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*N*-cinnamoylanthranilates as human TRPA1 modulators: Structure-activity relationships and channel binding sites

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1 *N*-Cinnamoylanthranilates as human TRPA1 Modulators: Structure-  
2 Activity Relationships and Channel Binding Sites.

3

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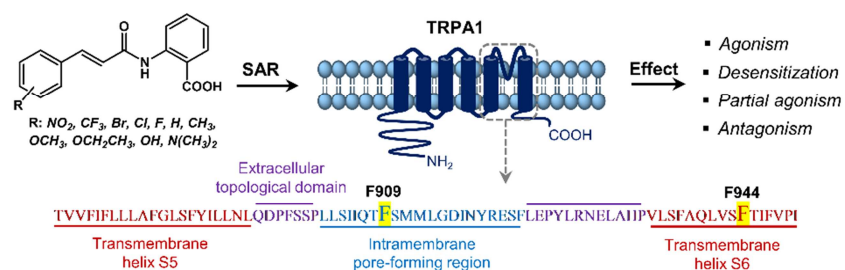
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17 **Graphical Abstract**

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21

22 **Highlights**23 *N*-Cinnamoyl anthranilate derivatives (CADs) are TRPA1 modulators

24 CADs with electron-withdrawing groups are TRPA1 agonists with desensitizing effects

25 CADs with strongly electron-donating groups are TRPA1 antagonists

26 CADs modulate TRPA1 through non-covalent interactions

27 F944A mutants show reduced sensitivity towards CADs and many other TRPA1 modulators

28

29 **Abstract**

30 The transient receptor potential ankyrin 1 (TRPA1) channel is a non-selective cation channel,  
31 which detects noxious stimuli leading to pain, itch and cough. However, the mechanism(s) of  
32 channel modulation by many of the known, non-reactive modulators has not been fully  
33 elucidated. *N*-cinnamoylanthranilic acid derivatives (CADs) contain structural elements from  
34 the TRPA1 modulators cinnamaldehyde and flufenamic acid, so it was hypothesized that  
35 specific modulators could be found amongst them and more could be learnt about modulation  
36 of TRPA1 with these compounds. A series of CADs was therefore screened for agonism and  
37 antagonism in HEK293 cells stably transfected with WT-human (h)TRPA1, or C621A,  
38 F909A or F944A mutant hTRPA1. Derivatives with electron-withdrawing and/or electron-  
39 donating substituents were found to possess different activities. CADs with inductive  
40 electron-withdrawing groups were agonists with desensitizing effects, and CADs with  
41 electron-donating groups were either partial agonists or antagonists. Site-directed  
42 mutagenesis revealed the CADs do not undergo conjugate addition reaction with TRPA1 and  
43 reveal that F944 is a key residue involved in the non-covalent modulation of TRPA1 by  
44 CADs, as well as many other structurally distinct non-reactive TRPA1 ligands already  
45 reported.

46 **Keywords:** transient receptor potential ankyrin 1; TRPA1; TRP; *N*-cinnamoylanthranilic  
47 acid; tranilast; calcium signaling; binding site; non-covalent

48

49 **Abbreviations Used**

50  $[Ca^{2+}]_i$ , intracellular calcium ion; CAD, *N*-cinnamoylanthranilic acid derivative; hTRPA1,  
51 human transient receptor potential ankyrin 1; hTRPM8, human transient receptor potential  
52 melastatin 8.

53

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## 55 1. Introduction

56 The human transient receptor potential ankyrin 1 (hTRPA1) channel [1] is predominantly  
57 expressed in the primary afferent (sensory) neurons [2] including trigeminal [3], dorsal root  
58 [4] and nodose ganglia, [5] and acts as a detector of chemical, mechanical and thermal stimuli  
59 [6]. TRPA1 is known to be involved in pain, itch, [7, 8] inflammatory diseases like arthritis  
60 [9], chronic obstructive pulmonary disease (COPD), and other respiratory diseases including  
61 chronic cough and asthma [6, 10]. There are many compounds known to modulate TRPA1,  
62 among them compounds containing reactive electrophilic groups that activate the channel  
63 through covalent modification of cysteine or lysine residues at the *N*-terminus of the protein  
64 [11, 12]. However, TRPA1 is also modulated by compounds through non-covalent  
65 interactions only. Mutagenesis and chimeric approaches have been utilized to elucidate the  
66 interactions of individual non-reactive chemicals with the channel [12-17]. However, the  
67 non-covalent mechanism(s) of modulation (activation or blocking) by compounds with  
68 distinct structures are currently much less well determined, hindering our understanding of  
69 the role of TRPA1 in diseases, and development of drugs targeting the channel.

70 Analogues of *N*-cinnamoylanthranilic acid (**1**) have shown a range of biological activities,  
71 including anti-allergic, anti-histaminic, anti-inflammatory, anti-asthmatic, [18, 19] anti-  
72 oxidant, [20] anti-fibrotic [21], anti-proliferative, [22] anti-cancer, [23] anti-platelet, [24]  
73 anti-coagulant [25] and as modulators of B-cells and T-cells, [26, 27], and are under  
74 investigations for several medicinal applications. The derivative *N*-(3,4-  
75 dimethoxycinnamoyl)anthranilic acid (**11**, Tranilast<sup>TM</sup> or Rizaben<sup>TM</sup>) is an anti-inflammatory  
76 drug that has been used in South Korea and Japan for over twenty years to treat allergic  
77 diseases such as bronchial asthma, allergic rhinitis, hypertrophic scars and scleroderma [18,  
78 21, 28]. *N*-cinnamoylanthranilate derivatives (CADs) also contain structural elements from  
79 two classes of known TRPA1 modulators, namely the  $\alpha,\beta$ -unsaturated carbonyl moiety found

80 in the agonist cinnamaldehyde (CA), [29] and the anthranilate moiety found in the agonist  
81 flufenamic acid (FFA) and related fenamates [30]. It was therefore anticipated that discovery  
82 of CAD agonists or antagonists of TRPA1 could be used to learn about the modulation of the  
83 channel by non-reactive compounds.

