improves efficacy and potency at mouse PIEZO1**

889

890 Previous work suggested that Yoda1 is more potent at mouse compared with human PIEZO1  
891 (Blythe et al., 2019; Evans et al., 2018; Syeda et al., 2015). We therefore studied cells  
892 overexpressing mouse PIEZO1. As with human PIEZO1, **KC159** and **KC289** evoke larger  
893 responses than Yoda1 and **KC157** is inactive (Figure 3A and 3B). **KC158**, **KC161** and **KC162**  
894 exhibit agonist effects in the mouse PIEZO1 overexpression system, although their effects are  
895 slower to develop and smaller than those of Yoda1 (Figure 3A and 3B). Concentration-  
896 response curves were generated in parallel to directly compare Yoda1, **KC159** and **KC289**.

897 Similar to human PIEZO1, the responses to **KC159** and **KC289** are less variable than those  
898 to Yoda1 (Figure 4A and 4B, Figure 4 SI1). In contrast to human PIEZO1, all compounds  
899 produce effects that are saturating or approaching saturation at the highest concentrations  
900 tested (Figure 4A and 4B). Therefore, EC<sub>50</sub>s were compared by fitting Hill equations (Figure  
901 4C). EC<sub>50</sub>s are 0.6 μM (Yoda1), 0.28 μM (**KC159**) and 0.15 μM (**KC289**). The data suggest  
902 that **KC159** and **KC289** are more efficacious and potent than Yoda1 at mouse PIEZO1 with a  
903 rank order of potency of **KC289**>**KC159**>Yoda1. **KC289** is approximately 4 times more potent  
904 than Yoda1.

905

### 906 **Agonism is confirmed by manual patch-clamp**

907

908 An important technique in ion channel studies is manual patch-clamp. Its throughput is limited  
909 and so we focused on a comparison of Yoda1 and **KC159** in cells overexpressing mouse  
910 PIEZO1. Mechanical stimulus due to fluid flow through the recording chamber causes a small  
911 current in PIEZO1-expressing but not control (i.e., null) cells that lacked transfection with  
912 PIEZO1 (Figure 5A-C i cf ii and iii). **KC159** increases current in PIEZO1-expressing but not  
913 null cells, seen as outward current at positive voltages and inward current at negative voltages,  
914 with reversal near 0 mV as expected for PIEZO1 currents (Figure 5B-Ci, iii). The effect is  
915 similar to that of Yoda1 (Figure 5B-Cii). Current reaches a peak and then declines to a plateau  
916 in the continuous presence of **KC159** or Yoda1 (Figure 5B-Cii, iii). The plateau current  
917 disappears when **KC159** or Yoda1 is washed from the chamber (Figure 5B-Cii, iii). The  
918 application of DMSO only (the solvent for Yoda1 and KC159) evoked no change in current in  
919 6 independent recordings from cells overexpressing mouse PIEZO1. The data further suggest  
920 that **KC159** is an agonist of PIEZO1. They suggest that the responses to a single high  
921 concentration are similar to those of Yoda1.

922

### 923 **Improved agonism is confirmed by high throughput patch-clamp**

924

925 For comparative electrophysiological studies, we performed high-throughput automated  
926 patch-clamp on cells overexpressing human or mouse PIEZO1 in comparison to null cells that  
927 lacked the overexpression of PIEZO1. Mechanical stimulation alone (M-Stim only) was caused  
928 by fluid flow from the compound application pipette, which we expected would cause shear  
929 stress on the cells and possibly also a compression force. M-Stim only elicits small transient  
930 inward currents at the holding potential of -80 mV in both human and mouse PIEZO1 cells  
931 (Figure 6A-E). Inclusion of 10 μM **KC159** greatly amplifies the currents (Figure 6A-E). In  
932 mouse PIEZO1 cells, the currents show a sustained component that is 26±8 % (mean ±  
933 standard deviation) of the peak current amplitude, which is not seen in human PIEZO1 cells  
934 (-4±2% of peak current) (Figure 6B, Figure 6 SI 1). There are little or no responses in empty  
935 cells (Figure 6C-E). **KC157** mostly fails to evoke responses (Figure 6D, Figure 6 SI 1).

936

937 We next performed paired comparisons with Yoda1. **KC159** causes more cells to respond  
938 compared with Yoda1, most notably for mouse PIEZO1 (Figure 7A and B). **KC157** is largely  
939 ineffective (Figure 7A and B). Yoda1 responses are highly variable and concentration-  
940 dependence is lacking (Figure 7C and 7D). **KC159** elicits concentration-dependent increases  
941 in current in human and mouse PIEZO1 cells (Figure 7E and 7F). Maximum responses are  
942 not certain and so EC<sub>50</sub>s are only estimates at 1.67 and 1.54 μM for human and mouse  
943 respectively. The data suggest that **KC159** is more reliable and effective as a PIEZO1 agonist  
944 than Yoda1 and more effective at mouse compared with human PIEZO1.

945

### 946 **KC159 and KC289 relax the mouse portal vein**

947

948 Previous studies have suggested that PIEZO1 channels signal to endothelial nitric oxide  
949 synthase (NOS3) to generate nitric oxide (NO) and thereby cause endothelium-dependent  
950 blood vessel relaxation (Beech & Kalli, 2019). We used this effect to investigate **KC159** and  
951 **KC289** in a physiological assay. The portal vein was studied because of evidence that PIEZO1

952 is important in the hepatic circulation (Caolo et al., 2020; Hilscher et al., 2019; Li et al., 2014;  
 953 Rode et al., 2017). Vessel wall tension was recorded ex vivo using wire myography and  
 954 smooth muscle contraction was evoked by the  $\alpha_1$ -adrenoceptor agonist phenylephrine (PE)  
 955 so that endothelium-dependent relaxation could be observed (e.g., Figure 8A). Yoda1, **KC159**  
 956 and **KC289** cause concentration-dependent relaxation (Figure 8A). **KC159** and **KC289**  
 957 concentration-response curves reach saturation, thus enabling calculation of EC<sub>50</sub>s of 1.14  
 958  $\mu$ M and 1.20  $\mu$ M respectively (Figure 8A). Yoda1 effects are more variable and no saturation  
 959 occurs (Figure 8A). The data suggest that **KC159** and **KC289** are suitable for use in a  
 960 physiological assay and are an improvement on Yoda1.

961

#### 962 **KC289-evoked relaxation is NO dependent**

963

964 To investigate if **KC289** causes relaxation via NOS3 we incubated portal vein with N<sup>G</sup>-Nitro- L-  
 965 Arginine Methyl Ester (L-NAME), which is a substrate inhibitor of NOS3. L-NAME abolishes  
 966 the relaxant effect of **KC289** (Figure 8B). Effects of L-NAME in the absence of **KC289** are  
 967 shown in Figure 8 SI 1. We had technical difficulty removing endothelium from portal vein  
 968 without damaging the smooth muscle layer and so we could not test the endothelium-  
 969 dependence of the KC289 response. The data suggest that **KC289** acts to stimulate NOS3  
 970 and NO production, as expected for a PIEZO1 agonist.

971

#### 972 **KC289-evoked relaxation is endothelial PIEZO1 dependent**

973

974 To determine the role of endothelial PIEZO1 in **KC289** responses we compared portal vein of  
 975 control mice with that of matched mice in which endothelial PIEZO1 expression was  
 976 conditionally deleted at the adult stage (PIEZO1<sup>ΔEC</sup>), as previously described (Caolo et al.,  
 977 2020; Rode et al., 2017). **KC289**-evoked relaxation is inhibited by endothelial PIEZO1 deletion  
 978 and a contractile response often becomes evident at 10  $\mu$ M (Figure 8C). Variable spontaneous  
 979 oscillatory contractions occurred in these experiments, regardless of PIEZO1 deletion. This  
 980 may explain the wide variability in the overall data and response to KC289. For transparency,  
 981 all original traces are provided (Figure 8 SI 2). The mechanism of the contractile effect of  
 982 KC289 was not investigated but it may relate to PIEZO1 expressed in smooth muscle cells or  
 983 another cell type of the vessel.

984

#### 985 **KC289-evoked relaxation is inhibited by Dooku1**

986

987 Dooku1 is an analogue of Yoda1 that antagonises the action of Yoda1 (Evans et al., 2018).  
 988 Pre-incubation with 10  $\mu$ M [Dooku1](#) reduces relaxations evoked by **KC159** and **KC289** (Figure  
 989 8 SI 3). Dooku1 also consistently inhibits the PE response (Figure 8 SI 3), as previously  
 990 reported in studies of mouse aorta (Evans et al., 2018). The data suggest that **KC159** and  
 991 **KC289** cause relaxation via a site that is the same as the one mediating effects of Yoda1.

992

#### 993 **Effects of 3- and 4-benzamide and 3-benzoic acid analogues**

994

995 In view of the effect of Dooku1 described above, we tested if there are inhibitory actions of the  
 996 new Yoda1 analogues by pre-treating cells at 10  $\mu$ M for 30 minutes prior to adding 2  $\mu$ M Yoda1  
 997 (Figure 9 and Figure 9 SI 1). All except **KC157** and **KC161** inhibit the action of Yoda1 on  
 998 human PIEZO1 (Figure 9A, Figure 9 SI 1). Only **KC157** fails to inhibit the action of Yoda1 on  
 999 mouse PIEZO1 (Figure 9B, Figure 9 SI 1). **KC159** and **KC289** are agonists and so they may  
 1000 inhibit the effect of Yoda1 simply by pre-activating the channels. **KC158**, **KC161** and **KC162**  
 1001 are also agonists of mouse PIEZO1 (Figure 3). **KC158** and **KC162** appear to inhibit the Yoda1  
 1002 response without pre-activating human PIEZO1 (Figure 9A, Figure 9 SI 1, Figure 1B and 1C)  
 1003 but we noticed an elevated baseline Ca<sup>2+</sup> signal for **KC161** and **KC162** in the pre-incubation  
 1004 protocol, suggesting that these compounds are actually slowly acting mild agonists (Figure 9  
 1005 SI 2). The data suggest that 3-benzamide (**KC162**), 4-benzamide (**KC161**) and 3-benzoic acid

1006 (**KC158**) analogues are mild, slowly acting, agonists that may also inhibit the effect of Yoda1,  
1007 as if they are partial agonists. Only the 2-benzoic acid analogue (**KC157**) lacks agonist or  
1008 antagonist properties.

1009

## 1010 Relationship to PIEZO2

1011

1012 Yoda1 was discovered in a chemical screen of HEK 293 cells overexpressing mouse PIEZO1  
1013 and mouse PIEZO2 but Yoda1 was subsequently found not to activate mouse PIEZO2  
1014 overexpressed alone (Syeda et al., 2015). To investigate **KC159** and **KC289** in relation to  
1015 PIEZO2 we compared HEK 293 cells overexpressing mouse PIEZO1, mouse PIEZO2 or  
1016 neither (“non-transfected cells”) (Figure 10, Figure 10 SI 1, Figure 10 SI 2). Ca<sup>2+</sup> elevations  
1017 evoked by **KC159** or **KC289** in HEK 293 cells overexpressing mouse PIEZO2 are not different  
1018 from background signals in non-transfected cells and contrast with the larger responses when  
1019 mouse PIEZO1 is overexpressed (Figure 10A). The background signals may have been due  
1020 to endogenous human PIEZO1 channels of these HEK 293 cells (Dubin et al., 2017). To  
1021 investigate the relevance to native PIEZO2, we studied HeLa cells because they natively  
1022 express PIEZO2 (TheHumanProteinAtlas, 2022). Yoda1, **KC159** and **KC289** evoked Ca<sup>2+</sup>  
1023 elevations in HeLa cells (Figure 10B). To determine the relevance to PIEZO2, we used RNA  
1024 interference to deplete its expression (Figure 10 SI 2). There is PIEZO1 in HeLa cells (Geng  
1025 et al., 2020) and so we depleted its expression too (Figure 10 SI 2). PIEZO2 depletion partially  
1026 suppresses responses to **KC159**, **KC289** and Yoda1, but with greater effect on responses to  
1027 **KC159** and **KC289** (Figure 10B). Responses to Yoda1, **KC159** and **KC289** are all partly  
1028 suppressed by PIEZO1 depletion (Figure 10B). Depletion of PIEZO1 and PIEZO2 further  
1029 suppresses the responses (Figure 10B). The data suggest that like Yoda1, **KC159** and **KC289**  
1030 are not agonists of overexpressed mouse PIEZO2 but may agonise native human PIEZO2, at  
1031 least when it is natively coexpressed with PIEZO1.

1032

## 1033 DISCUSSION AND CONCLUSIONS

1034

1035 In this study, we modified Yoda1 with the aim of improving capability for PIEZO1 modulation  
1036 in physiological settings. Our data suggest that modification of Yoda1’s pyrazine moiety is a  
1037 route to better agonism and physico-chemical properties. Specifically, benzoic acid  
1038 substitution with carboxylate is beneficial, with the 4-position being critical. Substitution at the  
1039 2-position generates an inactive compound while the 3-position leads to inferior agonism. The  
1040 2-position analogue can serve as a negative control comparator compound. When there is 4-  
1041 benzamide instead of 4-benzoic acid, there is weak or no agonism. Since 4-benzoic acid and  
1042 4-benzamide occupy a similar molecular volume, it may be the neutral charge of 4-benzamide  
1043 at physiological pH that impairs agonism. The potassium salt of the 4-benzoic acid analogue  
1044 (**KC289**) is superior to the analogue itself (**KC159**). Its improved aqueous solubility is likely to  
1045 be a factor in this. **KC159** and **KC289** are nevertheless both advances on Yoda1 in PIEZO1  
1046 agonism.

1047

1048 An important next step would be determination of a binding site or binding sites for Yoda1,  
1049 **KC159** and **KC289**, particularly if such a site or sites could be characterised at atomic  
1050 resolution. There could then be better rational design and understanding of PIEZO1  
1051 modulators. Surface plasmon resonance studies suggest binding of Yoda1 between residues  
1052 1 and 2190 of mouse PIEZO1 (in total it comprises 2547 residues) (Wang et al., 2018).  
1053 Computer simulations have suggested a pocket away from the C-terminal region of the central  
1054 ion pore and mutagenesis data point to importance of residues such as alanine at position  
1055 1718 (Botello-Smith et al., 2019). **KC289** could help future structural determination studies by  
1056 enabling incubation of PIEZO1 with a higher concentration of compound and perhaps  
1057 conferring stronger binding. Our data suggest greater effect and potency of **KC159** and **KC289**  
1058 at mouse compared with human PIEZO1, so mouse PIEZO1 may be the most promising route  
1059 to binding site determination. Moreover, comparisons of mouse and human sequences could  
1060 indicate what is optimal for binding and efficacy. However, we suggest caution in such

1061 inferences because the expression of mouse PIEZO1 may have been better than human  
1062 PIEZO1 in our studies, possibly creating an impression of greater efficacy and sensitivity  
1063 through greater receptor reserve.

1064  
1065 Various factors can explain differences in apparent efficacy and potency. In addition to  
1066 receptor reserve, aqueous solubility could be important. Our estimates suggest aqueous  
1067 solubility of Yoda1 only up to the low  $\mu\text{M}$  range. We used it up to 30  $\mu\text{M}$  and other groups  
1068 report use of higher concentrations. Inclusion of a solvent such as dimethylsulphoxide in the  
1069 buffer (as we did) may aid solubility but partial precipitation could still occur and potentially  
1070 explain variability in Yoda1 effects. **KC159** and **KC289** have better aqueous solubility and so  
1071 this may be why they show less variability in effect. Another factor explaining differences in  
1072 apparent efficacy and potency could be complexity in the transduction pathway between  
1073 channel activation and effect. When tested against over-expressed mouse PIEZO1 in  $\text{Ca}^{2+}$   
1074 assays, the  $\text{EC}_{50}$ s for **KC289** and **KC159** were 0.15 and 0.28  $\mu\text{M}$ , whereas in mouse portal  
1075 vein relaxation, the  $\text{EC}_{50}$ s were 1.2 and 1.14  $\mu\text{M}$ . The portal vein effects occur via PIEZO1, so  
1076 a high concentration of compound may be required to overcome non-linearity in the signalling.

1077  
1078 The  $\text{EC}_{50}$  we report for Yoda1 is lower (i.e., 'better') than that reported in some other studies  
1079 (Lacroix, Botello-Smith & Luo, 2018; Syeda et al., 2015). There could be several reasons,  
1080 including different channel expression levels and experiment conditions, but we note that we  
1081 found considerable variability in the effect of Yoda1, such that we also sometimes observed  
1082 high  $\text{EC}_{50}$ s in individual experiments. We suggest that such differences may arise due to the  
1083 limited aqueous solubility of Yoda1 at concentrations relevant to  $\text{EC}_{50}$  determination, causing  
1084 Yoda1 to variably precipitate out of solution. In support of this hypothesis, our new more  
1085 soluble analogues had less variable effects.

1086  
1087 Manual patch-clamp enables comparisons of the efficacy and potency of compounds but its  
1088 utility is restricted by the low throughput of recording from one cell at a time. Therefore, we  
1089 explored automated patch-clamp technology, which has the potential to increase throughput  
1090 greatly. The arising data support our findings from 96-well  $\text{Ca}^{2+}$  measurements by suggesting  
1091 that **KC159** has advantages over Yoda1 and that **KC157** (the 2-position analogue) is inactive.  
1092 Differences between mouse and human PIEZO1 are again evident. In this regard, we  
1093 observed an intriguing sustained response of mouse but not human PIEZO1. In these  
1094 experiments, there was a rapid small-volume application of compound followed by rapid  
1095 retraction of double the volume. Reversal of the **KC159** effect occurs after washout in manual  
1096 patch recordings, but these recordings were on a slower timescale. We speculate that the  
1097 rapid retraction in the automated system insufficiently removes **KC159** in the short timeframe  
1098 of the recordings, leaving residual compound at low concentration. This low concentration may  
1099 cause the sustained current evident in mouse but not human PIEZO1 because **KC159** has  
1100 apparent great efficacy and potency at mouse PIEZO1. This hypothesis is supported by failure  
1101 to observe sustained current when applying Yoda1, which is less effective than **KC159**.

1102  
1103 A limitation of our efficacy comparison of Figure 3 is that the effects of the various analogues  
1104 may not have been compared at equal saturation for all compounds. This is challenging to  
1105 overcome because of the limited aqueous solubility of this series of compounds, despite our  
1106 improvements. Therefore, we cannot exclude that some of the compounds would be more  
1107 effective if tested at higher concentrations, if they were to be retained in aqueous solution.

1108  
1109 Yoda1, **KC159** and **KC289** are tool compounds for laboratory and potentially in vivo animal  
1110 studies aimed at determining functions of PIEZO1 and informing potential drug discovery  
1111 projects. We do not yet know if they have therapeutic relevance or suitability (e.g., distribution  
1112 and safety features) for use in humans. Nevertheless, there is potential clinical value. A  
1113 disease area to consider is malaria because gain-of-function mutation in PIEZO1 is associated  
1114 with protection against malaria (Ma et al., 2018) and so this effect might be mimicked by a



1115 PIEZO1 agonist acting on wild-type PIEZO1. Another area to consider is lymphoedema  
1116 because loss-of-function mutations in PIEZO1 are associated with generalized lymphatic  
1117 dysplasia (Fotiou et al., 2015) and so PIEZO1 agonism might be beneficial if partially functional  
1118 channels are still available.

1119  
1120 Despite Yoda1's value and apparent selectivity, its use at relatively high concentrations could  
1121 make it especially vulnerable to as-yet unknown off-target effects. Moreover, **KC159** and  
1122 **KC289** are analogues of Yoda1 but they are not the same chemicals and may not have the  
1123 same selectivity profile. We provide evidence for their selectivity: there is small or no activation  
1124 of Ca<sup>2+</sup> or electrophysiological signals in control (null) HEK 293 cells without PIEZO1  
1125 overexpression; they do not activate mouse PIEZO2 or human TRPC5 overexpressed in HEK  
1126 293 cells; genetic disruption of PIEZO1 inhibits vasorelaxation; and a commercial selectivity  
1127 screen suggests little or no binding to a range of other proteins other than potential modest  
1128 effects on A2A and EP4 receptors (SI Table 1). We did not further investigate the relevance  
1129 of the latter receptors but the strong inhibitory effect of genetic disruption of PIEZO1 on the  
1130 effect of KC289 suggests that PIEZO1-independent effects do not contribute, or are minor  
1131 contributors, in portal vein.

1132  
1133 Although **KC159** and **KC289** did not activate overexpressed mouse PIEZO2 channels,  
1134 knockdown of native human PIEZO2 in HeLa cells partially inhibited responses to **KC159** and  
1135 **KC289**. These data suggest that the new Yoda1 analogues (such as KC289) may have the  
1136 ability to activate PIEZO2 channels in a species- or context- dependent manner. This warrants  
1137 further investigation using electrophysiological approaches and overexpressed human  
1138 PIEZO2 because there are currently limited possibilities for chemically modulating PIEZO2  
1139 channels. Although PIEZO2 siRNA (siPIEZO2) has no significant effect on PIEZO1 mRNA  
1140 abundance, there is a visual impression of a potential small off-target effect on PIEZO1 (Figure  
1141 10 SI 1B). This does not detract from the observation that siPIEZO2 is more effective against  
1142 responses evoked by **KC159** and **KC289** than Yoda1 (Figure 10B). Therefore, we cautiously  
1143 suggest that some Yoda1 analogues may cross over to PIEZO2 and thereby provide a route  
1144 to PIEZO2 agonism.

1145  
1146 In conclusion, this study supports the idea that Yoda1 provides a template for developing a  
1147 series of useful PIEZO1 modulators. The structure-activity requirements are quite strict and  
1148 seem to be relatively inflexible at the 2,6-dichlorophenyl moiety (Evans et al., 2018) but our  
1149 data suggest better opportunity at the pyrazine moiety. Here we show value of 4-benzoic acid  
1150 substitution and improvement on Yoda1 for PIEZO1 agonism in terms of efficacy, potency and  
1151 physico-chemical properties (e.g., aqueous solubility). We suggest calling the potassium salt  
1152 of this analogue (i.e., KC289) Yoda2 and propose its potential value as a tool compound in  
1153 physiological assays and for facilitating efforts to identify a binding site. In addition, we suggest  
1154 consideration of Yoda2 or a variant thereof in disease conditions such malaria and  
1155 lymphoedema.

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Figure 1

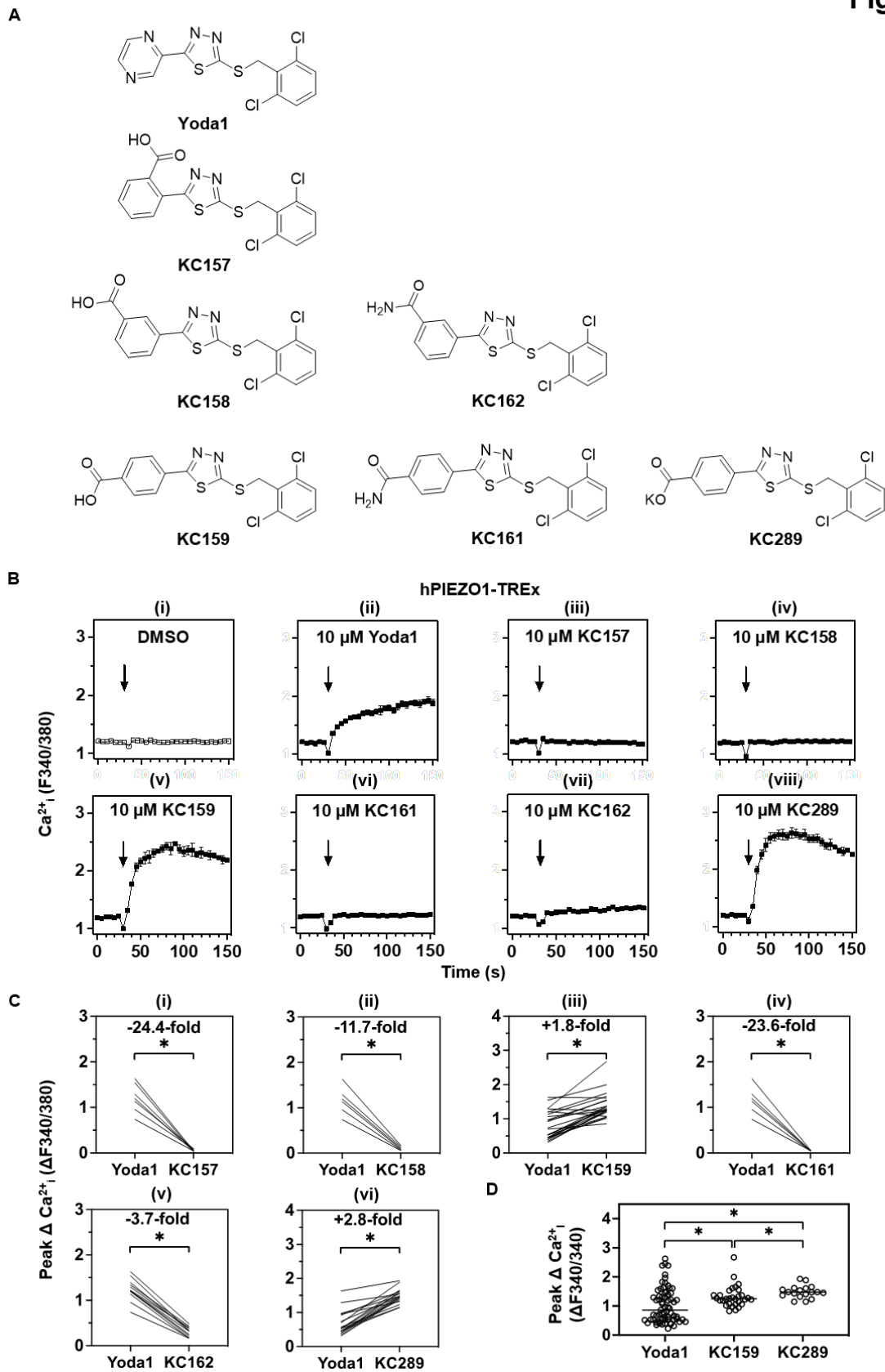


Figure 1. Yoda1 analogues and their effects on human PIEZO1

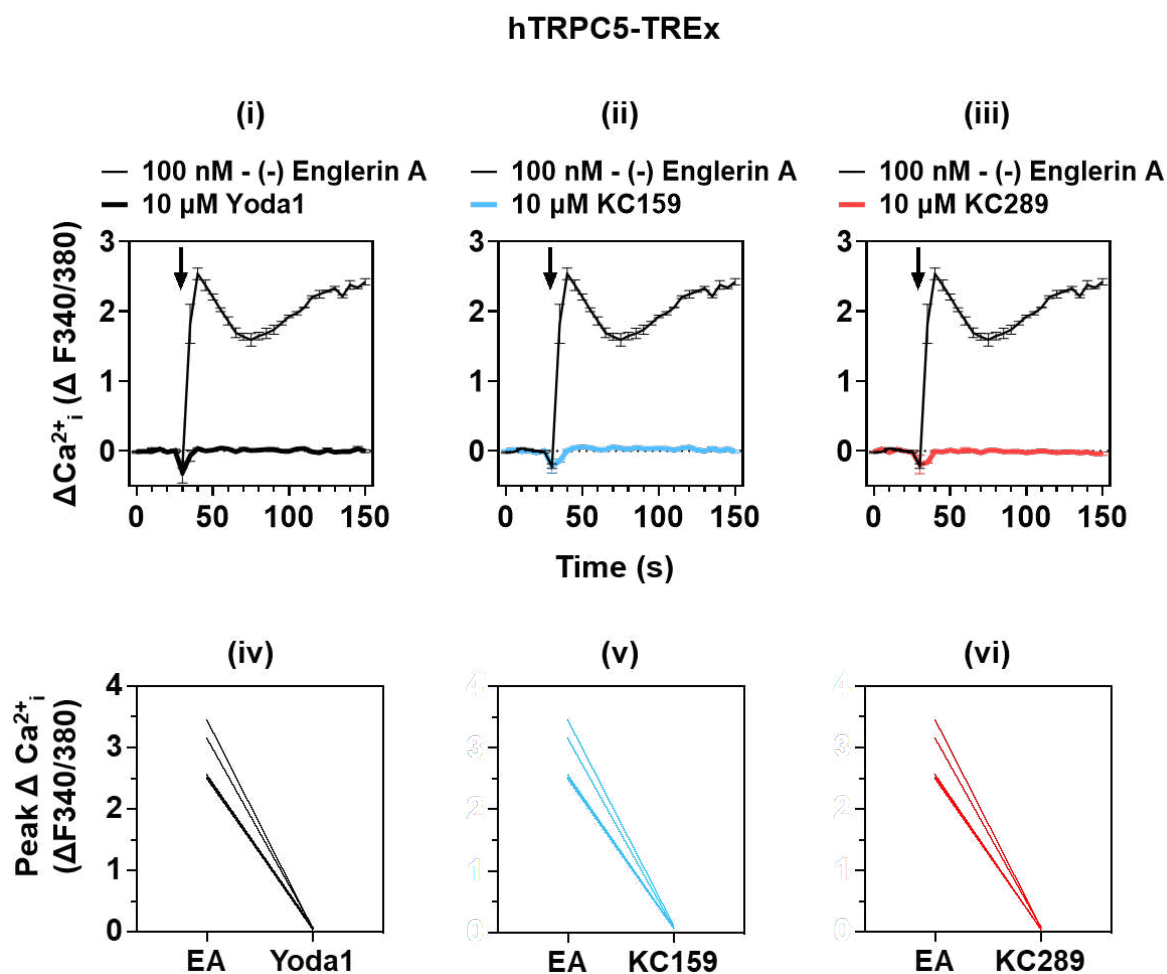
1298 (A) Structures of Yoda1 and analogues in which the pyrazine moiety has been altered. **KC157**,  
1299 2-benzoic acid; **KC158**, 3-benzoic acid; **KC159**, 4-benzoic acid; **KC162**, 3-benzamide;  
1300 **KC161**, 4-benzamide; **KC289**, 4-benzoic acid (potassium salt).

1301 (B) Intracellular  $\text{Ca}^{2+}$  measurements from a single experiment ( $n=1$ ) in which hPIEZO1-TREx  
1302 cells were acutely exposed to; (i) DMSO vehicle, or 10  $\mu\text{M}$ ; (ii) Yoda1, (iii) **KC157**, (iv) **KC158**,  
1303 (v) **KC159**, (vi) **KC161**, (vii) **KC162**, (viii) **KC289**. Arrows indicate the time at which the  
1304 indicated compound was added to the cells, following a 30 s background read. Mean  $\pm$  SEM  
1305 values from 3 technical replicates are shown.

1306 (C) Paired, background-subtracted (change above background,  $\Delta$ ), peak intracellular  $\text{Ca}^{2+}$   
1307 measurement comparisons of hPIEZO1-TREx cells treated with 10  $\mu\text{M}$  indicated compound.  
1308 Each plot shows mean peak values from independent experiments (**KC157**,  $n=7$ ; **KC158**,  $n=6$ ;  
1309 **KC159**,  $n=25$ ; **KC161**,  $n=6$ ; **KC162**,  $n=12$ , **KC289**,  $n=18$ ). Paired sample Wilcoxon signed  
1310 rank test results comparing the median values of the collated experiments are shown as: \*  $p$   
1311  $< 0.05$ . Median fold-differences compared to Yoda1 are also indicated.

1312 (D) Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$  measurements for hPIEZO1-TREx cells  
1313 treated with 10  $\mu\text{M}$  indicated compound. Each symbol shows a mean peak value from an  
1314 independent experiment (Yoda1,  $n=67$ ; **KC159**,  $n=30$ ; **KC289**,  $n=18$ ). Kruskal-Wallis ANOVA  
1315 results comparing the median values of each treatment pair are shown as: \*  $p < 0.05$ .  
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Figure 1 SI 1



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#### Figure 1 SI 1 Lack of agonism at TRPC5 channels

(upper panel) Background-subtracted ( $\Delta$ ) intracellular  $Ca^{2+}$  measurements from a single experiment in which hTRPC5-TREx cells were acutely exposed to 10  $\mu$ M (i) Yoda1, (ii) **KC159**, (iii) **KC289**. Arrows indicate the time at which the indicated compound was added to the cells, following a 30 s background read. The positive control stimulus (100 nM (-) Englerin A is shown for comparison. Mean  $\pm$  SEM values from 3 technical replicates are shown.

(lower panel) Paired, background-subtracted ( $\Delta$ ) peak intracellular  $Ca^{2+}$  measurement comparisons of hTRPC5-TREx cells treated with 100 nM (-)–Englerin A (EA) versus 10  $\mu$ M (iv) Yoda1, (v) **KC159**, and (vi) **KC289**. Each plot shows mean peak values from independent experiments ( $n=5$  for all compounds).

SI Table 1

Assay Name	Gene / Subfamily Name	Binding Assay Type	Catalogue #	Organism	% Inhibition
Acetylcholine M2 (Muscarinic)	CHRM2	GPCR (Antagonist Radioligand)	252710	Human	10
Acetylcholine M3 (Muscarinic)	CHRM3	GPCR (Antagonist Radioligand)	252810	Human	1
Adenosine A1	ADORA1	GPCR (Antagonist Radioligand)	200510	Human	-4
<b>Adenosine A2A</b>	<b>ADORA2A</b>	<b>GPCR (Agonist Radioligand)</b>	<b>200610</b>	<b>Human</b>	<b>40</b>
Adrenoceptor alpha1A	ADRA1A	GPCR (Antagonist Radioligand)	203100	Rat	3
Adrenoceptor alpha1B	ADRA1B	GPCR (Antagonist Radioligand)	203200	Rat	6
Adrenoceptor alpha2A	ADRA2A	GPCR (Antagonist Radioligand)	203630	Human	14
Adrenoceptor beta1	ADRB1	GPCR (Antagonist Radioligand)	204010	Human	11
Adrenoceptor beta2	ADRB2	GPCR (Antagonist Radioligand)	204110	Human	-2
Cav1.2 (L-type)	CACNA1C	Calcium Ion Channel (Dihydropyridine Site)	214600	Rat	20
CB1 Human Cannabinoid	CNR	GPCR (Antagonist Radioligand)	217050	Human	5
Dopamine D1	DRD1	GPCR (Antagonist Radioligand)	219500	Human	9
Dopamine D2S	DRD2	GPCR (Antagonist Radioligand)	219700	Human	1
GABA A	GABAA	(Non-Selective) Ion Channel [3H] Muscimol (Agonist Radioligand)	226500	Rat	12
GABA A	GABAA	(Non-Selective) Ion Channel [3H] Flunitrazepam (Agonist Radioligand)	226600	Rat	1
Glutamate	Ionotropic	(Non-Selective) Ion Channel [3H] TCP	233000	Rat	0
Histamine H1	HRH1	GPCR (Antagonist Radioligand)	239610	Human	-7
Imidazoline I2	Imidazoline	Central (Antagonist Radioligand)	241000	Rat	13
mu Opioid	OPRM1	GPCR (Antagonist Radioligand)	260410	Human	-10
nAChR (alpha1)	CHRNA1	Ion Channel, Bungarotoxin (Antagonist Radioligand)	258700	Human	-3
nAChR Nicotinic Acetylcholine	-	Ion Channel Binding, Epibatidine (Antagonist Radioligand binding)	258590	Human	-5
Norepinephrine Transporter	SLC6A2	Transporter (Antagonist Radioligand)	204410	Human	-9
PDE	Phosphodiesterase	(Non-Selective) [3H] Rolipram (Antagonist Radioligand)	270000	Rat	5
Phorbol Ester	-	Phorbol Ester	264500	Mouse	9
Potassium Channel (hERG)	KCNH2	Potassium Ion Channel [3H] Astemizole (Antagonist Radioligand)	265900	Human	3
Potassium Channel (KATP)	KCNJ11	Potassium Ion Channel (Antagonist Radioligand)	265600	Hamster	0
<b>Prostanoid EP4</b>	<b>PTGER4</b>	<b>GPCR (Agonist Radioligand)</b>	<b>268420</b>	<b>Human</b>	<b>63</b>
Serotonin (5-HT2B)	HTR2B	GPCR [3H]LSD (Agonist Radioligand)	271700	Human	-1
Sigma 1	SIGMAR1	Non-Selective (Antagonist Radioligand)	278110	Human	2
Sodium Ion Channel	Sodium Ion Channel	(Non-Selective) Sodium Ion Channel [3H] Batrachotoxinin (Site 2)	279510	Rat	-3

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SI Table 1. Binding results for 30 targets.

1332 Data are for KC289 at 5  $\mu$ M. 'Phorbol ester' is a binding assay that targets protein kinase Cs  
1333 and potentially other proteins. Data were produced by Eurofins Scientific  
1334 (<https://www.eurofins.co.uk/>).