84 CADs have previously been shown to inhibit other TRP channels. For example *N*-(*p*-  
85 amylcinnamoyl)anthranilic acid (ACA), a PLA<sub>2</sub> inhibitor, [31] was characterized as a novel  
86 TRPM2, TRPM8, TRPC3, TRPC6 and TRPV1 channel blocker [32]. Similarly, *N*-(3-  
87 methoxyphenyl)-4-chlorocinnamide (SB366791) selectively blocks TRPV1 [33], yet is  
88 inactive against TRPM8 [34]. The anthranilates 5-nitro-2-(phenethylamino)benzoic acid  
89 (NPEB), 5-nitro-2-(phenethylamino)benzamide (NPBA) and 5-nitro-2-(3-  
90 phenylpropylamino)benzoic acid (NPPB), the latter which bears some overall similarity to  
91 CAD 1), selectively activates TRPA1 [35]. Therefore, to investigate both the structure-  
92 activity relationship (SAR) and pharmacology of CADs as TRPA1 modulators, we set out to  
93 synthesize and screen a series of compounds against TRPA1-transfected HEK293 cells.

94

## 95 2. Materials and Methods

### 96 2.1 Materials.

97 **2.1.1 Commercial TRPA1 modulators.** Cinnamaldehyde (CA,  $\geq 95$  %, natural), acrolein  
98 (ACR, analytical standard), allyl isothiocyanate (AITC, analytical standard), menthol (99 %),  
99 (-)-menthol and 5-nitro-2-(3-phenylpropylamino)benzoic (NPPB) were purchased from  
100 Sigma-Aldrich. Calcimycin (calcium ionophore, A23187), thymol ( $> 99.5$  %), carvacrol (98  
101 %), eugenol (99 %), cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597,  $\geq$   
102 98 %), (-)-nicotine ( $\geq 99$  %), *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA,  $\geq 98$  %),  
103 probenecid, 4-(4-chlorophenyl)-3-methylbut-3-en-2-oxime (AP18) and 2-(1,3-dimethyl-2,6-  
104 dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide (HC030031) were

105 purchased from Sigma-Aldrich. (1*E*,3*E*)-1-(4-fluorophenyl)-2-methylpent-1-en-3-one oxime  
106 (A967079), *N*-(3-aminopropyl)-2-((3-methylbenzyl)oxy)-*N*-(thiophen-2-ylmethyl)benzamide  
107 hydrochloride (AMTB.HCl) and SB366791 were purchased from Tocris Bioscience. Ethyl  
108 ((1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexane-1-carbonyl)glycinate (WS5) was from  
109 Millennium Specialty Chemicals Inc., Procter & Gamble, flufenamic acid (analytical  
110 standard) was from Fluka Analytical, mefenamic acid (98 %) was from Johnson Matthey  
111 Company and diclofenac (> 98 %) was from Tokyo Chemical Industry. Nordihydroguaiaretic  
112 acid (NDGA) and farnesylthiosalicylic acid (FTS) were purchased from Santa Cruz  
113 Biotechnology. The structures of these various TRPA1 modulators are shown in Figure 1.  
114 The stock solutions of the compounds were made and serially diluted to lower half-log scale  
115 concentrations in dimethyl sulfoxide (DMSO, 100 %, analytical reagent grade, Fisher  
116 Scientific), and thus the concentration of DMSO was maintained constant in a given total  
117 volume of sample.

118

### 119 **2.1.2 Chemistry.**

120 The Orion Corporation-disclosed aryl sulfonamide derivative (*S*)-*N*-(4-chlorobenzyl)-1-((4-  
121 fluorophenyl)sulfonyl)pyrrolidine-2-carboxamide (ASD) [36], the series of CADs and related  
122 derivatives were synthesized in-house using standard methods (see Supporting Information  
123 for details). Briefly, three synthetic routes were followed to synthesize the CADs. In one  
124 approach, the cinnamic acid derivative was converted to its corresponding acid chloride,  
125 which was coupled to methyl anthranilate, and the resulting methyl *N*-cinnamoylanthranilate  
126 was then hydrolyzed to yield the corresponding *N*-cinnamoylanthranilic acid derivative  
127 (Scheme 1). In the second approach, Meldrum's acid was reacted with anthranilic acid to  
128 produce 2-[(carboxyacetyl)amino]benzoic acid, which was then condensed with a  
129 benzaldehyde derivative *via* a piperidine-catalysed Knoevenagel condensation, producing *N*-



130 cinnamoylanthranilate as the piperidinium salt which was acidified to yield the final product  
131 (Scheme 2). In the third approach, a secondary amino acid was reacted with cinnamoyl  
132 chloride under basic conditions, and the resulting salt was acidified to yield the product  
133 (Scheme 3). Compound purity was assessed by satisfactory CHN combustion elemental  
134 microanalysis,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy, and a constant melting point. Chemical and  
135 spectroscopic data obtained for compounds synthesized (see Supporting Information) agreed  
136 closely with that reported, where available, in the literature. Stock solutions of these  
137 compounds were prepared as described above (Section 2.1.1).

138  
139 **2.2 Cell culture.** HEK293 cells stably transfected with either pcDNA3.1(+) constructs  
140 containing cDNA for hTRPA1 [37], hTRPM8 [38], hTRPA1 mutants or pcDNA3 (mock)  
141 were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM 4-(2-  
142 hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 4.5 g l<sup>-1</sup> glucose, supplemented  
143 with 2 mM L-glutamine, 100 U ml<sup>-1</sup> penicillin, 100 U ml<sup>-1</sup> streptomycin (all from Lonza), 10  
144 % v/v heat inactivated Fetal Bovine Serum (Life Technologies) and 0.25 mg ml<sup>-1</sup> geneticin  
145 (G418 Sulfate, Corning). The cells were grown in T75 tissue culture flask (Greiner Bio-One  
146 CELLSTAR) in a humidified cell culture incubator with 5 % CO<sub>2</sub> at 37 °C. When the cells  
147 reached ~90 % confluence, they were harvested in phosphate buffered saline (PBS, Sigma-  
148 Aldrich) solution and centrifuged at 205 × g for 4 min for calcium signaling experiments.

149  
150 **2.3 Calcium signaling.** The modulatory effects of the CADs were evaluated on hTRPA1,  
151 hTRPM8 and pcDNA3 (mock) transfected-HEK293 cells using a fluorescence-based calcium  
152 signaling assays at room temperature, either in the cuvette-based system or a micro-well plate  
153 system as described below.

154

155 Cuvette-based system: A previously reported protocol was followed [38]. Briefly, the cell  
156 pellet obtained from ~90 % confluence in T75 flask was resuspended in 0.5 ml complete  
157 DMEM and incubated with 2.5  $\mu$ l of 2.5  $\mu$ g  $\mu$ l<sup>-1</sup> Fluo-3AM fluorescent dye ( $\lambda_{Ex/Em}$  506/526  
158 nm, Life Technologies) for 30 min at room temperature with gentle rotary mixing (50 rpm).  
159 The cells were washed with PBS by centrifuging as before, and the pellet was resuspended in  
160 isotonic assay buffer [38] (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>.6H<sub>2</sub>O, 1  
161 mM CaCl<sub>2</sub> and 10 mM D-(+)-glucose made in double distilled water, and pH was adjusted  
162 with NaOH) at a density of  $5 \times 10^6$  cells ml<sup>-1</sup>. The cell suspension (100  $\mu$ l) was added to  
163 cuvettes containing the assay buffer (1.9 ml). The assays were carried out using a PTI  
164 fluorometer with FelixGX version 4.2.2 software. The amount of [Ca<sup>2+</sup>]<sub>i</sub> released upon  
165 activation of TRP channels were measured as a real-time-based fluorescence spectrum. The  
166 baseline of a spectrum was recorded for 20 s before the addition of a test compound (agonist)  
167 and the response was recorded for another 90 s, followed by the addition of calcimycin (2  
168  $\mu$ M) and recording for further 30 s. In antagonist assays, the standard antagonist, the vehicle  
169 control or the test compounds were pre-incubated with cells for 10 min prior to the addition  
170 of a standard agonist, and the spectrum was recorded as before.

171  
172 Micro-well plate system: 96-Well cell culture microplates (black polystyrene, flat  $\mu$ Clear  
173 bottom, Greiner Bio-One) were coated with poly-D-lysine (PDL, MW 70-150 kDa, Sigma-  
174 Aldrich, 50  $\mu$ g ml<sup>-1</sup> prepared in sterile PBS) as follows: PDL was added to the wells (200  $\mu$ l  
175 cm<sup>-2</sup>) and incubated for an hour at room temperature, excess PDL solution was aspirated and  
176 the wells were washed with PBS (2  $\times$  100  $\mu$ l per well), and air-dried at room temperature for  
177 2 h in a safety cabinet. Cell suspension (200  $\mu$ l of 12500 cells per well in complete DMEM)  
178 was added to the wells and incubated in the cell culture incubator (95% air and 5% CO<sub>2</sub> at 37  
179 °C) for 48 h to yield a final concentration of  $5 \times 10^4$  cells per well. The culture medium was

180 replaced with 100  $\mu$ l of 2  $\mu$ M Fluo-4AM [ $\lambda_{\text{Ex/Em}}$  494/506 nm (Life Technologies), diluted in  
181 phenol red-free DMEM (Life Technologies) from DMSO stock] and incubated in the dark at  
182 room temperature for 45 min. The cells were rinsed with PBS ( $2 \times 100 \mu$ l per well), and the  
183 isotonic assay buffer (100  $\mu$ l per well for agonist assays or 50  $\mu$ l per well for antagonist  
184 assays) was added to the wells. In antagonist assays, the cells in 50  $\mu$ l buffer were incubated  
185 with 50  $\mu$ l antagonists for 10 min before assaying. The assays were carried out using a  
186 FlexStation3 Molecular Devices with the SoftMax Pro Software version 5. The protocol  
187 utilized was adapted from the literature [39, 40]. Briefly, the real-time-based fluorescence  
188 spectra were recorded using the read-mode/type: Flex fluorescence (RFUs) bottom-read. An  
189 excitation and emission wavelengths of 485 and 538 nm were used for measurements with  
190 530 nm auto cut-off. Each spectrum was recorded for a total run time of 240 s, where the  
191 baseline was recorded for 20 s, and at the end of which the first addition (compound) was  
192 made followed by the second addition (calcimycin) at 180 s.

193

194 To determine the reversibility of an antagonist, after pre-incubation, the cells were washed  
195 with the assay buffer and resuspended in fresh assay buffer, and the response for the TRPA1  
196 standard agonist CA (30  $\mu$ M) was examined. Competitiveness of an antagonist was  
197 determined by pre-incubating cells with the test antagonist compound of a known  
198 concentration for 10 min and by considering the shift in the dose-response curve of CA with  
199 and without the inhibitor.

200

201 **2.4 Graphical and statistical analyses.** The agonism and antagonism responses were  
202 calculated either as a percentage of calcimycin or a standard agonist respectively, using the  
203 differences in the maximum and minimum relative fluorescence unit ( $\Delta$ RFU) values. Initial  
204 screening results correspond to three independent experiments ( $N = 3$ ), and the errors

205 reported are the standard error of the mean (SEM). The dose-response curves were plotted  
206 and analyzed using GraphPad Prism version 5.03, and the values of logEC<sub>50</sub> and logIC<sub>50</sub> were  
207 obtained with their SEM. Each data point on a dose-response curve corresponds to the mean  
208 of three independent experiments ( $N = 3$ ), performed either in duplicates ( $n = 6$ ) or triplicates  
209 ( $n = 9$ ), with their SEM. The responses were normalized by subtracting the noise/response  
210 obtained for the vehicle control (DMSO). In antagonist assays, the maximum response was  
211 obtained by normalising the standard agonist response to 100 %. Thus, in antagonist assays, a  
212 higher percentage value indicates a lower inhibitory effect by the test compound. To  
213 determine statistical significance between groups, the one-way analysis of variance  
214 (ANOVA) at  $p < 0.05$  was carried out.

215

216 The standard agonists and antagonists for positive controls were chosen, based on the ion  
217 channel specificity and potency of the ligands, and a concentration near or above EC<sub>50</sub> or IC<sub>50</sub>  
218 were used in the assays. The standard agonists used for the hTRPA1 channels were CA (30  
219  $\mu\text{M}$ ), ACR (30  $\mu\text{M}$ ) or AITC (10  $\mu\text{M}$ ), and for hTRPM8 was WS5 (1  $\mu\text{M}$ ). The standard  
220 antagonists used were the potent TRPA1 selective A967079 (100 or 300 nM) and TRPM8  
221 selective AMTB.HCl (1 or 3  $\mu\text{M}$ ).