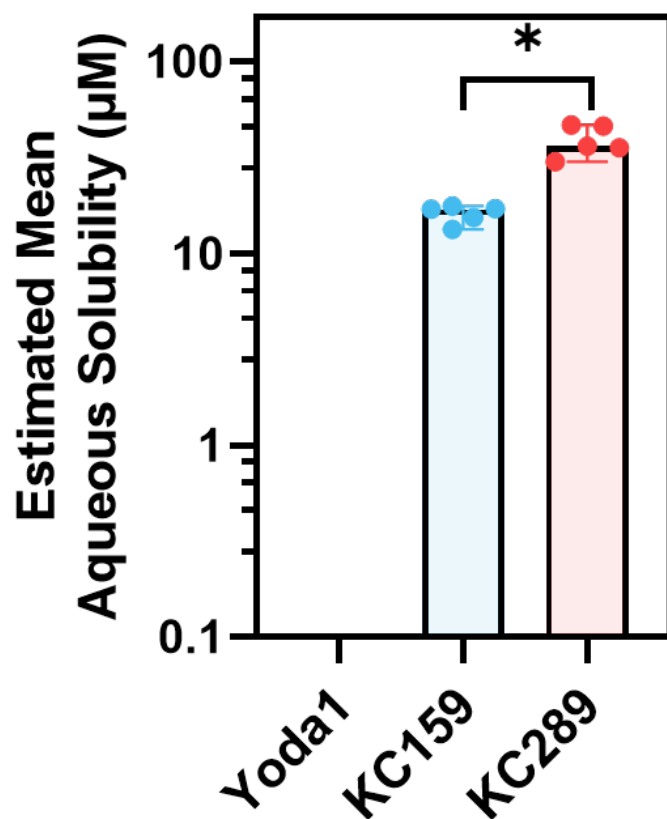
SI Table 2

Assay	Repeat	Yoda1 Values	Yoda1 Mean	KC159 Values	KC159 Mean	KC289 Values	KC289 Mean
Kinetic solubility ( $\mu\text{M}$ ) (0.1 M phosphate buffer, pH 7.4)	1	0.2	<b>2</b>	76.6	<b>39.1</b>	17.8	<b>14</b>
	2	3.8		1.5		10.2	
Thermodynamic solubility ( $\mu\text{M}$ ) (0.1 M phosphate buffer, pH 7.4)	1	0.44	<b>0.37</b>	11.9	<b>9.8</b>	46	<b>27.5</b>
	2	0.3		7.7		8.9	
Mouse Microsomal $t_{1/2}$ (min)	1	1.0	<b>1.1</b>	37.2	<b>29.6</b>	31.8	<b>24.6</b>
	2	1.2		21.9		17.3	
Mouse Plasma Protein Binding (% Fraction Unbound)	1	<0.1	<b>&lt;0.1</b>	0.4	<b>0.6</b>	0.5	<b>0.65</b>
	2	0.1		0.8		0.8	
Mouse plasma stability (% compound remaining after 2 hours)	1	nd	<b>117</b>	69	<b>85.7 ± 14.6 (S.D.)</b>	125	<b>111 ± 13.5 (S.D.)</b>
	2	119		96		110	
	3	115		92		98	

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**SI Table 2. Physico-chemical properties of Yoda1, KC159 and KC289.**  
Data were produced by Malvern PanAnalytical (<https://www.malvernpanalytical.com/>).

Figure 1 SI 2

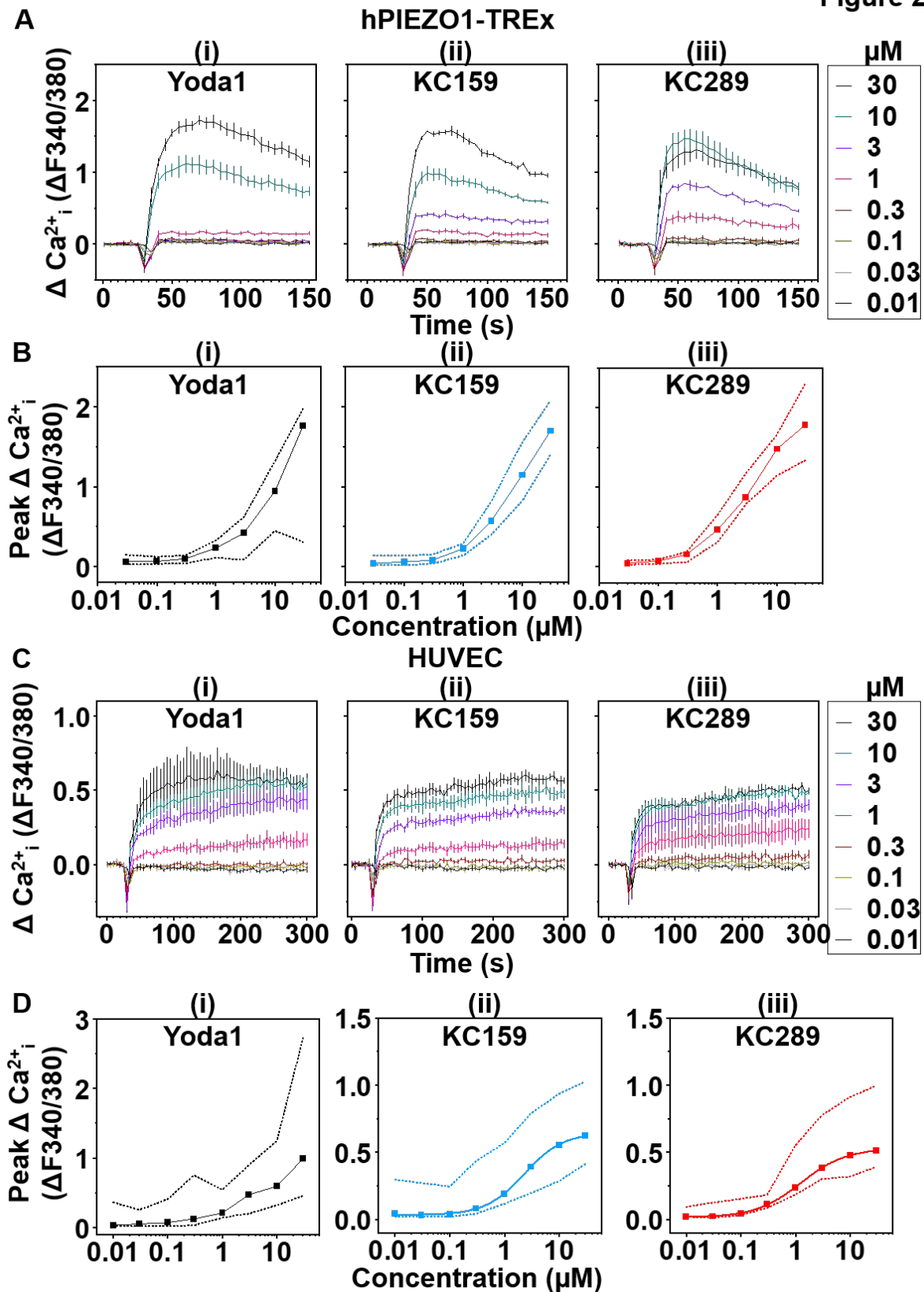


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**Figure 1 SI 2. In-house solubility comparisons for Yoda1, KC159 and KC289.**

Estimated solubility of Yoda1, **KC159**, and **KC289** in universal aqueous buffer. Median values with data range from 5 independent experiments are shown (n=5). The lower limit of detection for the assay was 0.1 µM. Data for Yoda1 were below this limit, suggesting that Yoda1 was poorly soluble in these conditions. Results of a two-tailed Mann-Whitney rank comparison of the median values of the collated experiments are shown as: \* p < 0.05.

Figure 2



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1349 **Figure 2. 4-benzoic acid improves concentration-response data for human PIEZO1**1350 (A) Background-subtracted ( $\Delta$ ) intracellular  $Ca^{2+}$  measurements from a single experiment

1351 (n=1) in which hPIEZO1-TREx cells were exposed to the indicated concentrations of; (i)

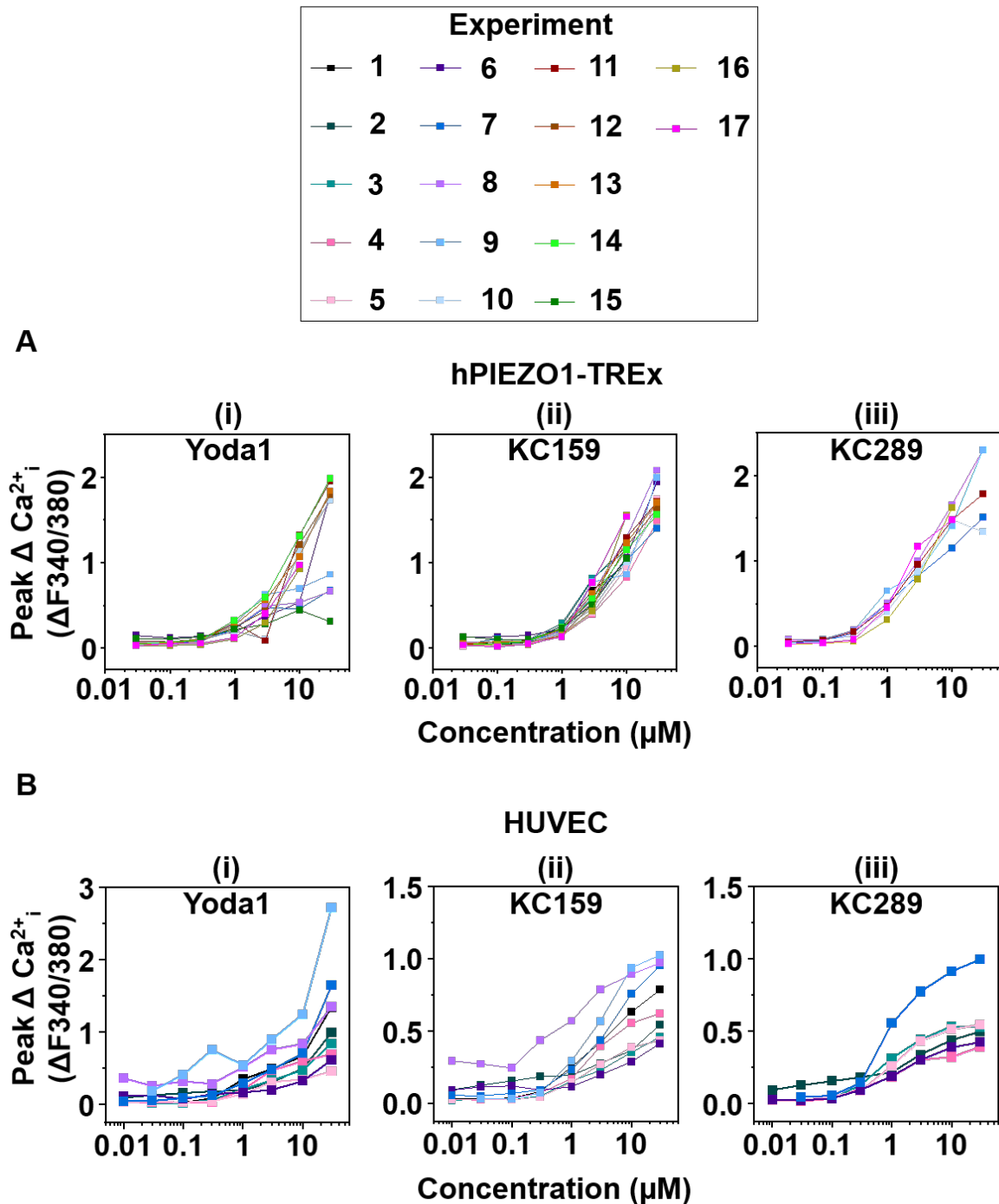
1352 Yoda1, (ii) **KC159**, (iii) **KC289**. Mean  $\pm$  SEM values from 3 technical replicates are shown.

1352 (B) Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$  measurements of hPIEZO1-TREx cells  
1353 treated with the indicated concentration range of; (i) Yoda1, (ii) **KC159**, (iii) **KC289**. Median  
1354 peak values and the data range from collated experiments are shown (Yoda1, n=12; **KC159**,  
1355 n=17; **KC289**, n=7).

1356 (C) Background-subtracted ( $\Delta$ ) intracellular  $\text{Ca}^{2+}$  measurements from a single dose-response  
1357 experiment (n=1) in which HUVEC were exposed to the indicated concentrations of; (i) Yoda1,  
1358 (ii) **KC159**, (iii) **KC289**. Mean  $\pm$  SEM values from 3 technical replicates are shown.

1359 (D) Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$  measurements of HUVEC treated with  
1360 the indicated concentration range of; (i) Yoda1, (ii) **KC159**, (iii) **KC289**. Median peak values  
1361 and the data range from collated experiments are shown (Yoda1, n=9; **KC159**, n=9, **KC289**,  
1362 n=6). Fitted curves constructed using the Hill equation are shown in (ii) and (iii).  
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Figure 2 SI 1



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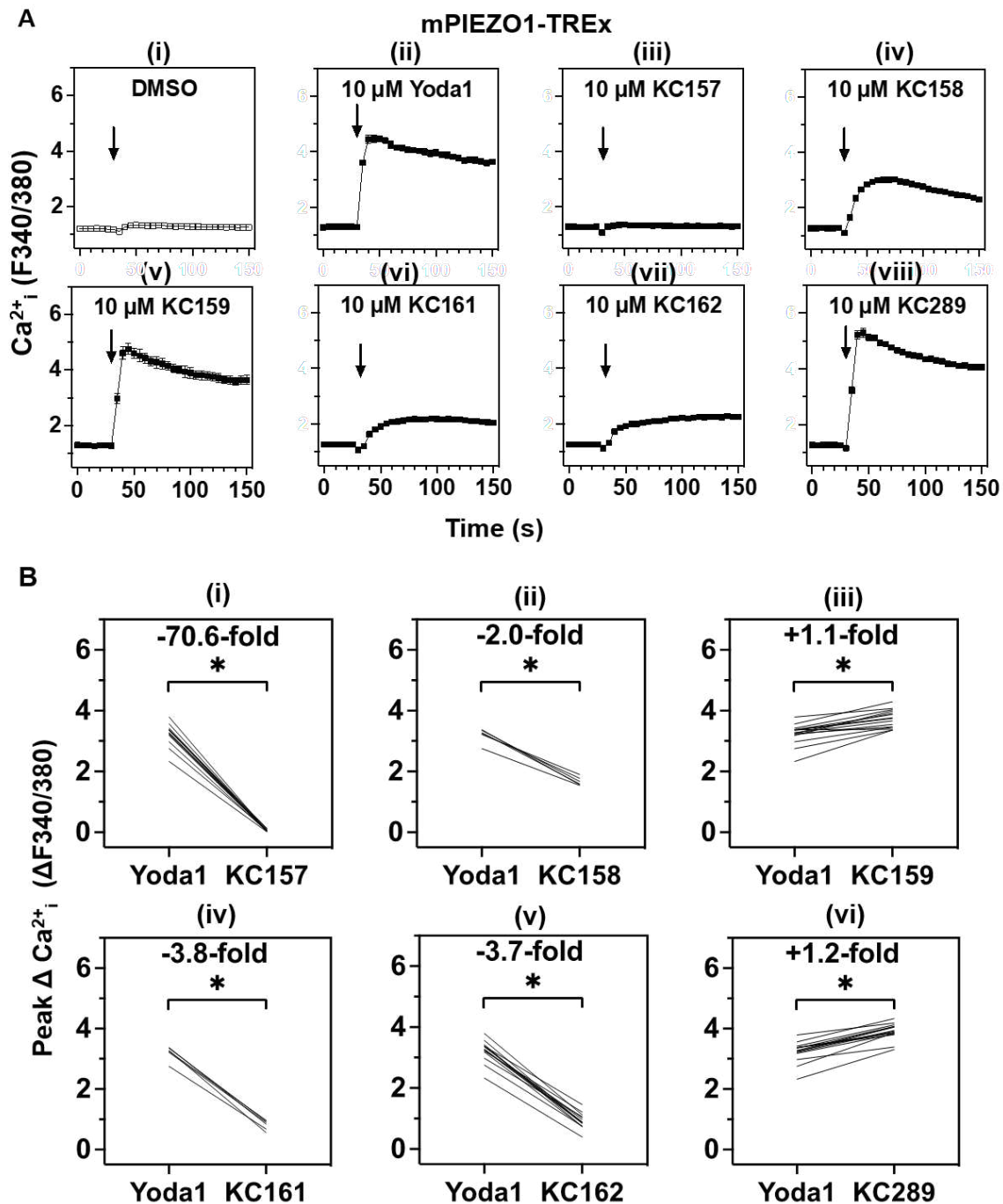
**Figure 2 SI 1 Supporting data for Figure 2**

1366 (A) Background-subtracted ( $\Delta$ ) peak intracellular  $Ca^{2+}$  measurements from individual  
 1367 experiments in which hPIEZO1-TREx cells were exposed to the indicated concentrations of:  
 1368 (i) Yoda1 (n=12); (ii) **KC159** (n=17); (iii) **KC289** (n=7). Mean values from 2 to 5 technical  
 1369 replicates are shown for each of the individual experiments.

1370 (B) Background-subtracted ( $\Delta$ ) peak intracellular  $Ca^{2+}$  measurements from individual  
 1371 experiments in which HUVECs were exposed to the indicated concentrations of: (i) Yoda1  
 1372 (n=9); (ii) **KC159** (n=9); (iii) **KC289** (n=6). Mean values from 2 to 5 technical replicates are  
 1373 shown for each of the individual experiments.

1374

Figure 3



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1378 **Figure 3. 4-benzoic acid improves efficacy at mouse PIEZO1**

1379 (A) Intracellular  $\text{Ca}^{2+}$  measurements from a single experiment in which mPIEZO1-TREx cells

1380 were acutely exposed to; (i) DMSO vehicle, or 10  $\mu\text{M}$ ; (ii) Yoda1, (iii) **KC157**, (iv) **KC158**, (v)

1381 **KC159**, (vi) **KC161**, (vii) **KC162**, (viii) **KC289**. Arrows indicate the time at which the indicated

1382 compound was added to the cells, following a 30 s background read. Mean  $\pm$  SEM values

1383 from 5 technical replicates are shown. (B) Paired, background-subtracted ( $\Delta$ ), peak

1384 intracellular  $\text{Ca}^{2+}$  measurement comparisons of mPIEZO1-TREx cells treated with 10  $\mu\text{M}$

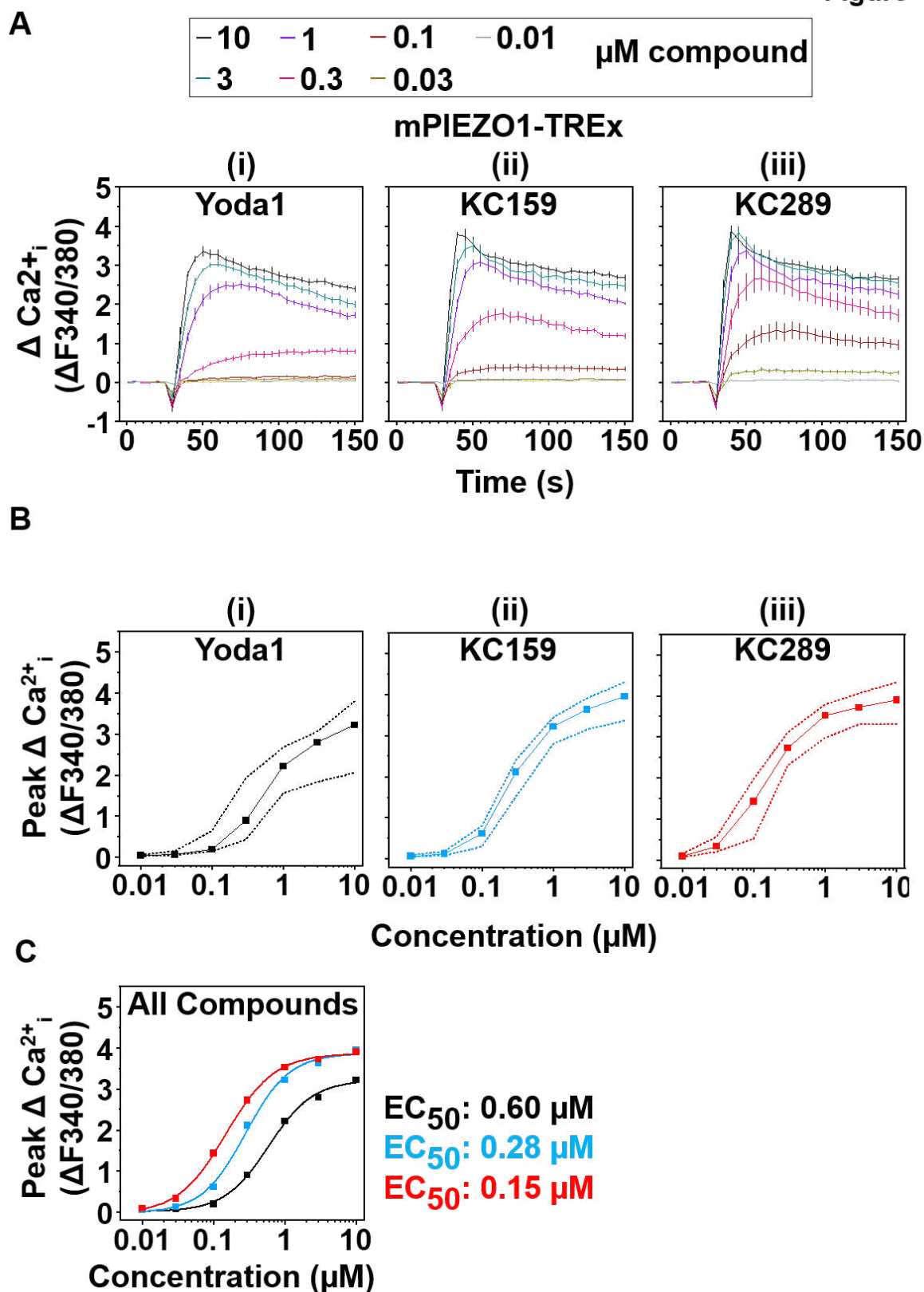
1385 indicated compound. Each plot shows mean peak values from independent experiments

1386 (**KC157**, n=15; **KC158**, n=6; **KC159**, n=15; **KC161**, n=6; **KC162**, n=15; **KC289**, n=15). Paired

sample Wilcoxon signed rank test results comparing the median values of the collated

1387 experiments are shown as: \*  $p < 0.05$ . Median fold-differences compared to Yoda1 are also  
1388 indicated.  
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Figure 4



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**Figure 4. 4-benzoic acid improves potency at mouse PIEZO1**

(A) Intracellular Ca<sup>2+</sup> measurements from a single experiment (n=1) in which mPIEZO1-TREx cells were exposed to the indicated concentrations of; (i) Yoda1, (ii) **KC159**, (iii) **KC289**. Mean ± SEM values from 4 technical replicates are shown.

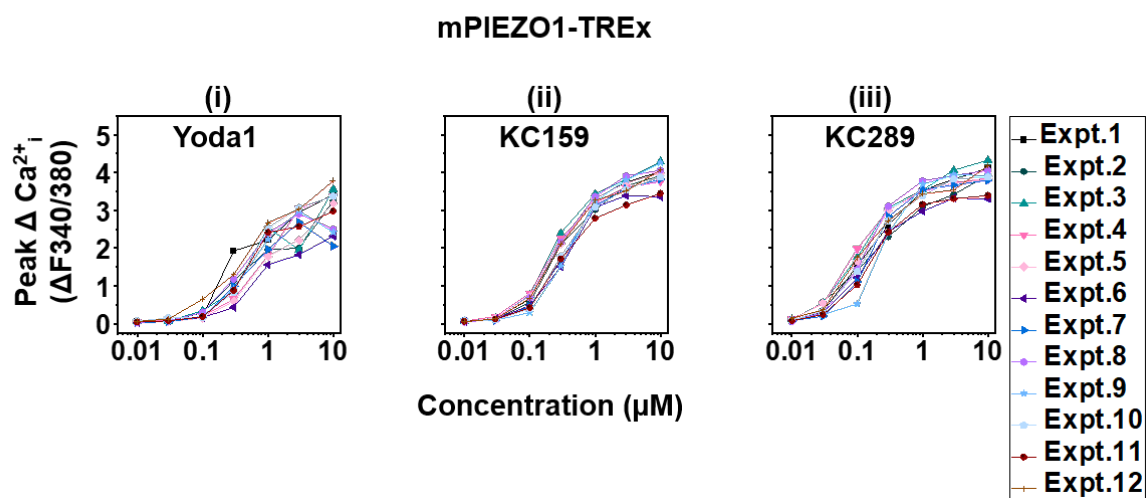


1395 (B) Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$  measurements of mPIEZO1-TREx cells  
1396 treated with the indicated concentration range of; (i) Yoda1, (ii) **KC159**, (iii) **KC289**. Median  
1397 peak values and the data range from collated experiments are shown (n=12 for all  
1398 compounds).

1399 (C) Median peak values for each compound were used to construct fitted curves from the Hill  
1400 equation, and the respective  $\text{EC}_{50}$  values are indicated.

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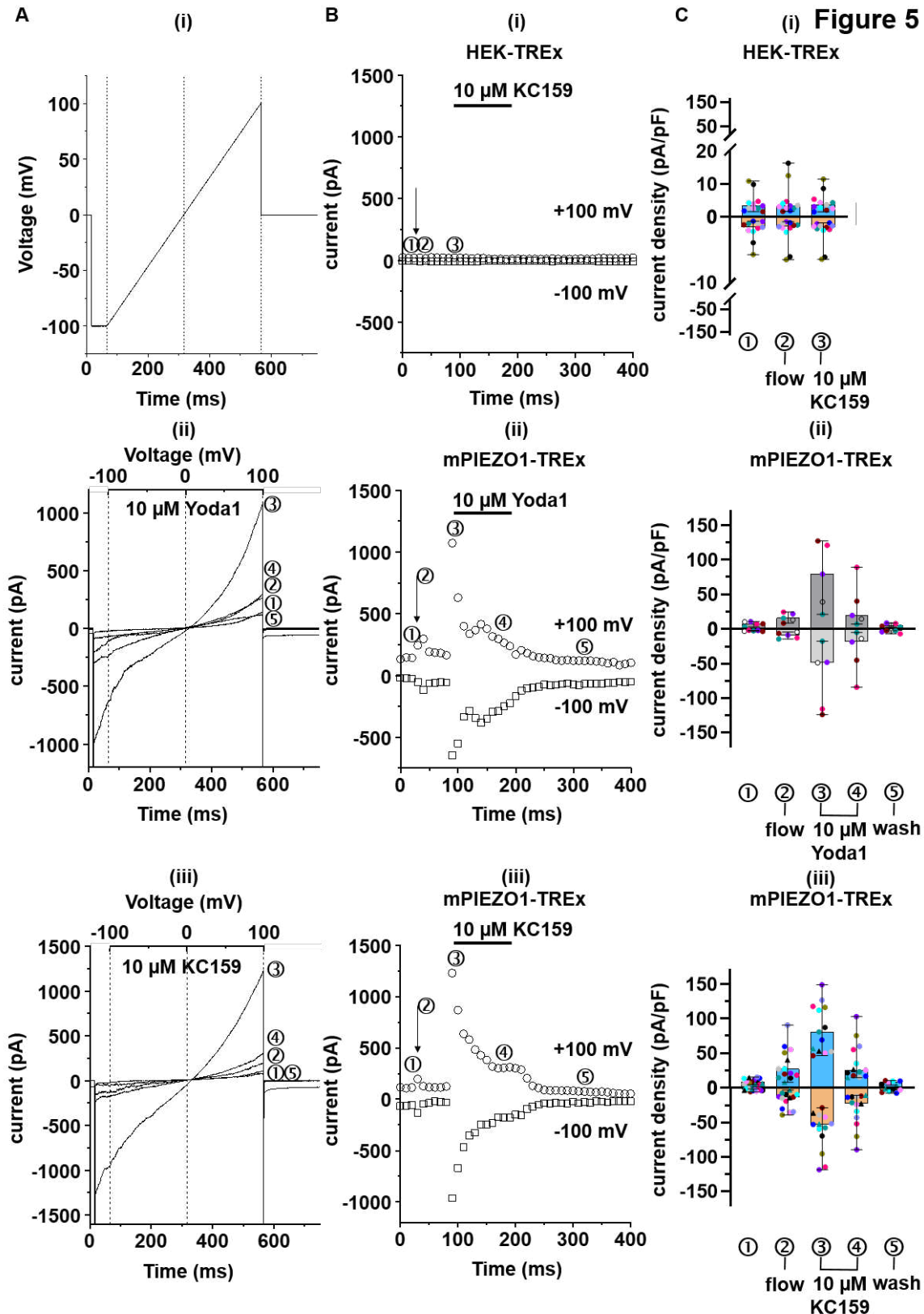
Figure 4 SI 1



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#### Figure 4 SI 1 Supporting data for Figure 4

Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$  measurements from individual experiments in which mPIEZO1-TREx cells were exposed to the indicated concentrations of; (i) Yoda1, (ii) **KC159**, (iii) **KC289**. Mean values from 2 or 3 technical replicates are shown for each of the individual experiments (n=12 for all compound tests).



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### Figure 5. PIEZO1 agonism confirmed by manual patch-clamp

(A) (i) Ramp voltage protocol used for cell stimulation. Dashed gridlines show time points where the ramp starts (-100 mV), reverses (0 mV) and ends (+100 mV). They also match the lines in (ii) and (iii) which show representative (N=1) whole-cell currents activated in mPIEZO1-TREx channels by 10  $\mu$ M (ii) Yoda1, or (iii) **KC159**. The circled numbers indicate currents at

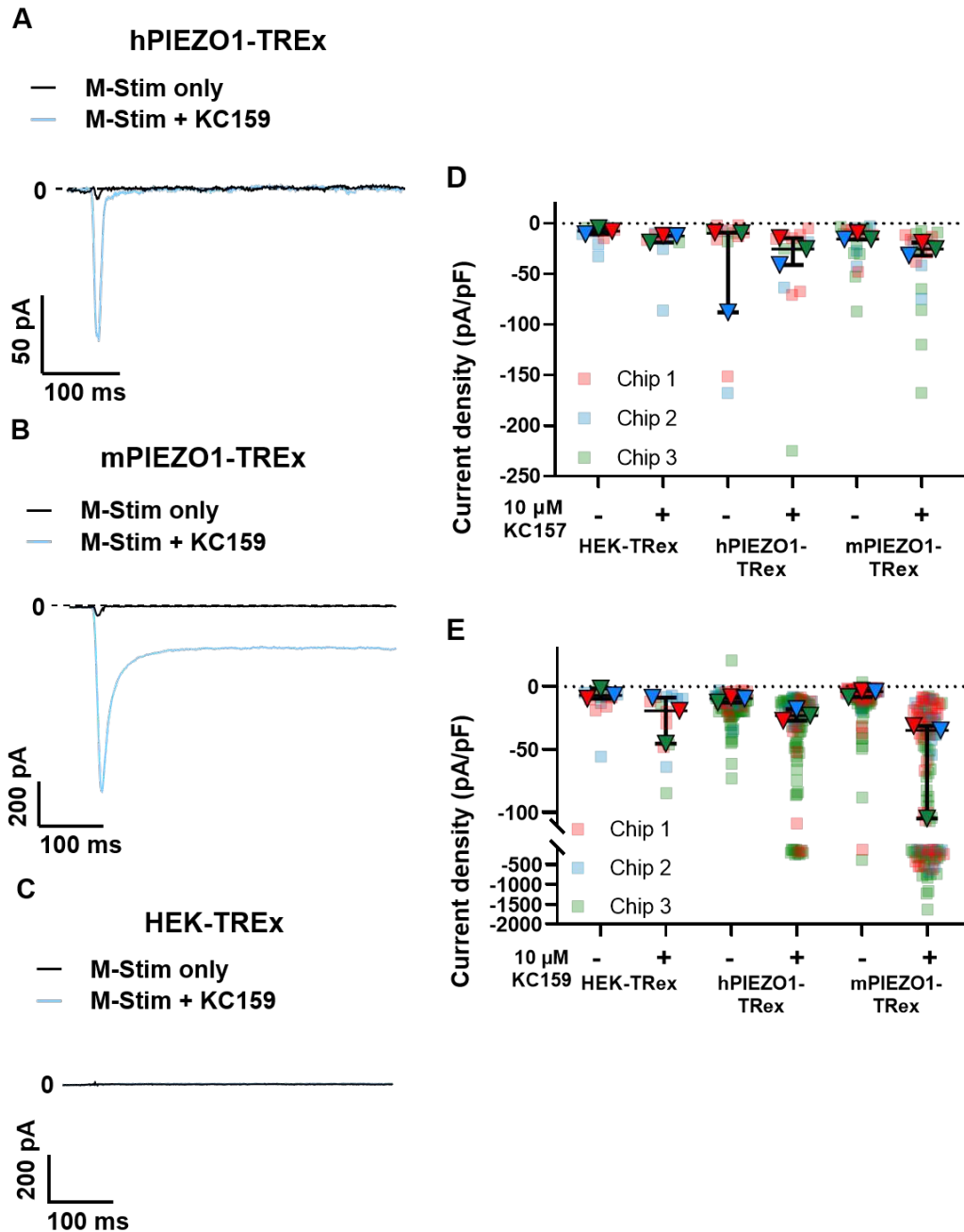
1415 the following stages of the experiment; ① control; ② after the start of SBS flow; ③ (maximal)  
1416 after application of the indicated compound; ④ 100 ms after application of compound; ⑤  
1417 after bath solution (SBS) washout of compound. The circled numbers correspond to those  
1418 shown in all subsequent plots.

1419 (B) Time-resolved plots of representative experiments (N=1); (i) HEK-TREx control (null) cell  
1420 treated with fluid flow and then flow plus 10  $\mu$ M **KC159**; (ii) mPIEZO1-TREx cell treated with  
1421 flow and then flow plus 10  $\mu$ M Yoda1; (iii) mPIEZO1-TREx cell treated with flow and then flow  
1422 plus 10  $\mu$ M **KC159**. Arrow indicates the start of SBS flow. Circles indicate currents measured  
1423 at +100 mV of the ramp, and squares those measured at -100mV. The currents were recorded  
1424 every 10 s.

1425 (C) Collated data for experiments as exemplified in (A) and (B). Current densities (pA/pF)  
1426 measured at +100 mV are shown on the upper y-axis (blue for KC159, dark grey for Yoda1),  
1427 and those measured at -100 mV on the lower y-axis (orange for KC159, light grey for Yoda1).  
1428 Individual data points of different cells are shown with different colours. Bars indicate the  
1429 median  $\pm$  range. (i) HEK-TREx control cells treated with 10  $\mu$ M **KC159**: ① (N=11); ② (N=11);  
1430 ③ (N=11). (ii) mPIEZO1-TREx cells treated with 10  $\mu$ M Yoda1: ① (N=5); ② (N=5); ③ (N=5);  
1431 ④ (N=5) and; ⑤ (N=5). (iii) mPIEZO1-TREx cells treated with 10  $\mu$ M **KC159**: ① (N=13); ②  
1432 (N=13); ③ (N=13); ④ (N=13) and; ⑤ (N=6).  
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Figure 6



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**Figure 6. Improved agonism seen by automated patch-clamp**

1437 (A, B, C) Averaged traces for PIEZO1-mediated currents activated by mechanical stimulation

1438 (M-Stim) on the SyncroPatch384 in the absence (black traces) and presence of 10  $\mu$ M **KC159**

1439 (blue traces) from: (A) hPIEZO1-TREx (N=44); (B) mPIEZO1-TREx (N=46) and (C)

1440 untransfected HEK-TREx (N=55).

1441 (D and E) Current densities elicited by M-Stim in the absence and presence of 10  $\mu$ M: (D)1442 **KC157** or; (E) **KC159** in untransfected HEK-TREx, hPIEZO1-TREx and mPIEZO1-TREx cells.

1443 Each square represents the peak current density of a single cell, with median values and

1444 ranges indicated in black. Only data for cells defined as responding (see Methods) are

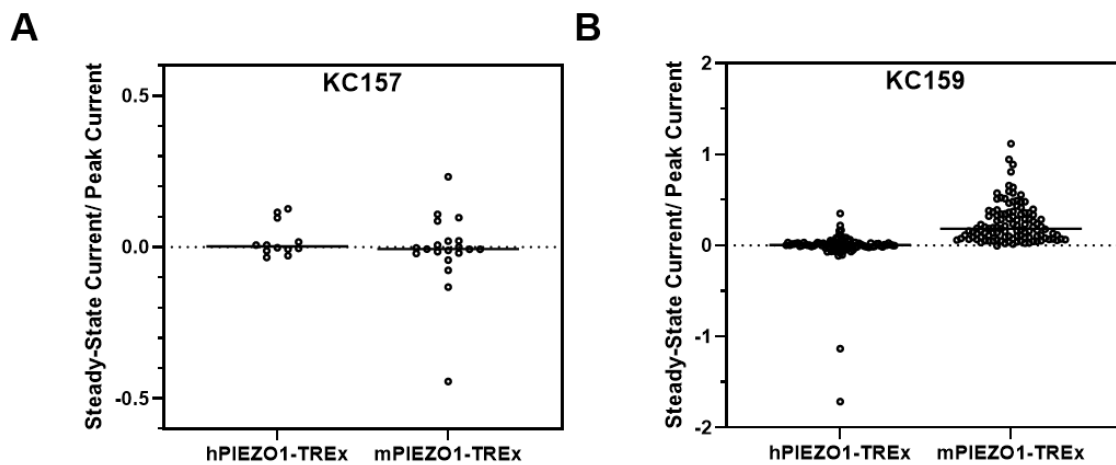
1445 included in the plots. Recordings were made from cells on 3 different 384-well recording chips

1446 and the data from these chips are distinguished by red, blue and green colour with the filled

1447 triangles indicating the median values for each chip. (D) HEK-TREx M-Stim (N=10), HEK-

1448 TREx M-Stim + **KC157** (N=10), hPIEZO1-TREx M-Stim (N=12), hPIEZO1-TREx M-Stim +  
1449 **KC157** (N=12), mPIEZO1-TREx M-Stim (N=20), mPIEZO1-TREx M-Stim + **KC157** (N=20).  
1450 (E) HEK-TREx M-Stim (N=13), HEK-TREx M-Stim + **KC159** (N=13), hPIEZO1-TREx M-Stim  
1451 (N=94), hPIEZO1-TREx M-Stim + **KC159** (N=94), mPIEZO1-TREx M-Stim (N=113),  
1452 mPIEZO1-TREx M-Stim + **KC159** (N=113).  
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Figure 6 SI 1



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**Figure 6 SI 1. Quantification of sustained current after application of KC157 and KC159.** Ratio of sustained current to peak current after application of (A) **KC157** and (B) **KC159** for mouse PIEZO1 and human PIEZO1. (A) hPIEZO1-TREx M-Stim + **KC157** (N=12), mPIEZO1-TREx M-Stim + **KC157** (N=20). (B) hPIEZO1-TREx M-Stim + **KC159** (N=94), mPIEZO1-TREx M-Stim + **KC159** (N=113). Bars indicate median values.