222

223 **2.5 Indirect measure of inertness towards covalent modification.** As CADs could  
224 conceivably react with the TRPA1 *via* a conjugate addition processes, we wanted first to  
225 disprove that they reacted with a model nucleophile in the absence of the channel (see similar  
226 studies in [41]). Thus compound **1a** (4 equiv., 226 mM) and *N*-acetyl-L-cysteine methyl ester  
227 (1 equiv., 56 mM) or *N*-acetyl-L-cysteine (1 equiv., 61 mM) in *d*<sub>6</sub>-DMSO were mixed  
228 together, and the progress of any reaction was monitored using <sup>1</sup>H NMR spectra recorded at  
229 25 °C and at known time intervals.

230

231 **2.6 Site-directed mutagenesis.** hTRPA1 mutants C621A (site of covalent modification) [13],  
232 F909A or F944A (sites of non-covalent interaction identified by [16, 17]) cDNAs were  
233 produced using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent  
234 Technologies) as instructed in their manual, using pcDNA3.1(+) as the template and hTRPA1  
235 primers containing the mutants (synthetic oligonucleotides purchased from Eurofins  
236 Genomics MWG Operon). Briefly, hTRPA1 mutants strands were synthesized by performing  
237 18 cycles of PCR, followed by digestion of the parental- and hemi-methylated supercoiled  
238 dsDNA in the amplified products with *Dpn* I restriction enzyme. The amplified DNA was  
239 then transformed into XL10-Gold ultracompetent cells (*E. coli*) treated with  $\beta$ -  
240 mercaptoethanol. Plasmid DNA was isolated from the *E. coli* and purified using a  
241 NucleoSpin<sup>®</sup> Plasmid DNA purification miniprep kit (Macherey-Nagel) according to the  
242 manufacturer's protocol. The dsDNA samples were quantified (NanoDrop Lite  
243 Spectrophotometer, Thermo Scientific), and sequenced (Eurofins Genomics DNA sequencing  
244 service) and compared against the parent hTRPA1 sequence (sequence ID: Y10601) to  
245 confirm the mutations.

246

247 HEK293 cells were transfected with the hTRPA1 mutants dsDNA ( $0.3 \mu\text{g} \mu\text{l}^{-1}$ ) separately  
248 using FuGENE<sup>®</sup> 6 Transfection Reagent (Promega) as per the manufacturer's instructions.  
249 The transfection reagent FuGENE and DNA were added in 3:1 ratio. G418 ( $0.5 \text{ mg ml}^{-1}$ )  
250 resistance cells were then expanded in T25 and T75 flasks sequentially. The transfected cells  
251 were single cell cloned by serial dilution in a 96-well cell culture plate following Corning's  
252 procedure. Monoclonal cells were transferred to a 6-well cell culture plate and then into T75  
253 flasks for expansion. The cells were characterized with known hTRPA1 specific agonists and  
254 antagonists using the calcium signaling technique and compared against the WT-hTRPA1

255 responses to determine the level of TRPA1 protein stably expressed in the transfected  
256 HEK293 cells. The known TRPA1 ligands characterized in the hTRPA1 mutants were CA,  
257 AITC, ACR, menthol, thymol, carvacrol, eugenol, FFA, MFA, DCF, NPPB, URB597, FTS,  
258 (-)-nicotine, NDGA, probenecid, HC030031, AP18, A967079 and ASD.

259

260 **2.7 Electrophysiology.** Experiments involving animals were performed by IR Pharma  
261 (London, UK). Experiments were performed in accordance with the U.K. Home Office  
262 Guidelines for Animal Welfare based on the Animals (Scientific Procedures) Act of 1986 and  
263 the ARRIVE guidelines [42]. Vagus nerves of eight male Dunkin-Hartley guinea pigs  
264 (300-400 g) were isolated, characterized and experimented as described previously [43, 44].  
265 Compound stock solutions were prepared in DMSO and diluted 1000× in Krebs-Henseleit  
266 (KH) buffer. The depolarization was recorded in mV. To determine if a compound caused  
267 activation of the nerve, non-cumulative concentration responses to potential tussive stimuli  
268 were carried out. A control response to the TRPV1 agonist capsaicin (1  $\mu\text{M}$ ) was carried out  
269 to determine nerve viability, following which the nerve was stimulated with single  
270 concentrations of a test compound for 2 min. After application of each concentration of the  
271 compound, the nerve was washed with KH buffer until it returned to baseline, and this was  
272 repeated with the full range of concentrations. A similar stimulation was also carried out with  
273 the vehicle control (DMSO). A further control response to TRPA1 agonist acrolein (300  $\mu\text{M}$ )  
274 was carried out at the end of the experiment to determine nerve viability.

275

276 In order to study antagonism, the nerve was exposed to the agonist ACR (300  $\mu\text{M}$ ) for 2 min  
277 and washed with KH buffer until the response returned to baseline. This was repeated to  
278 provide two control agonist responses. The nerve was then pre-treated with a test compound  
279 for 10 min, and then re-stimulated with ACR (300  $\mu\text{M}$ ) for 2 min 20 s (the additional 20 s

280 was to allow for the changeover of stimuli) to assess if the compound was able to affect the  
281 magnitude of the depolarization induced by the ACR. Following a brief washout, the nerve  
282 was exposed to ACR (300  $\mu\text{M}$ ) for 2 min to provide a recovery response to ensure nerve  
283 viability and that the compound was washed off.

284

### 285 **3. Results**

286 **3.1 Calcium signaling.** The CADs were evaluated in HEK293 cells stably transfected with  
287 hTRPA1, hTRPM8 or pcDNA3 using a fluorescence-based calcium signaling technique.  
288 Agonism of the compounds were measured by assessing the  $\text{Ca}^{2+}$  influx in TRPA1-HEK293  
289 cells upon exposure to the test compounds relative to calcimycin (calcium ionophore,  
290 A23187), and antagonism of the compounds were measured by the ability of the test  
291 compounds to antagonize the agonism of a standard agonist. Hence, the agonism and  
292 antagonism responses of the compounds are presented as the percentage of calcimycin and  
293 standard agonist, respectively (Tables 1 and 2). The vehicle control (0.2 or 0.4 % DMSO) had  
294 no significant activity in hTRPA1 and in mock transfected-HEK293 cells (data not shown),  
295 but had a slight antagonising effect (inhibits ~20 % of WS5 response) in hTRPM8.