Figure 7

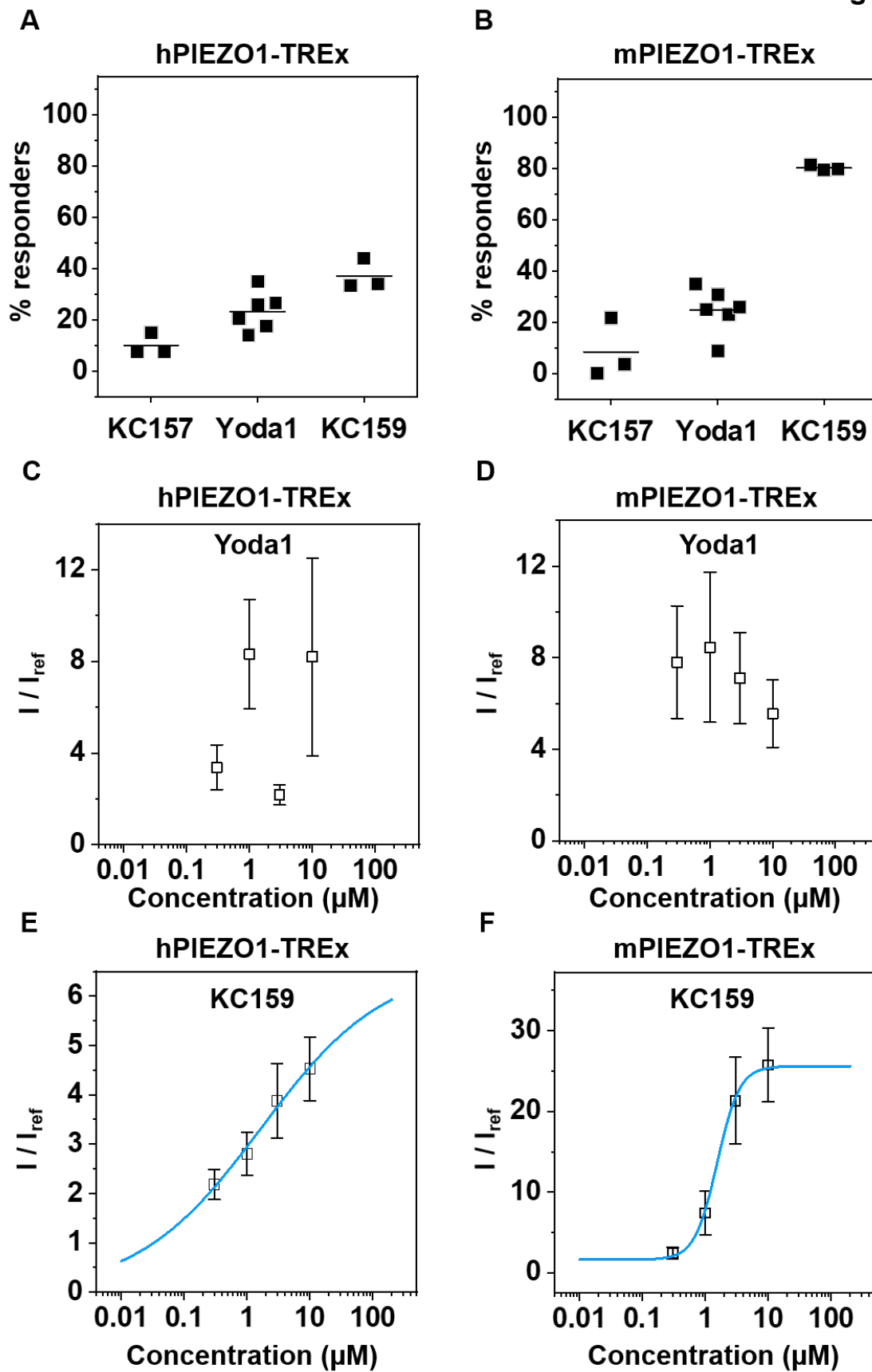
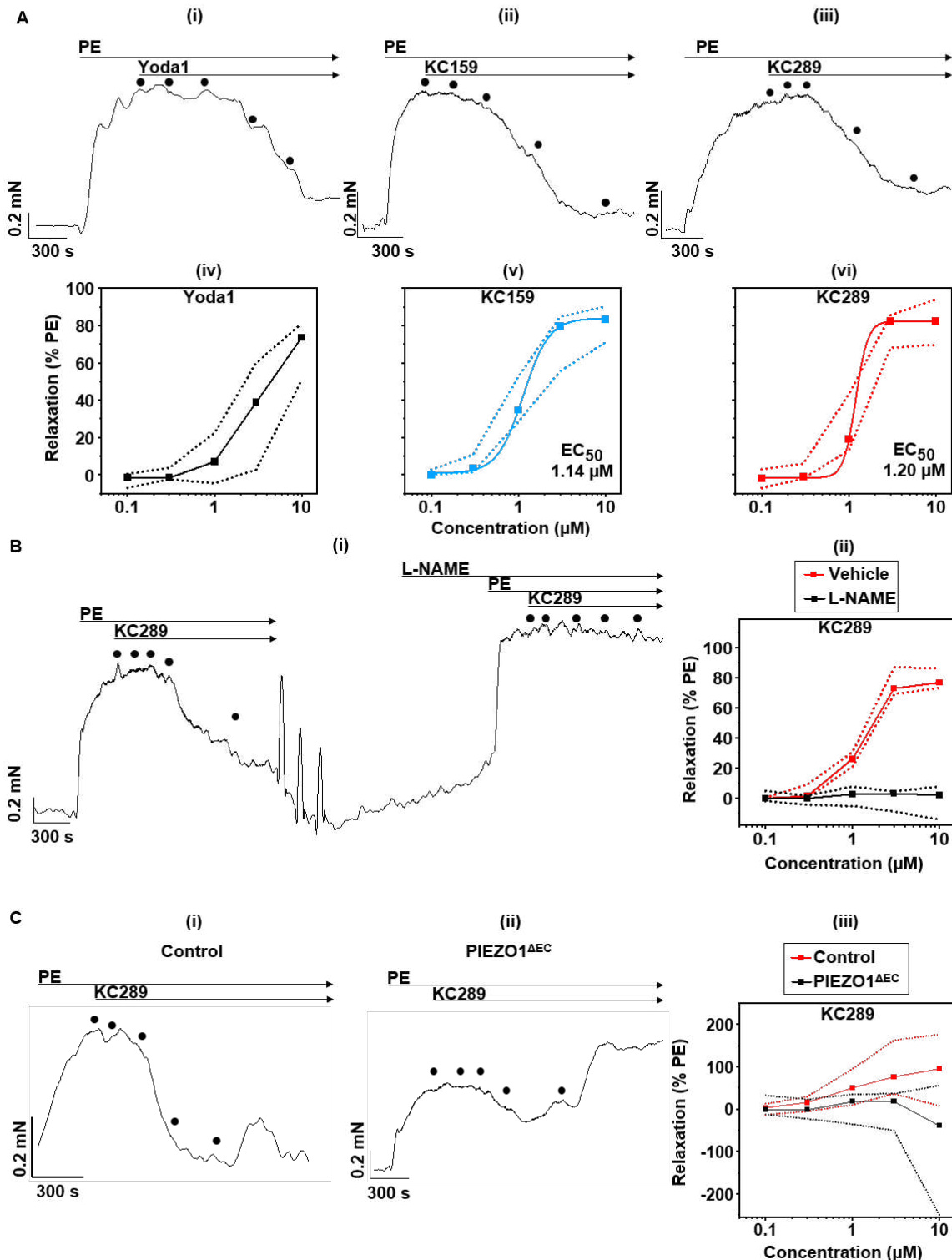
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Figure 7. Automated patch-clamp comparison with Yoda1



1462 (A and B) Percentages of; (A) hPIEZO1-TREx and; (B) mPIEZO1-TREx cells responding to  
1463 activation by 10  $\mu$ M **KC157**, 10  $\mu$ M Yoda1 and 5  $\mu$ M **KC159**. Each square represents an  
1464 independent experiment with mean values indicated by black bars. All groups contained 64  
1465 cells per condition tested on the same chip, and the following numbers of valid cells were  
1466 assessed for responsiveness in each repeat: (A) **KC157**, N=40, 40, 53; **Yoda1**, N=37, 50, 51,  
1467 46, 39, 49; **KC159**, N=42, 50, 41. (B) **KC157**, N=48, 55, 46; **Yoda1**, N=42, 48, 56, 48, 43, 54;  
1468 **KC159**, N=34, 25, 43. Because of the number of independent 384-well chips used was less  
1469 than 5 in some cases (i.e., 3), we did not apply statistical testing to these data.  
1470 (C and D) Dose-response data expressed as mean normalized activation  $\pm$  SEM, obtained  
1471 from; (C) hPIEZO1-TREx (N=108) and; (D) mPIEZO1-TREx (N=134) cells exposed to  
1472 increasing concentrations (0.3, 1, 3 and 10  $\mu$ M) of Yoda1. hPIEZO1 0.3  $\mu$ M (N=27), 1  $\mu$ M  
1473 (N=25), 3  $\mu$ M (N=28), 10  $\mu$ M (N=28); mPIEZO1 0.3  $\mu$ M (N=34), 1  $\mu$ M (N=34), 3  $\mu$ M (N=34),  
1474 10  $\mu$ M (N=32) The peak current in the presence of the agonist was normalized to the peak  
1475 current in the presence of reference only.  
1476 (E and F) Dose-response data expressed as mean normalized activation  $\pm$  SEM, obtained  
1477 from; (E) hPIEZO1-TREx (N=316) and; (F) mPIEZO1-TREx (N=250) cells exposed to  
1478 increasing concentrations (0.3, 1, 3 and 10  $\mu$ M) of **KC159**. hPIEZO1 0.3  $\mu$ M (N=79), 1  $\mu$ M  
1479 (N=86), 3  $\mu$ M (N=73), 10  $\mu$ M (N=78); mPIEZO1 0.3  $\mu$ M (N=65), 1  $\mu$ M (N=58), 3  $\mu$ M (N=63),  
1480 10  $\mu$ M (N=64). The peak current in the presence of the agonist was normalized to the peak  
1481 current in the presence of reference only. Fitted curves (blue) were generated from the Hill  
1482 equation.  
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Figure 8



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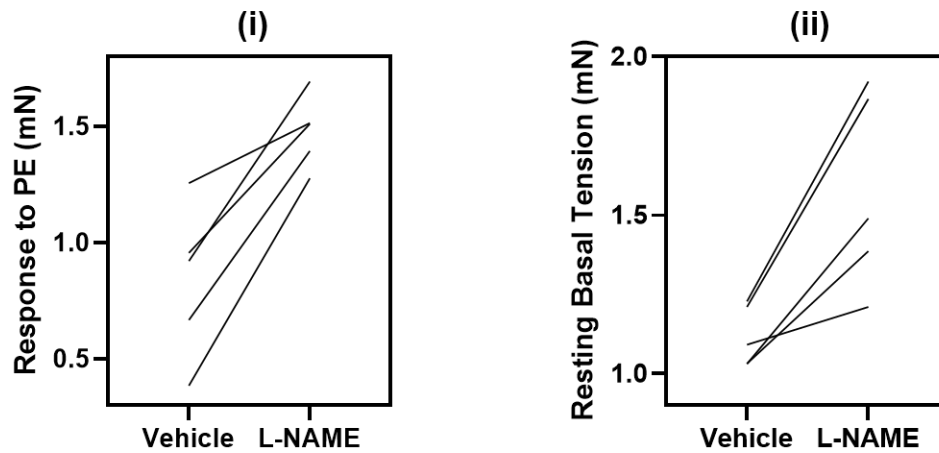
### Figure 8. NO- and endothelial PIEZO1- dependent vasorelaxation

Isometric tension of mouse portal vein possessing intact endothelium was measured following pre-constriction with 10  $\mu\text{M}$  phenylephrine (PE) and exposure to increasing concentrations (0.1, 0.3, 1, 3 and 10  $\mu\text{M}$ ) of indicated compounds.

(A) (upper panel) Isometric tension traces from single experiments ( $n=1$ ) in which mouse portal vein segments were exposed to increasing concentrations of: (i) Yoda1; (ii) **KC159**; (iii) **KC289**. The dots indicate the sequential addition of rising concentrations of compounds.

1492 (lower panel) Corresponding dose-response data for: (iv) Yoda1 (n=8); (v) **KC159** (n=5); (vi)  
1493 **KC289** (n=5) expressed as a % of the maximal PE-induced tension. Squares indicate median  
1494 values and dotted lines indicate the data range from collated experiments. For Yoda1, curve  
1495 fitting was not successful due to the lack of upper plateau. For KC159 and KC289, fitted curves  
1496 generated from the Hill equation (Hill1 in OriginPro 2020 software) and their corresponding  
1497 EC<sub>50</sub> values are shown. (B) (i) Isometric tension response trace from a single experiment (n=1)  
1498 in which increasing concentrations of **KC289** were added before and after a 30-minute pre-  
1499 incubation of the vessel with 100 μM Nω-nitro-L-arginine methyl ester (L-NAME). (ii)  
1500 Corresponding collated dose-response data expressed as a % of the maximal PE-induced  
1501 tension (n=5). Median and range are shown.  
1502 (C) (i-ii) Isometric tension response traces from a single experiment (n=1) in which increasing  
1503 concentrations of **KC289** were added to portal veins from; (i) control and; (ii) PIEZO1<sup>ΔEC</sup> mice.  
1504 (iii) Corresponding dose-response data expressed as a % of the maximal PE-induced tension  
1505 (control, n=9; PIEZO1<sup>ΔEC</sup>, n=9). Median and range from collated experiments are shown.  
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Figure 8 SI 1



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**Figure 8 SI 1 Effects of L-NAME on vessel response to PE and resting basal tension**

1510 Paired comparisons of (i) tension responses to PE and; (ii) isometric basal tension

1511 measurements before and after a 30-minute pre-incubation of vessels with 100  $\mu$ M N $\omega$ -nitro-

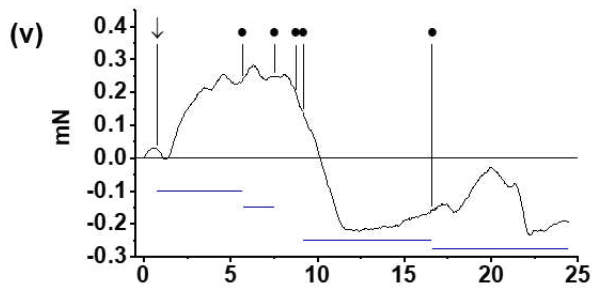
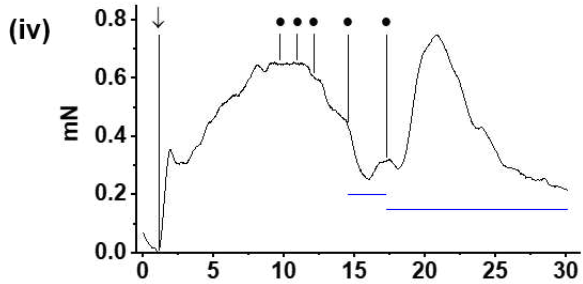
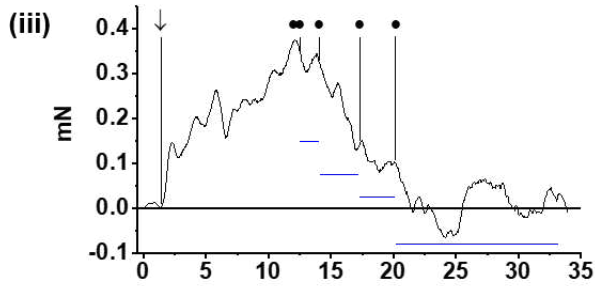
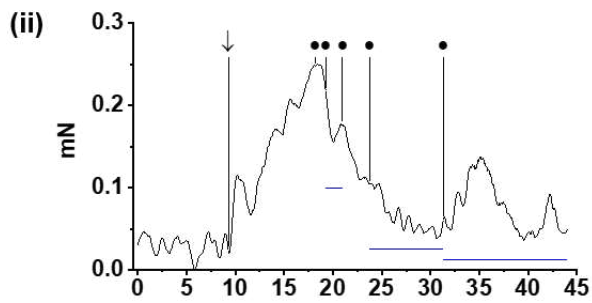
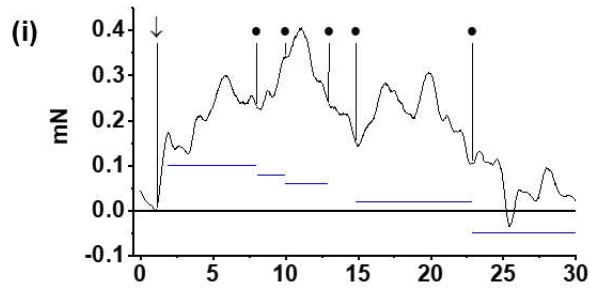
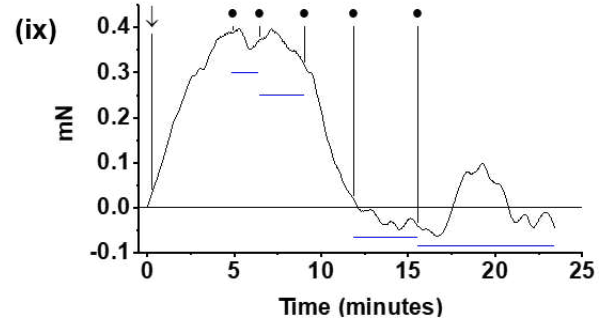
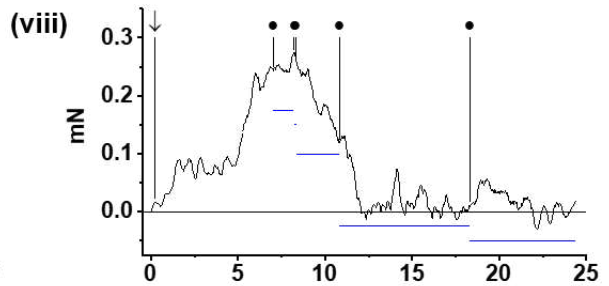
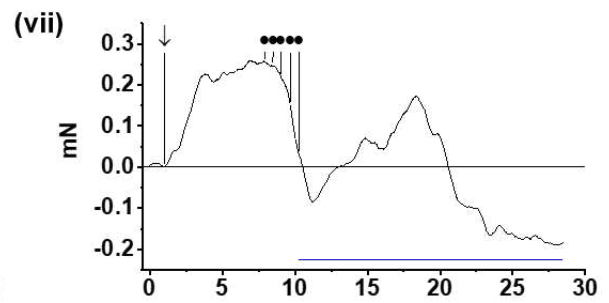
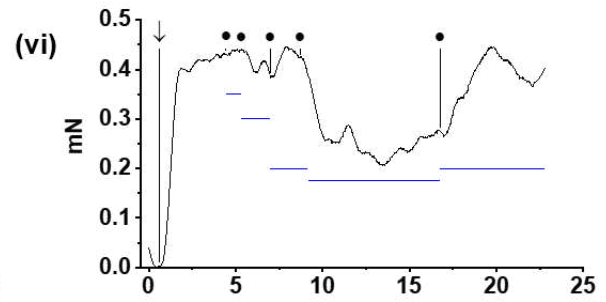
1512 L-arginine methyl ester (L-NAME). Each plot shows values from independent experiments

1513 (n=5).