296

297 Carboxylic acid-containing compounds were evaluated as their corresponding carboxylate  
298 salt due to the isotonic assay buffer (pH 7.4). Even so, for some derivatives insolubility in the  
299 buffer at higher concentrations (300  $\mu\text{M}$ , or in some cases  $>100 \mu\text{M}$ ) prevented completion of  
300 the dose-response curve (Fig. 3). Most of the CAD esters were partially soluble or insoluble  
301 above 3  $\mu\text{M}$ , and hence screening at higher concentrations was not possible. The CADs, **14**  
302 and **15** autofluoresced in the wavelength region of Fluo-3 and Fluo-4 dyes, so screening was  
303 not possible. Due to broad absorption ranges for these two CADs other long-wavelength  
304 calcium dyes were found not to be suitable either.

305

306 **3.2 CAD Structure-Activity Relationship in hTRPA1.** In the initial screening of *N*-  
307 cinnamoylanthranilic acids (Table 1), the CADs **1-10**, **20** and **21** showed  $\geq 25$  % agonism at  
308 30  $\mu\text{M}$  and  $\geq 50$  % inhibition at 100  $\mu\text{M}$ . The agonists **2**, **3**, **20** and **21** showed weak  
309 antagonism in the initial screen, relative to other antagonists (e.g. **4**, **5** and **6**), so full  
310 inhibition curves were not done and  $\text{IC}_{50}$  values were not calculated. The CADs **11**, **12**, and  
311 **16-19** showed weak agonism ( $< 25$  %) whereas CAD **13** showed no agonism. However, the  
312 CADs **11-13** showed  $\geq 40$  % inhibition, and **16-19** showed weak inhibitory effect ( $< 30$  %). In  
313 the initial screening of methyl *N*-cinnamoylanthranilates (Table 2), all the evaluated esters,  
314 including **1a**, **3a-8a**, **13a-15a**, and **20a-22a**, were agonists (Table 2). Most of the methyl *N*-  
315 cinnamoylanthranilate esters at 3  $\mu\text{M}$  showed a similar level of agonism to that of their  
316 corresponding *N*-cinnamoylanthranilic acid derivatives at 30  $\mu\text{M}$ . However, apart from **20a**  
317 and **21a**, none of the esters showed antagonism. Dose-response curves were carried out for *N*-  
318 cinnamoylanthranilic acids that showed higher than 25 % agonism at 30  $\mu\text{M}$ , and for those  
319 which had high antagonism and partial agonism. As can be seen from the curves, the effects  
320 observed were dose-dependent (Fig. 3).

321

322 The halogenated CADs **4** and **5**, with a bromo or chloro substituent at the *para*-position,  
323 had similar potency, whereas the fluorinated CAD **7** showed an increase in  $\text{EC}_{50}$  and  $\text{IC}_{50}$ ,  
324 relative to other halogens in the series. As the halogenated CADs **4-7** showed potent agonism  
325 and antagonism, it was thought that they might possess desensitising effects following  
326 activation of the channel. This was confirmed by recording the real-time spectra for 10 min  
327 (Fig. 4a,b). Since CA and FFA are known to have desensitising effects, [30, 45] they were  
328 also evaluated along with the CADs for comparison. At the concentration eliciting a  
329 maximum response, the elevated  $\text{Ca}^{2+}$  level caused by the agonism was sustained (Fig. 4b),



330 and at a submaximal concentration the effect dropped over 200-300 s. On continuous  
331 exposure to an agonist a diminished response was obtained (Fig. 4c-f), due to desensitization  
332 of the channel (see [30]). For the CADs tested at 10  $\mu$ M the agonism response dropped back  
333 nearer to the spectral baseline within 10 min of administration and desensitized the agonism  
334 of the CA (30  $\mu$ M) standard agonist (Fig. 4c-f). Due to this bimodal activity, potent agonism  
335 and desensitising effect, a washout experiment was carried out to examine the reversibility of  
336 the compounds. The desensitising effect shown by the halogenated CADs on hTRPA1 was  
337 found to be reversible up to 10  $\mu$ M for **4** and **5**, and up to 30  $\mu$ M for **6** and **7**, and were  
338 irreversible at higher concentrations (Fig. 5).

339

340 The CADs with a methyl (**8**) or methoxy (**10**) group possessed similar agonist activity at  
341 30  $\mu$ M in the initial screening. However, on comparing the EC<sub>50</sub> of **8** and **10**, CAD **8**  
342 appeared to be less potent. Disubstituted CADs **11** and **12** with electron donating groups  
343 (EDGs) exhibited partial agonist and antagonist activities, whilst **13** showed only an  
344 antagonistic effect and no agonism below 100  $\mu$ M. Nevertheless, the disubstituted CADs **11**  
345 and **12** were less potent antagonists compared to the monosubstituted **13**.

346

347 *Ortho*-substituted CADs **6** and **9** were less efficacious, relative to their corresponding  
348 *para*-substituted analogues **5** and **10**. In addition,  $\alpha,\beta$ -saturated derivatives **16** and **17** showed  
349 poor activity at 30  $\mu$ M compared to the corresponding CADs **1** and **10** with an  $\alpha,\beta$ -  
350 unsaturation. The compounds **18** and **19** with a non-planar ring replacing the anthranilate  
351 moiety showed weak responses relative to the parent compound **1**. The  $\alpha$ -substituted CADs  
352 either with a methyl or phenyl group were more potent agonists (**20** and **21**) relative to the  
353 unsubstituted CAD (**1**). Among the  $\alpha$ -substituted CADs **20** and **21**, and unsubstituted CAD **1**,  
354 the derivative **21** showed pronounced agonist potency.

355

356 The *N*-cinnamoylanthranilic acid methyl esters evaluated in hTRPA1 at 3  $\mu$ M (Table 2)  
357 showed a similar level of agonism to their corresponding *N*-cinnamoylanthranilate derivatives  
358 at 30  $\mu$ M (Table 1), but there were no considerable antagonism shown by the esters, except  
359 for **20a** and **21a** with desensitising effects.

360

361 **3.3 Channel selectivity.** To determine the channel selectivity of CADs, they were assessed in  
362 pcDNA3 mock-HEK293 cells for agonism, and in hTRPM8-HEK293 cells for both agonism  
363 and antagonism. The CADs, evaluated at 30 and 100  $\mu$ M, and CAD methyl esters, at 3  $\mu$ M,  
364 did not show any response in the negative control pcDNA3 mock-transfected HEK293 cells  
365 ( $N = 3$ , data not shown).

366

367 There was no agonism seen for any CADs in hTRPM8-transfected HEK293 at the  
368 concentrations 30 and 100  $\mu$ M of *N*-cinnamoylanthranilates, and 3  $\mu$ M of methyl *N*-  
369 cinnamoylanthranilates (data not shown), except for **21** and **21a** which exhibited weak  
370 agonism (Tables 1 and 2). The majority of the compounds which were hTRPA1 agonists,  
371 were weak hTRPM8 antagonists at high concentrations. Dose-response curves were carried  
372 out for, **3**, **4**, **6**, **9**, **20** and **22a**, that showed high antagonism (Tables 1 and 2), amongst which  
373 **22a** was the most potent hTRPM8 antagonist with  $IC_{50}$  10  $\mu$ M (Fig. 3g). In addition, it was  
374 observed from the  $IC_{50}$  values that the CAD **4**, which showed antagonism in both hTRPA1  
375 and hTRPM8, was more potent in hTRPA1. However, the CADs **6** and **9** had only a 10  $\mu$ M  
376 difference in the  $IC_{50}$  values obtained against hTRPM8 and hTRPA1 (Table 1).

377

378 **3.4 Determination of possible binding sites through mutagenesis studies.**

379 **Binding site.** CADs could undergo covalent modification due to the presence of an  $\alpha,\beta$ -  
380 unsaturated carbonyl group. Previously we have used NMR scale model reactions of reactive  
381 molecules with cysteine as a quick way of gauging susceptibility of a molecule towards  
382 covalent modification of a TRPA1 cysteine residue, and good correlation with agonism levels  
383 has been observed [41]. Therefore, the propensity of CADs to react through conjugate  
384 addition of the thiol group in cysteine (*N*-acetyl-L-cysteine methyl ester or *N*-acetyl-L-  
385 cysteine) to the  $\alpha,\beta$ -unsaturated double bond was evaluated for the representative CAD **1a**  
386 (Scheme S4, Supporting information) using a proton NMR time study. This CAD showed no  
387 reactivity to the thiol group of cysteine in *d*<sub>6</sub>-DMSO, as monitored by changes in intensity or  
388 appearance of new peaks in the NMR spectrum over 6 h (data not shown). However, to rule  
389 out covalent modification of the channel completely, studies of CADs with C621A mutants  
390 were conducted as part of a study to determine the binding site of the CADs.

391 CADs and a range of other known hTRPA1 active ligands were characterized in HEK293  
392 cells stably transfected with hTRPA1 mutants C621A, F909A or F944A at concentrations  
393 corresponding to the maximum response or closer to the EC<sub>50</sub> or IC<sub>50</sub> obtained in wild-type  
394 (WT)-hTRPA1 HEK293 cells. The EC<sub>50</sub> and IC<sub>50</sub> values obtained in the WT-hTRPA1 (Table  
395 3) were broadly consistent with the values found in the original reports, given possible  
396 variations in the assay methods, conditions, species and/or cell lines. Significantly reduced  
397 agonism was obtained, as expected, for the electrophilic compounds, CA, AITC and ACR in  
398 the mutant C621A-hTRPA1. Reduced agonism was also observed for the non-reactive  
399 compounds menthol, carvacrol, FFA, DCF, NPPB, FTS, probenecid and **22a**, relative to the  
400 responses obtained in WT-hTRPA1-HEK293 (Fig. 6a-e). With the exception of the saturated  
401 analogue **22a**, CADs produced increased responses relative to that observed in the WT-  
402 hTRPA1 (Fig. 6e).

403

404 A967079, AP18, CAD **13** and ASD all had reduced antagonistic responses in F909A-  
405 hTRPA1 expressing HEK293 cells relative to WT-TRPA1 expressing cells (Fig. 6f). No  
406 reduction in antagonism was observed for HC030031 in the F909 mutant. Menthol and  
407 carvacrol also showed a reduced agonist effect in the absence of F909, however most of the  
408 other compounds showed a significantly increased agonist response in this mutant compared  
409 to the WT (Fig. 6a-e).

410

411 In the mutant F944A-hTRPA1 HEK293 cells a significant drop in activity was found for  
412 ACR, menthol, carvacrol, eugenol and FFA, NDGA, probenecid, CADs **3** and **22a**, **8** and **20**,  
413 ACA, A967079 and AP18 and ASD, relative to the responses in WT-hTRPA1 (Fig. 6). In the  
414 case of ACA and **3**, reduced responses were obtained only at a lower concentration (10  $\mu$ M)  
415 and not at a higher concentration (30  $\mu$ M), as shown in Fig. 6e.

416

417 **3.5 Electrophysiology.** The agonist and antagonist effects of the compounds, **3** and **5** at 100  
418  $\mu$ M, and **13** at 300  $\mu$ M, were tested on fully characterized isolated guinea pig vagus nerve  
419 preparations. The compounds showed a small degree of activation of the vagus nerve (Fig.  
420 7a-c), with **3** having the largest effect as in the hTRPA1-HEK293 cells. However, the  
421 potency of the responses obtained in tissue (nerve) did not compare to those obtained in  
422 HEK293 cells overexpressing hTRPA1.

423

#### 424 **4. Discussion**

425 TRPA1 is activated by a wide range of stimuli. Some of the chemical modulators activate the  
426 channel *via* covalent modification of specific residues on the *N*-terminal of the channel [6, 11,  
427 12], however a large number do not possess the reactive groups to do likewise [12]. Here we  
428 have studied CADs as modulators of TRPA1 activity, and investigated where these  
429 compounds bind to the channel. Comparing trends in the SAR of the acid CADs against

430 hTRPA1, we noted the parent (unsubstituted) compound **1** had a moderate agonistic effect,  
431 and the electron withdrawing group (EWG)-substituted CADs **2** and **3** were more potent  
432 agonists. The halogenated CADs (**4** - **7**), with inductive electron withdrawing but lone pair  
433 donating properties showed potent agonism with desensitising effect, and the CADs **8** - **10**,  
434 with a weak/moderate electron donating group (EDG), showed bimodal activity, that is they  
435 possessed partial agonism and antagonism. As the electron donating nature of the EDG  
436 becomes stronger the agonism of the compounds decreased and became antagonists (**11** - **13**).  
437 The potent agonism and antagonism of the halogenated CADs was due to a desensitising  
438 effect following activation of the channel, as was also shown for FFA [30]. This was  
439 reversible at low CAD concentration (<10 $\mu$ M) but irreversible at higher concentrations.