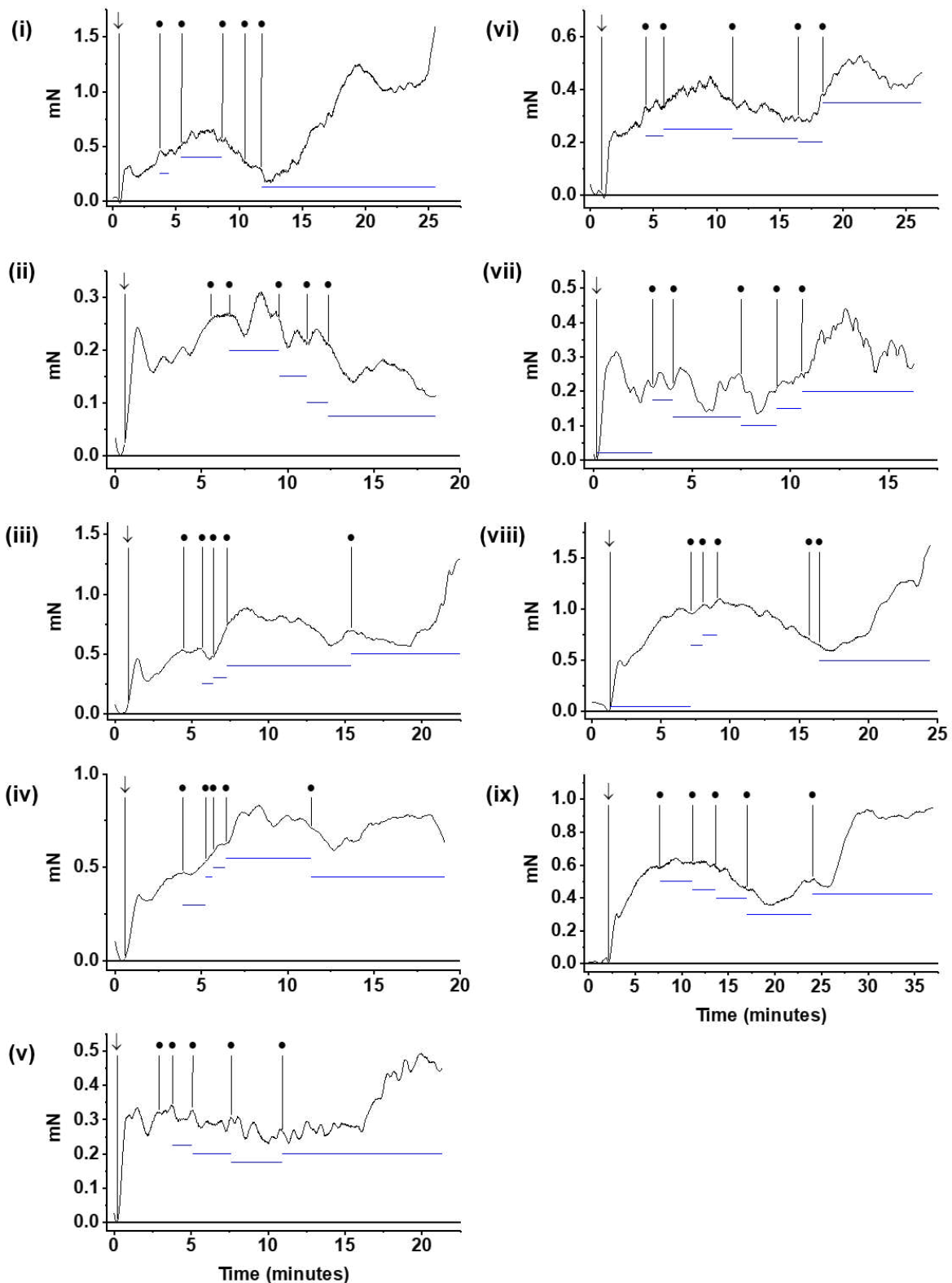
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**A Control****Figure 8 SI 2 (A)**

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**B** **PIEZO1<sup>ΔEC</sup>****Figure 8 SI 2 (B)**

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1521 **Figure 8 SI 2 All original traces for the data of Figure 8C.**

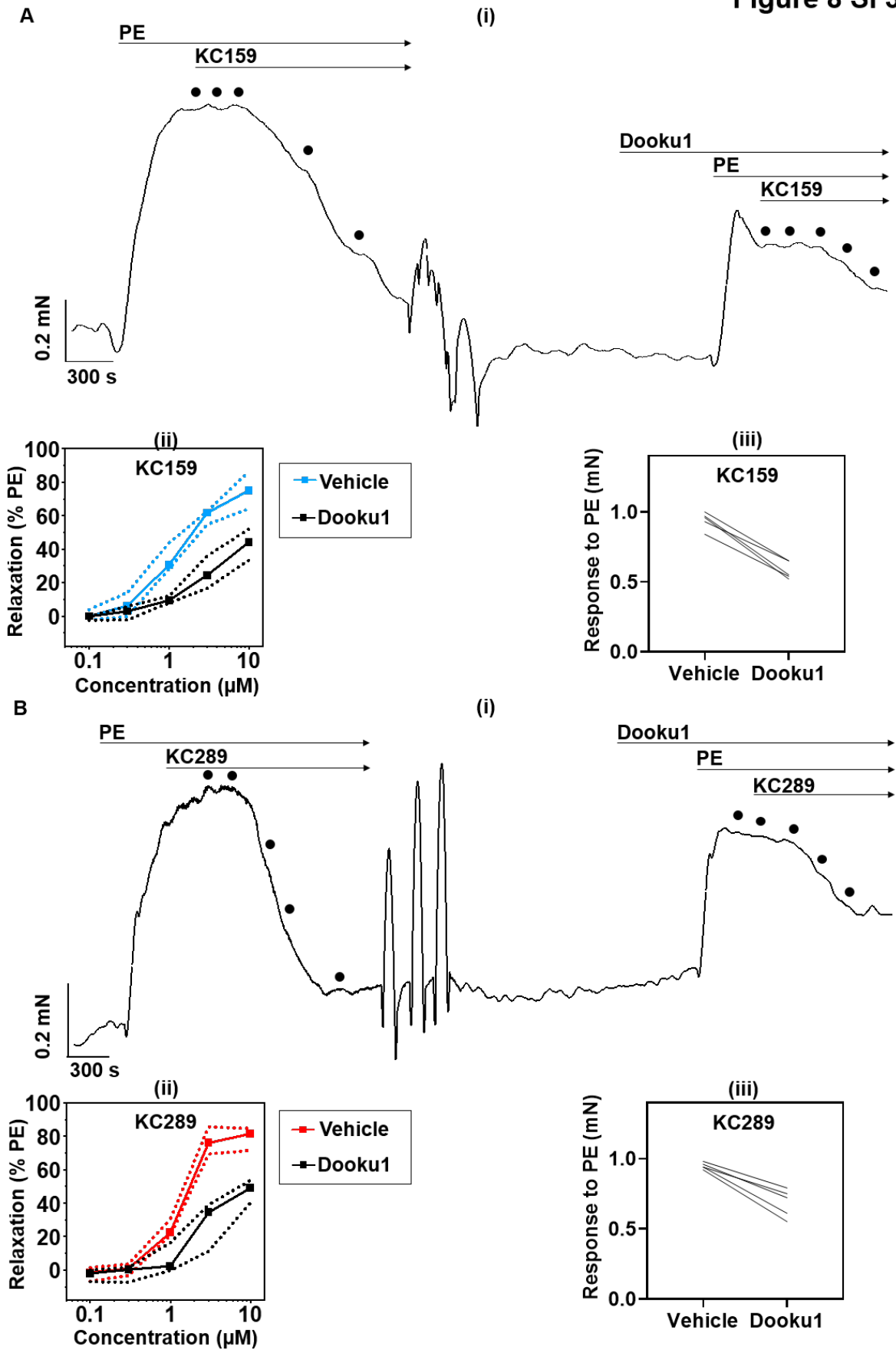
1522 Isometric tension traces from single experiments in which (A) (i-ix) control mouse, or (B) (i -

1523 ix) portal vein segments possessing intact endothelium were exposed to increasing

concentrations (0.1, 0.3, 1, 3 and 10  $\mu\text{M}$ ) of KC289 after pre-constriction with 10  $\mu\text{M}$

1524 phenylephrine (PE). The arrows indicate the addition of PE; dots indicate the sequential  
1525 addition of rising concentrations of KC289. Blue lines annotate treatment periods in which  
1526 spontaneous activity of the vessel made it difficult to determine if there were effects of the  
1527 compound.  
1528

Figure 8 SI 3

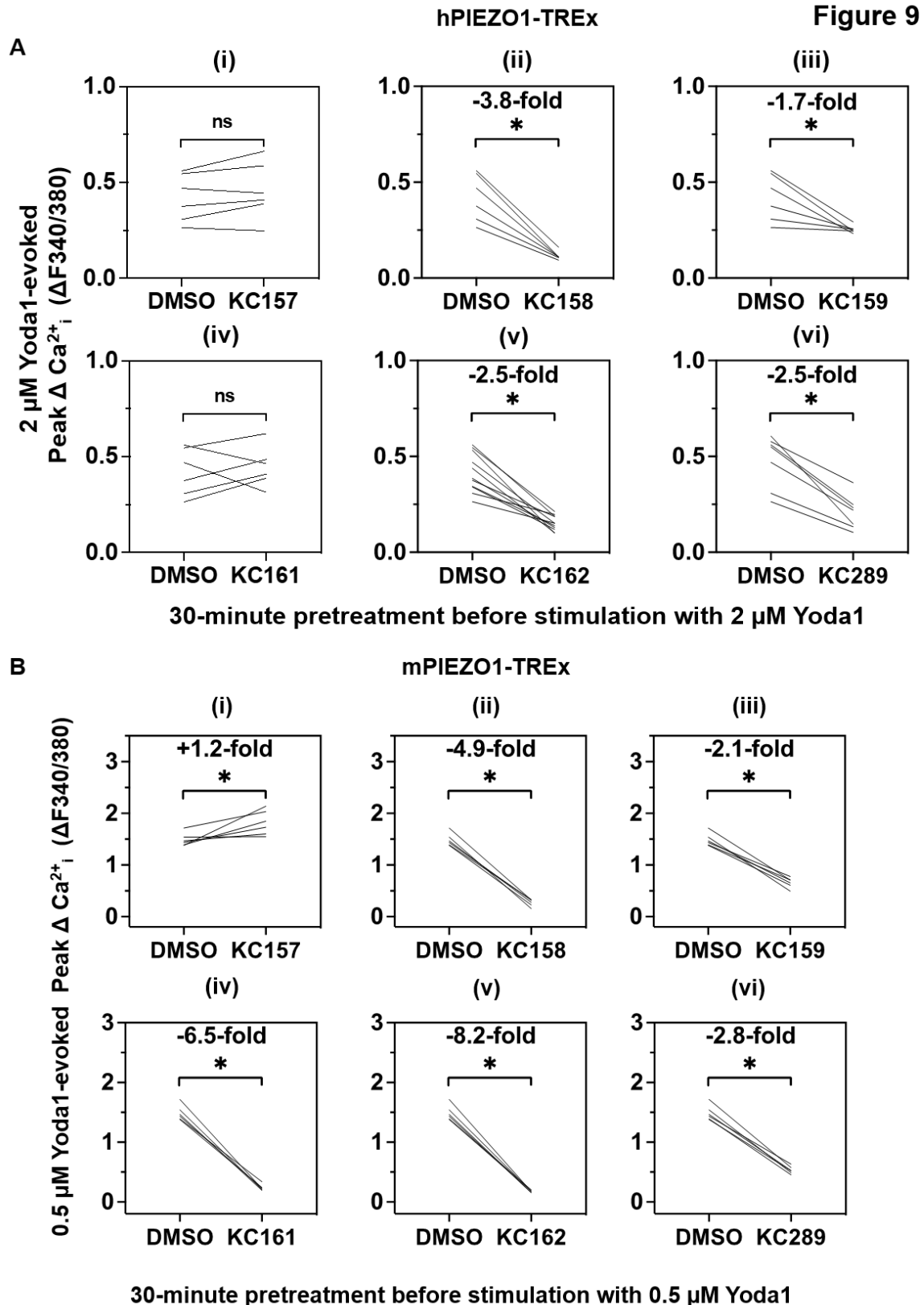


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Figure 8 SI 3 Dooku1 antagonises vasorelaxant effects of KC159 and KC289



1531 Isometric tension responses observed on addition of increasing concentrations of: (A) **KC159**  
1532 or; (B) **KC289** before and after a 30-minute pre-incubation of the vessel with 10  $\mu$ M Dooku1  
1533 or DMSO vehicle control. (A)(i) and (B)(i) show traces from individual experiments (n=1 each).  
1534 The dots indicate the sequential addition of rising concentrations of compounds. (A)(ii) and  
1535 (B)(ii) show corresponding dose-response data expressed as a % of the maximal PE-induced  
1536 tension (n=5 for each compound). Median and range from collated experiments are shown.  
1537 (A)(iii) and (B)(iii) show paired comparisons of vessel tension responses to PE. Each plot  
1538 shows values from independent experiments (n=5).  
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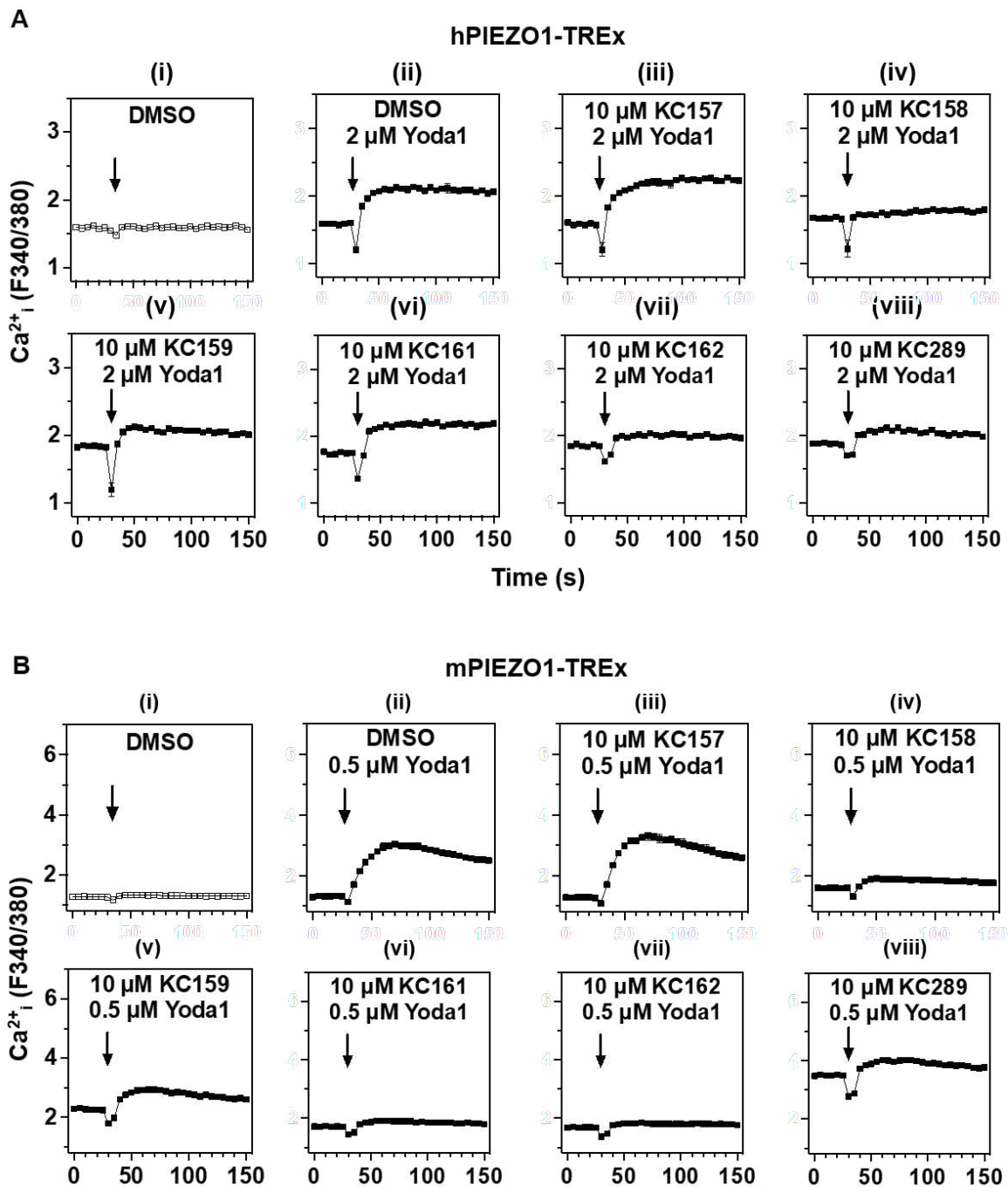
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**Figure 9. Effects of 3- and 4-benzamide and 3-benzoic acid analogues**

(A and B) Paired, background-subtracted ( $\Delta$ ), peak intracellular  $\text{Ca}^{2+}$  measurement comparisons of: (A) hPIEZO1-TREx and (B) mPIEZO1-TREx pretreated with 10  $\mu\text{M}$  indicated compound for 30 minutes prior to acute exposure to (ii – vi) the indicated Yoda1 stimulus. Each plot shows mean peak values from independent experiments. Independent experiment numbers for hPIEZO1-TREx experiments (A): **KC157**, n=6; **KC158**, n=6; **KC159**, n=6; **KC161**,

1548 n=6; **KC162**, n= 11, **KC289**, n=7). Independent experiment numbers for mPIEZO1-TREx  
1549 experiments (B): (n=6 for all compounds). Paired sample Wilcoxon signed rank test results  
1550 comparing the median values of the collated experiments are shown as: n.s ( $p > 0.05$ ), \*  $p <$   
1551 0.05. Median fold-differences compared to DMSO-pretreatment/ indicated Yoda1-stimulation  
1552 are also shown.  
1553

Figure 9 SI 1

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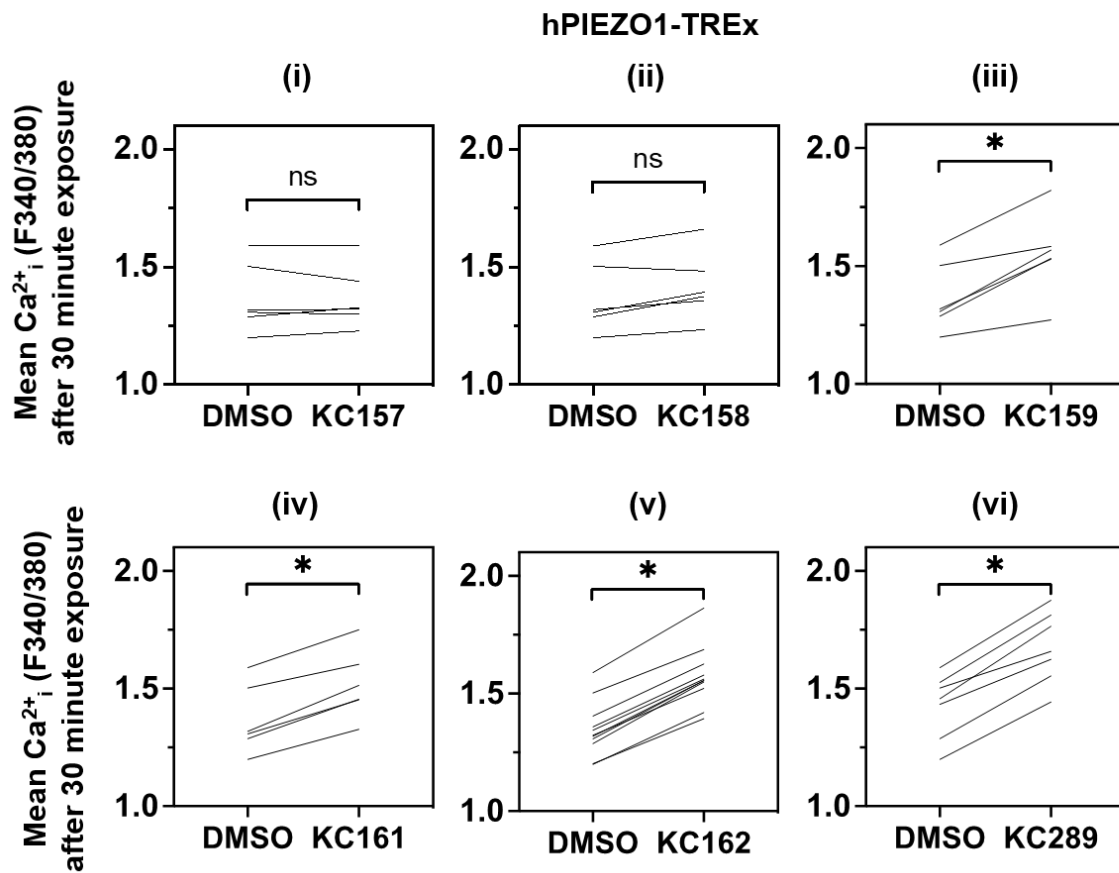
1557 **Figure 9 SI 1 Example single experiment data in support of Figure 9**1558 Intracellular  $Ca^{2+}_i$  measurements from single experiments (n=1) in which; (A) hPIEZO1-TREx1559 and; (B) mPIEZO1-TREx cells were pretreated with; (i - ii) DMSO vehicle, or 10  $\mu$ M; (iii)1560 **KC157**, (iv) **KC158**, (v) **KC159**, (vi) **KC161**, (vii) **KC162**, (viii) **KC289** for 30 minutes before

1561 stimulation with the indicated concentration of Yoda1. Arrows indicate the time at which Yoda1

1562 was added to the cells, following a 30 s background read. Mean  $\pm$  SEM values from between

1563 3 and 5 technical replicates are shown.