440  $\alpha,\beta$ -Saturated analogue **16** and **17** showed poor activity compared to the  
441 corresponding  $\alpha,\beta$ -unsaturated CADs. However it is interesting to note that the related,  
442 flexible derivative NPPB, a classic chloride ion channel antagonist, is a sub-micromolar  
443 agonist of TRPA1 [35]. Unlike the CADs studied, NPPB possesses a nitro group in the  
444 anthranilate ring, and replacing the anthranilate moiety in our CADs with a saturated cyclic  
445 amino acid (**18** and **19**) produced weak responses. This suggests that the anthranilate moiety  
446 probably plays a key role in the activity of the CADs.

447 The agonism of structurally related compounds in the literature including ACA, an  
448 inhibitor of several TRP channels, [32], and SB366791, a selective TRPV1 antagonist [33,  
449 34], were evaluated against hTRPA1-HEK293. ACA [32], with a 4-pentyl substituent,  
450 activated hTRPA1 potently relative to the 4-methyl substituted CAD **8**. Despite being similar  
451 to CAD **5** (but lacking COOH on the *N*-aryl ring), SB366791 had no agonism in hTRPA1  
452 (data not shown). A similar trend to that observed for the EWG and EDG substituted CADs,  
453 has also been reported for modulation of hTRPA1 by substituted benzylidenemalononitriles.  
454 [46]

455

456 HEK293 cells endogenously express a number of ion channels and receptors, including  
457 purinoceptors (P2Y<sub>1</sub> and P2Y<sub>2</sub>) [47], voltage-gated potassium channels [48, 49], sodium  
458 channels ( $\beta$ 1A and Na<sub>v</sub>1.7) [50, 51], sphingosine-1-phosphate receptors (Edg-1, Edg-3 and  
459 Edg-5) [52], calcium channels [53] including TRPC1, TRPC3, TRPC4, TRPC6 and TRPC7  
460 [54, 55], and M<sub>3</sub> muscarinic acetylcholine receptor [56]. The CADs and methyl *N*-  
461 cinnamoylanthranilate, did not activate the negative control mock-transfected HEK293 cells,  
462 proving that in TRPA1-HEK293 cells the compounds were selective to TRPA1.

463

464 TRPM8, a cold-sensitive channel [57], is stimulated by a number of TRPA1 modulators  
465 [58, 59]. Therefore, CADs were evaluated against stably transfected hTRPM8-HEK293 cells  
466 for selectivity between these two channels. CADs active in hTRPM8 contained either a  
467 halogen, an *ortho*- or an  $\alpha$ -substituent. Interestingly, most of the TRPA1 agonists were  
468 hTRPM8 antagonists at higher concentrations. However assays against a range of other ion  
469 channels are necessary to confirm overall channel selectivity.

470

471 Looking at the physicochemical properties (Table S1) among similar analogues,  
472 compounds possessing a higher clogP were the more potent agonists, as also seen in  
473 examples in the literature [35, 60]. Generally, the CADs with a higher number of hydrogen  
474 bond donors and acceptors, larger topological polar surface area and logS, and lower logP  
475 had greater inhibitory effects with poor or no hTRPA1 agonism (Table S1).

476

477 To determine the binding site(s) of the ligands, mutant TRPA1-expressing HEK293 cell  
478 lines were created. The residues mutated were chosen based on the functional groups on the  
479 ligands and the residues that could participate in either covalent modification (C621 [13]) or

480 through non-covalent aromatic interactions (F909 [16] and F944 [17]). C621A-hTRPA1  
481 showed significantly reduced agonism responses for electrophilic compounds, in line with  
482 previous reports [11, 12]. We also observed significantly diminished agonism with non-  
483 reactive compounds. A similar type of effect had been previously described for menthol,  
484 NPPB and FTS [35]. However the reduced effects observed with NPPB and FTS in the  
485 mutant C621A were not statistically significant, though a significant reduction in agonism  
486 was observed with the mutations of different cysteine residues at the *N*-terminus [35, 61]. The  
487 TRPA1 antagonists could not be evaluated in this mutant, since the hTRPA1 standard  
488 agonists CA, AITC and ACR bind to the mutated site. In addition to the <sup>1</sup>H NMR study  
489 carried out with CAD **1a**, the findings with the C621A mutant further suggest that CADs do  
490 not undergo covalent modification at the TRPA1 *N*-terminal cysteine. The reduced agonism  
491 observed for CAD **22a** is however an anomaly, but this can be explained by chemical  
492 reactivity observed for this ester. When attempting to hydrolyse **22a**, to get the free acid, the  
493 compound eliminated the phenoxy group, presumably *via* an E1cb mechanism, to yield an  
494 acrylamide derivative. With reduced conjugation and no  $\beta$ -substituent, this compound would  
495 be expected to be much more likely to undergo covalent addition reaction compared to a  
496 cinnamide. Thus, the change in modulation of **22a** in the C621A mutant could be explained if  
497 such a process also takes place inside the cell.