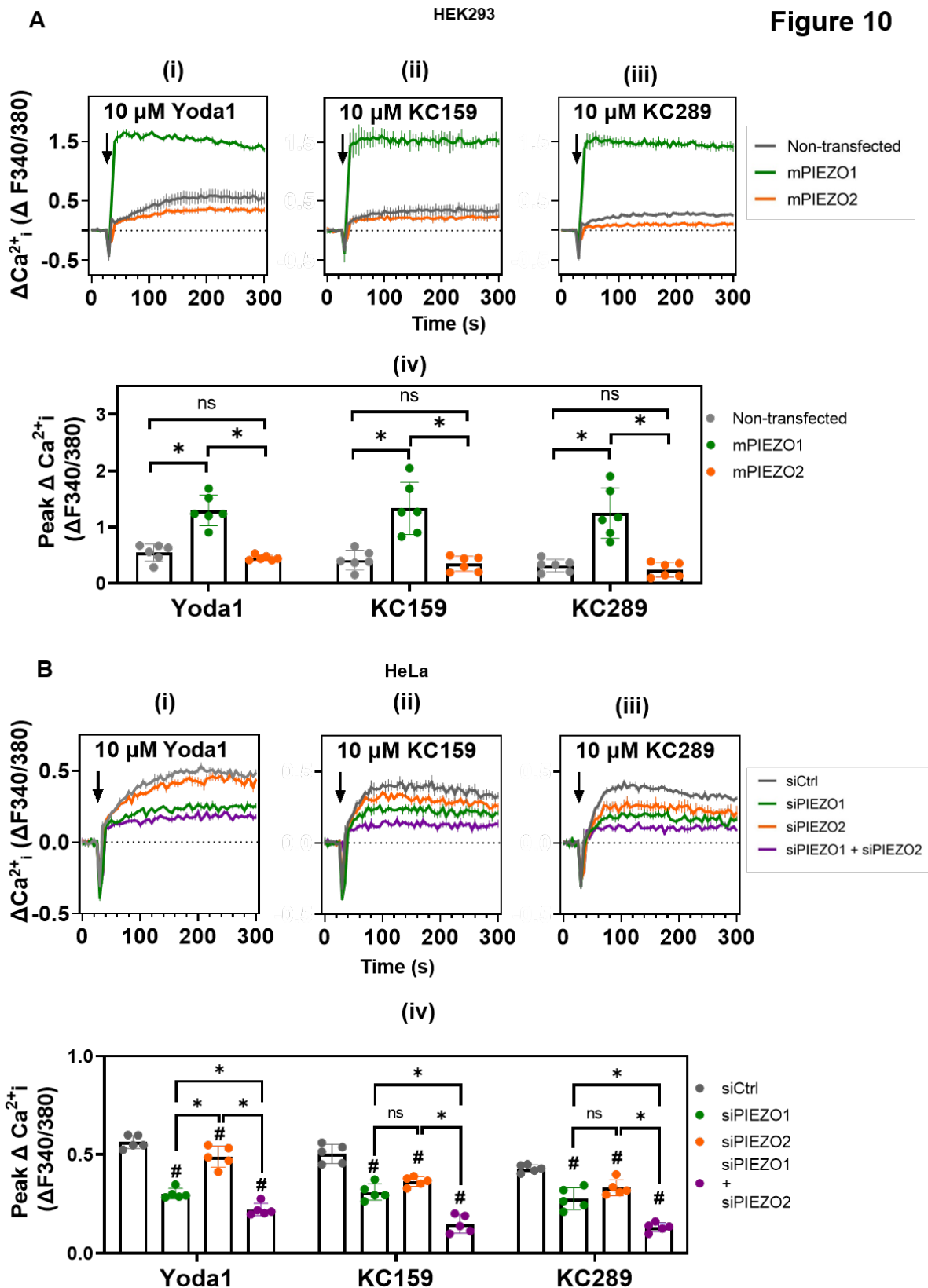
Figure 9 SI 2



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**Figure 9 SI 2 Further analysis of data in Figure 9 – effects of long exposure**

Paired mean intracellular  $\text{Ca}^{2+}$  measurement comparisons of hPIEZO1-TREx treated with 10  $\mu\text{M}$  indicated compound for 30 minutes. Each plot shows mean values from independent experiments. Independent experiment numbers: (i) **KC157**, n=6; (ii) **KC158**, n=6; (iii) **KC159**, n=6; (iv) **KC161**, n=6; (v) **KC162**, n= 11; (vi) **KC289**, n=7. Paired sample Wilcoxon signed rank test results comparing the median values of the collated experiments are shown as: n.s ( $p > 0.05$ ), \*  $p < 0.05$ .



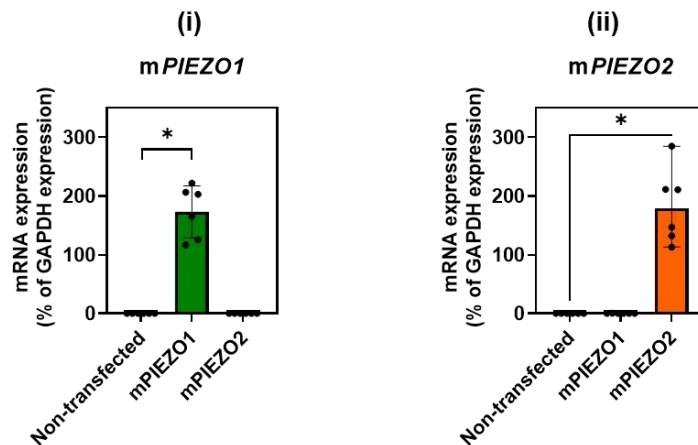
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### Figure 10 Relevance to PIEZO2

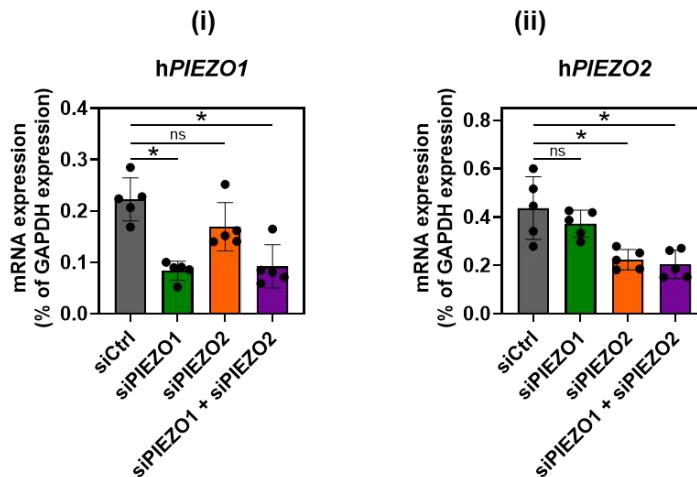
(A) Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$  measurements of HEK 293 cells overexpressing mPIEZO1, mPIEZO2 or non-transfected cells. Cells were exposed to 10  $\mu\text{M}$ : (i) Yoda1; (ii) **KC159**; (iii) **KC289**. In each case, mean  $\pm$  SEM values from 4 technical replicates are shown (n=1 each). (iv) Mean  $\pm$  SD (n=6) for peak  $\text{Ca}^{2+}$  signals evoked by 10  $\mu\text{M}$

1579 Yoda1, **KC159** and **KC289**. (B) Background-subtracted ( $\Delta$ ) peak intracellular  $\text{Ca}^{2+}$   
1580 measurements of HeLa cells after siRNA mediated knockdown of hPIEZO1 (siPIEZO1),  
1581 hPIEZO2 (siPIEZO2), both (siPIEZO1 + siPIEZO2) or transfected with a non-targeting  
1582 (Control, Ctrl) siRNA (siCtrl). Cells were exposed to 10  $\mu\text{M}$ : (i) Yoda1; (ii) **KC159**; (iii) **KC289**.  
1583 Mean  $\pm$  SEM values from 4 technical replicates are shown (n=1 each). (iv) Mean  $\pm$  SD (n=5)  
1584 for peak  $\text{Ca}^{2+}$  signals evoked by 10  $\mu\text{M}$  Yoda1, **KC159** and **KC289**. RM-One way ANOVA  
1585 followed by a Tukey's post-hoc test for multiple comparison performed on the mean values of  
1586 the collated experiments are shown as: n.s ( $p > 0.05$ ), # ( $p < 0.05$  vs siCtrl) \*  $p < 0.05$ .  
1587

A HEK293 **Figure 10 SI 1**



B HeLa



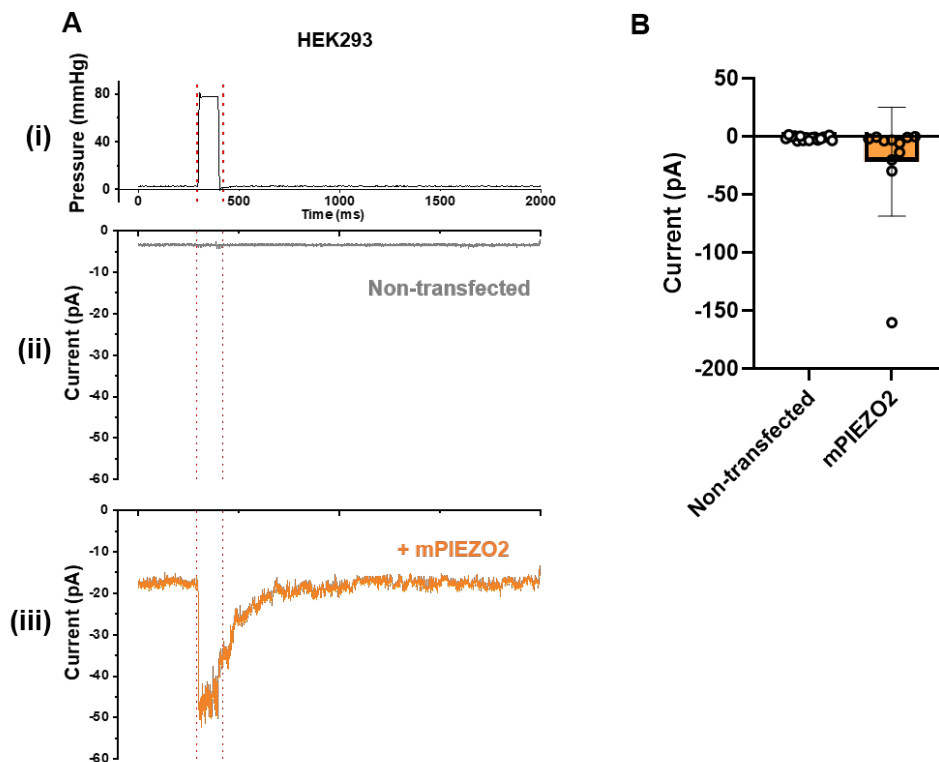
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**Figure 10 SI 1 Validation of transfection efficiency in HEK 293 and HeLa cells**

(A) mRNA abundance of (i) *mPIEZO1* and (ii) *mPIEZO2* ( $n=5$ ) transcripts relative to *hGAPDH* mRNA in HEK 293 cells overexpressing *mPIEZO1*, *mPIEZO2* or neither (“non-transfected cells”). (B) mRNA abundance of (i) *hPIEZO1* or (ii) *hPIEZO2* transcripts ( $n=5$ ) relative to *hGAPDH* expression in HeLa cells after siRNA mediated knockdown of *hPIEZO1* (*siPIEZO1*), *hPIEZO2* (*siPIEZO2*), both (*siPIEZO1* + *siPIEZO2*) or transfected with a non-targeting (Control, Ctrl) siRNA (*siCtrl*). Data expressed as Mean  $\pm$  SD except in Aii, which is Median with Range because the data could not be confirmed as normally distributed. One way ANOVA followed by a Tukey’s post-hoc test for multiple comparison was performed on the mean values of the collated experiments (Ai, Bi and Bii) and Friedmann test followed by a Dunn’s post-hoc test for multiple comparison for (Aii): n.s ( $p > 0.05$ ), \*  $p < 0.05$  for comparison against non-transfected and *siCtrl* only.



Figure 10 SI 2



1602

1603

1604 **Figure 10 SI 2 Detection of mechanically activated ionic current in HEK 293 cells**1605 **overexpressing mouse PIEZO2**

1606 (A) Example (n=1) outside-out patch current recordings showing the response to (i) 100-ms

1607 75 mmHg positive pressure pulse for (ii) non-transfected HEK 293 cells and (iii) HEK 293 cells

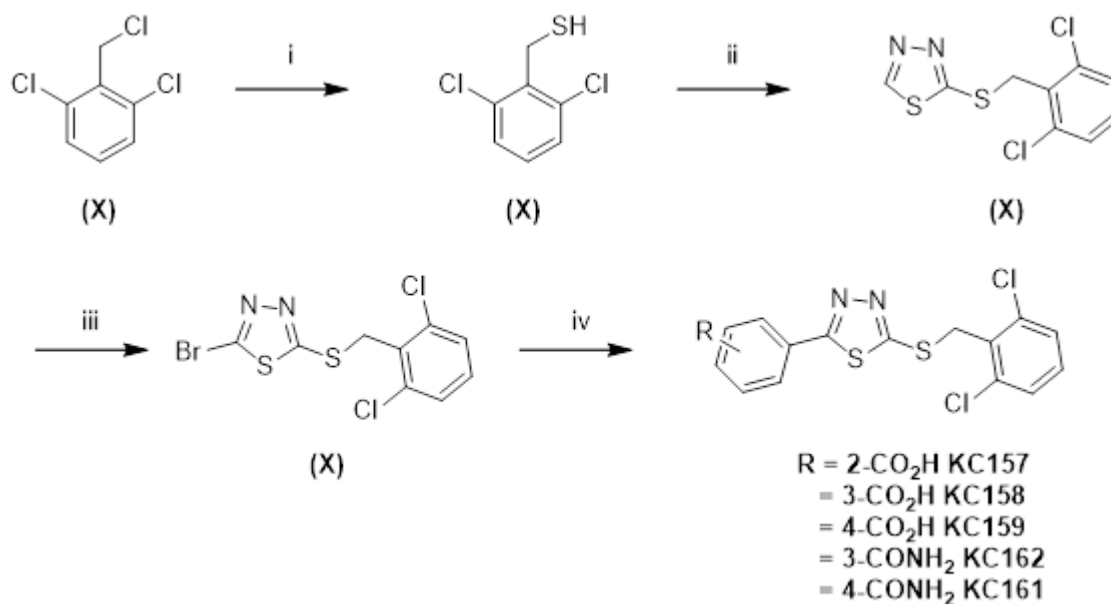
1608 overexpressing mPIEZO2. Traces in (ii) and (iii) are average currents from 3 consecutive

1609 pressure pulses. (B) Mean  $\pm$  SD for peak inward currents activated by the pressure pulse as

1610 exemplified in (A): non-transfected cells (N=17 independent recordings) and HEK 293 cells

overexpressing mPIEZO2 (N=11 independent recordings).

1611 SUPPLEMENTARY INFORMATION (SI)  
1612



1613  
1614  
1615 **Scheme X:** i) a) thiourea (1.1 eq.), EtOH, reflux, 3h b) NaOH, EtOH, reflux, N<sub>2</sub>, 3 h c) HCl  
1616 (98%) ii) 2-bromo 1,3,4-thiadiazole (1.0 eq.), K<sub>2</sub>CO<sub>3</sub> (1.2 eq.), DMF, 90 °C, 18 h (57%) iii) NBS  
1617 (1.4 eq.), DCM, reflux, N<sub>2</sub>, 48 h. (78%) iv) Appropriate boronic acid (1.0-3.5 eq.), K<sub>2</sub>CO<sub>3</sub> (4.0  
1618 eq.), Pd(PPh<sub>3</sub>)<sub>4</sub> (0.1-2 eq.) dioxane, H<sub>2</sub>O, 90 °C, N<sub>2</sub>, 2-24 h (7-68%)

1619  
1620 **Materials**

1621  
1622 All purchased chemicals and solvents were used without further purification unless otherwise  
1623 stated. All compounds were at least 95% pure by <sup>1</sup>H NMR.

1624  
1625 **Physical Methods**

1626  
1627 <sup>1</sup>H Nuclear Magnetic Resonance spectra were recorded at 500 MHz using a Bruker DRX 500  
1628 instrument or at 400 MHz using a Bruker DPX 400. <sup>1</sup>H spectra are referenced based on the  
1629 residual proton in the solvent (e.g. the CHCl<sub>3</sub>, 0.01 % in 99.99 % CDCl<sub>3</sub>). Coupling constants  
1630 (*J*) are reported to the nearest 0.1 Hz. <sup>13</sup>C NMR spectra were recorded at 125 MHz on 500  
1631 MHz spectrometers or at 100 MHz on 400 MHz spectrometers. HRMS was performed on a  
1632 Bruker Daltonics micrOTOF using positive electrospray ionisation (ES+). Automated column  
1633 chromatography (ACC) was carried out using a Biotage Isolera Four EXP with Spektra.

1634  
1635 **General Procedure A**

1636  
1637 The desired aromatic halide (0.28 mmol, 1.0 eq. the desired boronic acid (0.28-0.98 mmol, 1-  
1638 3.5 eq) and K<sub>2</sub>CO<sub>3</sub> (1.12 mmol, 4.0 eq.) were dissolved in dioxane (2 mL) and H<sub>2</sub>O (2 mL)  
1639 then degassed with N<sub>2</sub> for 30 minutes. Pd(PPh<sub>3</sub>)<sub>4</sub> (0.04 mmol, 0.15 eq.) was then added and  
1640 the reaction was then heated to 90 °C for 2-24 h. Upon completion, the reaction was diluted  
1641 with H<sub>2</sub>O (20 mL), extracted with DCM (3 × 10 mL), dried over Na<sub>2</sub>SO<sub>3</sub>, filtered and reduced  
1642 *in vacuo* to afford the crude or pure product.

1643  
1644 **2,6-dichlorobenzyl thiol (X)**

1645  
1646 2,6-dichlorobenzyl chloride (3.00 g 16.40 mmol) and thiourea (1.38 g, 18.40 mmol) were  
1647 dissolved in EtOH (36 mL) and heated to reflux for 3 h. The solution was cooled to room  
1648 temperature and dissolved in 2 M aq. NaOH (30 mL) and EtOH (10 mL) and the reaction was

1649 refluxed for a further 3 hours. The solution was then cooled, quenched with 1 M HCl (60 mL),  
 1650 reduced *in vacuo*, and extracted with EtOAc (3 × 20 mL). The combined organic layers were  
 1651 then washed with sat. NaCl solution (2 × 20 mL), dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness  
 1652 to afford an off-white crystalline solid without further need for purification (2.90 g, 15.00 mmol,  
 1653 98%). Rf 0.7 (9:1 Petroleum ether (40-60 °C):EtOAc (v/v)); δ<sub>H</sub> (500 MHz, CDCl<sub>3</sub>): 7.33 (2H, d,  
 1654 J = 8.0 Hz, benzyl 3-H), 7.15 (1H, t, J = 8.0 Hz, benzyl 4-H), 4.02 (2H, d, J = 8.5 Hz, benzyl  
 1655 CH<sub>2</sub>), 2.12 (1H, t, J = 8.5 Hz, SH); δ<sub>C</sub> (125 MHz, CDCl<sub>3</sub>): 137.3 (benzyl 1-C), 134.6 (benzyl 2-  
 1656 C), 128.5 (benzyl 4-C), 128.5 (benzyl 3-C), 24.4 (benzyl CH<sub>2</sub>).

### 1657 **2-((2,6-dichlorobenzyl)thio)-1,3,4-thiadiazole (X)**

1658  
 1659  
 1660 2,6-dichlorobenzyl thiol (2.85 g, 14.77 mmol), 2-bromo-1,3,4-thiadiazole (2.43 g, 14.77  
 1661 mmol), & K<sub>2</sub>CO<sub>3</sub> (2.38 g, 17.72 mmol) were dissolved in DMF (10 mL) and heated to 90 °C for  
 1662 18 h. The reaction was diluted with H<sub>2</sub>O (100 mL), extracted with EtOAc (3 × 40 mL) and the  
 1663 organic layers combined. These were washed with brine (3 × 40 mL), 10% LiCl (3 × 40 mL),  
 1664 dried over MgSO<sub>4</sub>, filtered and reduced *in vacuo* to give brown residue (4.33 g). This was  
 1665 purified by ACC (0-30% EtOAc in petroleum ether (40-60 °C)) to afford white crystalline solid  
 1666 (2.33 g, 8.43 mmol, 57%) Rf 0.60 (7:3 Petroleum ether (40-60 °C):EtOAc (v/v)); δ<sub>H</sub> (400 MHz,  
 1667 CDCl<sub>3</sub>): 8.99 (1H, s, thiadiazole 5-H), 7.27 (2H, d, J = 8.5 Hz, benzyl 3-H), 7.13 (1H, t, J = 8.5  
 1668 Hz, benzyl 4-H), 4.89 (2H, s, benzyl CH<sub>2</sub>); δ<sub>C</sub> (100 MHz, CDCl<sub>3</sub>): 164.7 (thiadiazole 2-C), 152.1  
 1669 (thiadiazole 5-C), 136.3 (benzyl 2-C), 131.7 (benzyl 1-C), 129.8 (benzyl 4-C), 128.5 (benzyl 3-  
 1670 C), 34.73 (benzyl CH<sub>2</sub>); m/z ES+ Found MNa<sup>+</sup> 298.9235, C<sub>9</sub>H<sub>6</sub>Cl<sub>2</sub>N<sub>2</sub>S<sub>2</sub> requires MNa<sup>+</sup>  
 1671 298.9242.

### 1672 **2-Bromo-5-((2,6-dichlorobenzyl)thio)-1,3,4-thiadiazole**

1673  
 1674  
 1675 2-((2,6-dichlorobenzyl)thio)-1,3,4-thiadiazole (2.33 g, 8.43 mmol), & N-bromosuccinimide  
 1676 (2.10 g, 11.80 mmol) were dissolved in DCM (10 mL) and refluxed for 48 h. The reaction was  
 1677 then cooled, quenched with sat. Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (20 mL), partitioned and the aqueous extracted with  
 1678 DCM (2 × 15 mL). The organic layers were combined, dried over MgSO<sub>4</sub>, filtered and reduced  
 1679 *in vacuo* to give an orange, oily crystals (3.15 g). This was purified by ACC (0-30% EtOAc in  
 1680 Petroleum ether (40-60 °C)) to afford a white crystalline solid (2.80 g, 7.87 mmol, 78 %) Rf  
 1681 0.80 (7:3 Petroleum ether (40-60 °C):EtOAc (v/v)); δ<sub>H</sub> (400 MHz, CDCl<sub>3</sub>): 7.37 (2H, d, J = 8  
 1682 Hz, benzyl 3-H), 7.24 (1H, dd, J = 8.5 & 7.5 Hz, benzyl 4-H), 4.92 (2H, s benzyl CH<sub>2</sub>); δ<sub>C</sub> (100  
 1683 MHz, CDCl<sub>3</sub>): 168.1 (thiadiazole 5-C), 138.1 (thiadiazole 2-C), 136.3 (benzyl 2-C), 131.5  
 1684 (benzyl 1-C), 129.9 (benzyl 4-C), 128.6 (benzyl 3-C), 34.6 (benzyl CH<sub>2</sub>); m/z ES+ Found MH<sup>+</sup>  
 1685 356.8462, C<sub>9</sub>H<sub>5</sub>BrCl<sub>2</sub>N<sub>4</sub>S<sub>2</sub> requires MH<sup>+</sup> 356.8512.

### 1686 **2-(5-((2,6-Dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzoic acid (KC157)**

1687  
 1688  
 1689 General procedure A was followed using 2-bromo-5-((2,6-dichlorobenzyl)thio)-1,3,4-  
 1690 thiadiazole (100 mg, 0.28 mmol), 2-(methoxycarbonyl)phenylboronic acid (43 mg, 0.28 mmol),  
 1691 K<sub>2</sub>CO<sub>3</sub> (155 mg, 1.12 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (35 mg, 0.03 mmol), dioxane (2 mL) and water (2 mL)  
 1692 to afford a crude orange solid. This was triturated with DCM to afford a yellow powder (16 mg,  
 1693 0.04 mmol, 14%). δ<sub>H</sub> (400 MHz, D<sub>6</sub>-DMSO): 7.91-7.88 (1H, m, benzoatyl 6-H), 7.73-7.67 (3H,  
 1694 m, benzoatyl 3, 4 & 5-H), 7.56 (2H, d, J = 8.0 Hz, benzyl 3-H), 7.42 (1H, t, J = 8.0 Hz, benzyl  
 1695 4-H), 4.85 (2H, s, benzyl CH<sub>2</sub>); δ<sub>C</sub> (100 MHz, D<sub>6</sub>-DMSO): 168.4 (carbonyl C), 167.9  
 1696 (thiadiazolyl 2-C), 164.7 (thiadiazolyl 5-C), 135.7 (benzoatyl 6-C), 133.1 (benzoatyl 1 or 2-C  
 1697 or benzyl 1-C), 132.1 (benzoatyl 3, 4 or 5-C), 131.9 (benzoatyl 1 or 2-C or benzyl 1-C), 131.6  
 1698 (benzoatyl 3, 4 or 5-C) 131.4 (benzyl 4-C), 131.3 (benzoatyl 3, 4 or 5-C), 130.3 (benzyl 2-C),  
 1699 129.4 (benzyl 3-C), 128.9 (benzoatyl 3, 4 or 5-C), 34.9 (benzyl CH<sub>2</sub>); m/z ES+ Found MH<sup>+</sup>  
 1700 396.9635, C<sub>16</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub> requires MH<sup>+</sup> 396.9639.

### 1701 **3-(5-((2,6-Dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzoic acid (KC158)**

1702  
 1703

1704 General procedure A was followed using 2-bromo-5-((2,6-dichlorobenzyl)thio)-1,3,4-  
 1705 thiadiazole (100 mg, 0.28 mmol), 3-(methoxycarbonyl)phenylboronic acid (43 mg, 0.28 mmol),  
 1706 K<sub>2</sub>CO<sub>3</sub> (155 mg, 1.12 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (35 mg, 0.03 mmol), dioxane (2 mL) and water (2 mL)  
 1707 to afford a crude orange solid. This was triturated with DCM to afford a yellow powder (6 mg,  
 1708 0.02 mmol, 7%) δ<sub>H</sub> (400 MHz, D<sub>6</sub>-DMSO): 8.43 (1H, s, benzoatyl 2-H), 8.17 (1H, d, *J* = 8.0 Hz,  
 1709 benzoatyl 4 or 6-H), 8.13 (1H, d, *J* = 8.0 Hz, benzoatyl 4 or 6-H), 7.71 (1H, ap. t, *J* = 8.0 Hz,  
 1710 benzoatyl 5-H), 7.57 (2H, d, *J* = 8.0 Hz, benzyl 3-H), 7.43 (1H, t, 8.0 Hz, benzyl 4-H), 4.87 (2H,  
 1711 s, benzyl CH<sub>2</sub>); δ<sub>C</sub> (100 MHz, D<sub>6</sub>-DMSO): 169.0 (carbonyl C), 166.9 (thiadiazolyl 2-C), 164.2  
 1712 (thiadiazolyl 5-C), 135.7 (benzyl 2-C), 132.4 (benzyl 1-C or benzoatyl 3-C), 132.2 (benzoatyl  
 1713 4 or 6-C), 132.0 (benzoatyl 4 or 6-C), 131.4 (benzyl 4-C), 130.5 (benzoatyl 5-C), 130.0 (benzyl  
 1714 1-C or benzoatyl 3-C), 129.4 (benzyl 3-C), 128.3 (benzoatyl 2-C), 35.1 (benzyl CH<sub>2</sub>); m/z ES+  
 1715 Found MH<sup>+</sup> C<sub>16</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S<sub>s</sub> requires MH<sup>+</sup> 396.9639.

1716

#### 1717 **4-(5-(2,6-Dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzoic acid (KC159)**

1718

1719 General procedure A was followed using 2-bromo-5-((2,6-dichlorobenzyl)thio)-1,3,4-  
 1720 thiadiazole (100 mg, 0.28 mmol), 4-(methoxycarbonyl)phenylboronic acid (43 mg, 0.28 mmol),  
 1721 K<sub>2</sub>CO<sub>3</sub> (155 mg, 1.12 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (35 mg, 0.03 mmol), dioxane (2 mL) and water (2 mL)  
 1722 to afford a crude orange solid. This was triturated with DCM to afford an orange powder (77  
 1723 mg, 0.19 mmol, 68%) δ<sub>H</sub> (400 MHz, D<sub>6</sub>-DMSO): 8.09-8.04 (4H, m, benzoate 2 & 3-H), 7.56  
 1724 (2H, d *J* = 8.0 Hz, benzyl 3-H), 7.43 (1H, t, *J* = 8.0 Hz, benzyl 4-H), 4.88 (2H, s, benzyl CH<sub>2</sub>); δ<sub>C</sub>  
 1725 (100 MHz, D<sub>6</sub>-DMSO): 168.8 (thiadiazolyl 2-C), 167.0 (thiadiazolyl 5-C), 164.8 (carbonyl C),  
 1726 135.7 (benzyl 2-C), 133.6 (benzoatyl 1 or 4-C), 133.3 (benzoatyl 1 or 4-C), 132.0 (benzyl 1-  
 1727 C), 131.4 (benzyl 4-C), 130.1 (benzoatyl 2 or 3-C), 129.4 (benzyl 3-C) 128.3 (benzoatyl 2 or  
 1728 3-C), 35.1 (benzyl CH<sub>2</sub>); m/z ES+ Found MH<sup>+</sup> 396.9627, C<sub>16</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S<sub>s</sub> requires MH<sup>+</sup>  
 1729 396.9639.

1730

#### 1731 **4-(5-((2,6-Dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzamide (KC161)**

1732

1733 General procedure A was followed using 2-bromo-5-((2,6-dichlorobenzyl)thio)-1,3,4-  
 1734 thiadiazole (100 mg, 0.28 mmol), 4-(aminocarbonyl)-phenylboronic acid (46 mg, 0.28 mmol),  
 1735 K<sub>2</sub>CO<sub>3</sub> (155 mg, 1.12 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (35 mg, 0.03 mmol), dioxane (2 mL) and water (2 mL)  
 1736 to afford a crude brown solid. This was purified by triturations with EtOAc and then DCM to  
 1737 afford a yellow solid (25 mg, 0.06 mmol, 21%). δ<sub>H</sub> (400 MHz, D<sub>6</sub>-DMSO): 8.03 (4H, bs,  
 1738 benzamidyl 2 & 3-H), 7.57 (2H, d, *J* = 8.0 Hz, benzyl 3-H), 7.43 (1H, t, *J* = 8.0 Hz, benzyl 4-  
 1739 H), 4.87 (2H, s, benzyl CH<sub>2</sub>); δ<sub>C</sub> (100 MHz, D<sub>6</sub>-DMSO): 169.0 (carbonyl C), 167.4 (thiadiazolyl  
 1740 2-C), 164.3 (thiadiazolyl 5-C), 137.2 (benzamidyl 1 or 4-C), 135.7 (benzyl 2-C), 132.0 (benzyl  
 1741 1-C or benzamide 1 or 4-C), 131.9 (benzyl 1-C or benzamidyl 1 or 4-C), 131.4 (benzyl 4-C),  
 1742 129.4 (benzamidyl 2 or 3-C), 129.0 (benzyl 3-C), 128.0 (benzamidyl 2 or 3-C), 35.1 (benzyl  
 1743 CH<sub>2</sub>); m/z ES+ Found MNa<sup>+</sup> 417.9608, C<sub>16</sub>H<sub>11</sub>Cl<sub>2</sub>N<sub>3</sub>OS<sub>2</sub> requires MNa<sup>+</sup> 417.9612.

1744

#### 1745 **3-(5-(2,6-Dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzamide (KC162)**

1746

1747 General procedure A was followed using 2-bromo-5-((2,6-dichlorobenzyl)thio)-1,3,4-  
 1748 thiadiazole (100 mg, 0.28 mmol), 3-(aminocarbonyl)-phenylboronic acid (46 mg, 0.28 mmol),  
 1749 K<sub>2</sub>CO<sub>3</sub> (155 mg, 1.12 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (35 mg, 0.03 mmol), dioxane (2 mL) and water (2 mL)  
 1750 to afford a crude brown solid. This was purified by triturations with EtOAc and then DCM to  
 1751 afford a yellow solid (44 mg, 0.10 mmol, 36%). δ<sub>H</sub> (400 MHz, D<sub>6</sub>-DMSO): 8.39 (1H, s,  
 1752 benzamidyl 2-H), 8.10 (1H, d, *J* = 8.0 Hz, benzamidyl 4 or 6-H), 8.07 (1H, d, *J* = 8.0 Hz,  
 1753 benzamidyl 4 or 6-H), 7.99 (1H, ap. t, *J* = 7.5 Hz, benzamidyl 5-H), 7.56 (2H, d, *J* = 8.0 Hz,  
 1754 benzyl 3-H), 7.43 (1H, t, *J* = 8.0 Hz, benzyl 4-H), 4.86 (2H, s, benzyl CH<sub>2</sub>); δ<sub>C</sub> (100 MHz, D<sub>6</sub>-  
 1755 DMSO): 169.3 (carbonyl C), 167.3 (thiadiazolyl 2-C), 164.0 (thiadiazolyl 5-C), 136.9  
 1756 (benzamidyl 3-C), 135.6 (benzyl 2-C), 132.0 (benzamidyl 1-C), 131.3 (benzyl 4-C), 130.8  
 1757 (benzamidyl 4 or 6-C), 130.6 (benzamidyl 4 or 6-C), 130.1 (benzamidyl 5-C), 129.8 (benzyl 1-

1758 C), 129.4 (benzyl 3-C), 127.1 benzamidyl 2-C), 35.1 (benzyl CH<sub>2</sub>); m/z ES+ Found MNa<sup>+</sup>  
1759 417.9609, C<sub>16</sub>H<sub>11</sub>Cl<sub>2</sub>N<sub>3</sub>OS<sub>2</sub> requires MNa<sup>+</sup> 417.9612.

1760

1761 **Potassium 4-(5-((2,6-dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzoate (KC289)**

1762

1763 4-(5-((2,6-dichlorobenzyl)thio)-1,3,4-thiadiazol-2-yl)benzoic acid (20 mg, 0.05 mmol) and KOH  
1764 (2.81 mg, 0.05 mmol) were dissolved in MeOH (3 mL) and heated to 60 °C for 18 h. The  
1765 reaction was then reduced under pressure to give a white solid without further need for  
1766 purification (20.92 mg, 0.05 mmol, 100%). δ<sub>H</sub> (500 MHz, D<sub>6</sub>-DMSO): 7.97 (2H, d, *J* = 8.0 Hz,  
1767 benzoatyl 2-H), 7.85 (2H, d, *J* = 8.0 Hz, benzoatyl 3-H), 7.56 (2H, d, *J* = 8.0 Hz, benzyl 3-H),  
1768 7.43 (1H, dd, *J* = 8.5 & 7.5 Hz, benzyl 4-H), 4.85 (2H, s, benzyl CH<sub>2</sub>); δ<sub>C</sub> (125 MHz, D<sub>6</sub>-DMSO):  
1769 170.0 (carbonyl-C), 167.5 (thiadiazolyl 2-C), 162.7 (thiadiazolyl 5-C), 135.6 (benzyl 2-C),  
1770 132.1 (benzyl 1-C), 131.3 (benzyl 4-C), 130.4 (benzoatyl 1/2-C), 129.4 (benzyl 3-C), 127.2  
1771 (benzoatyl 3/4-C), 35.1 (benzyl CH<sub>2</sub>); m/z ES+ Found MH<sup>+</sup> 396.9628, C<sub>16</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S<sub>s</sub>  
1772 requires MH<sup>+</sup> 396.9639.

1773

1774