498  
499 For F909A-hTRPA1, significantly reduced inhibition was observed as expected for  
500 A967079 relative to the WT-hTRPA1 response [16]. Significantly reduced antagonism was  
501 also observed for the structurally similar oxime AP18. Since the tested antagonists with  
502 distinct structures, including CAD **13** and ASD, also showed significant differences in this  
503 mutant, F909 residue appears to be a key residue for antagonist binding in hTRPA1. Both  
504 CAD **13**, ASD and HC030031 incorporate two aromatic rings joined by a linking group. The

505 reduced antagonism was not observed for HC030031, but this could be explained by the fact  
506 that the aromatic methyl xanthine half of the molecule is a larger bicyclic heteroaromatic ring  
507 structure, as opposed to a single benzenoid rings in CAD **13** and ASD; it is therefore likely to  
508 have a different binding site. There was also a drop in the agonism of menthol and carvacrol  
509 in the absence of the F909. As menthol lacks aromaticity and cannot participate in aromatic  $\pi$   
510 interactions, it appears a more general hydrophobic interaction may be involved here,  
511 possibly also involving residues neighbouring F909, such as T874. For most of the other  
512 compounds the responses were significantly higher than that observed in WT-hTRPA1. This  
513 could be due to changes in the structural conformation of hTRPA1 as a result of the  
514 substitution of the aromatic residue F909 to alanine.

515

516 The mutant F944A-hTRPA1-HEK293 cells displayed a significantly reduced response to  
517 many of the ligands evaluated, including the non-aromatic compounds ACR and menthol that  
518 cannot participate in  $\pi$ - $\pi$  interactions. The cryo-EM structure of TRPA1 shows that F944 is  
519 buried within the channel structure [16], and apparently closed to binding without  
520 reorganization of the channel structure. Samanta *et al* [62] used limited proteolysis and mass  
521 spectrometry to evaluate the effect of a small number of non-electrophilic channel modulators  
522 (menthol, antagonist A967079, and agonist PF-4840154) on mouse TRPA1. In that work,  
523 several regions of TRPA1 were proposed as experiencing conformational change upon  
524 binding of the ligands studied. However, F944 and the S5 and S6 helices were not amongst  
525 those implicated. Nevertheless our results, using a wider range of modulators and with  
526 hTRPA1, indicate that it is possible that the reduced activities observed for our F944A  
527 mutant is also indicative of significant structural reorganizations of the S5 and S6 helices  
528 upon ligand binding. The responses obtained for A967079 in both F909 and F944 mutants  
529 were equal. However, the analogue AP18 had significant differences in response despite the



530 close similarity in their structures; the AP18 inhibition was nearly abolished in the mutant  
531 F944A, but not in F909A. ASD, however, preferentially binds F909 over F944, possibly due  
532 to its greater size, site accessibility and location of the amino acid in the channel structure.

533 The residues F909 and F944 from each TRPA1 subunit are located in a 'ring' amongst the  
534 S5 and S6 helices, a portion of the channel which is thought to be membrane bound [16] (Fig.  
535 8a-c). F909 is located at the bottom of a shallow pocket, whereas F944 is buried within the  
536 tertiary structure of the channel (Fig. 8c-d). Whilst previously EM studies have suggested the  
537 location of the A967079 binding site [16], this area is buried behind the F909 residue in the  
538 atomic model 3J9P. The drop-in activities of modulators in F909A and F944A demonstrated  
539 that the corresponding compounds may possibly bind to the aromatic phenylalanine in the  
540 hTRPA1 putative pore region non-covalently *via*  $\pi$ - $\pi$  stacking and/or hydrophobic  
541 interactions. Whilst further structural biology will be needed to elucidate detailed ligand  
542 binding, derivatives with hydroxy groups, anthranilic acid derivatives such as non-steroidal  
543 anti-inflammatory drugs, CADs and aryl sulfonamide derivatives, showed significant  
544 reductions in responses in the mutant F944A, suggesting this residue might serve as a general  
545 determinant in the modulation of hTRPA1. Complete elimination of the responses were not  
546 observed with most of the ligands suggesting they may also interact with other nearby  
547 residues.

548

549 The most potent CADs (**3**, **5** and **13**) were also characterized on isolated guinea pig vagus  
550 nerves. Unfortunately, the potency seen in the TRPA1-HEK293 cells was not observed in the  
551 nerve tissue. ACR was used as the TRPA1 standard agonist to determine the inhibitory  
552 effects of the compounds in guinea pig vagus nerve tissue, as desensitization of responses  
553 were observed with the use of CA in depolarization measurements [45, 63]. As a positive  
554 control, incubation with the TRPA1 standard antagonist HC030031 inhibited the

555 depolarization induced by ACR. However, contrary to the antagonistic effects observed in  
556 hTRPA1-HEK293 cells, neither of the compounds **5** and **13** exhibited any antagonism against  
557 depolarization of the guinea pig vagus nerve by ACR. This discrepancy in results could be  
558 due to, but not limited to, experimental differences or a consequence of species difference.  
559 There is only 79% identity between the amino acid sequences of hTRPA1 and guinea pig  
560 TRPA1. The low sequence homology with 21% variation could cause differences in  
561 responses. Caffeine, menthol and thioaminals (e.g. 4-methyl-*N*-[2,2,2-trichloro-1-(4-nitro-  
562 phenylsulfanyl)-ethyl]-benzamide), are potent antagonists in hTRPA1 but showed reduced  
563 potency, agonism or inactivity in rodent TRPA1 [64-66]. Similarly, rodent TRPA1 is  
564 activated by cold, whereas human and rhesus monkey TRPA1 are not [67]. The menthol and  
565 cold species difference were attributed to a single residue, V875 in primates and G878 in  
566 rodents [67].

567  
568 A possible correlation is also noted between the various observed biological activities of  
569 CADs and TRP channels. This is plausible due to the known participation of TRP channels in  
570 a wide range of cellular functions [68]. Key to this suggestion is a study [69] which reports  
571 that TRPA1 is necessary for TGF- $\beta$  signaling. Loss of the receptor significantly suppresses  
572 the mRNA expression of the inflammatory cytokines, IL-6,  $\alpha$ -smooth muscle actin involved  
573 in fibrosis, substance P involved in inflammation, VEGF, collagen I $\alpha$ 1 and the  
574 phosphorylation of kinases induced by TGF- $\beta$ , and thereby results in attenuation of  
575 fibrogenic and inflammatory reactions. Likewise, in the literature, the anti-allergic, anti-  
576 inflammatory [18, 19], anti-oxidant [20], anti-fibrotic [70], anti-proliferative [22] and anti-  
577 cancer [23] properties of CADs were related to inhibition of cytokines, chemokines and  
578 growth factors. Therefore the fundamental reason behind attenuation of cytokines and growth  
579 factors by CADs, might be the consequence of effects imposed by CADs on TRP channels.

580

581 **5. Conclusions**

582 The series of CADs possess various activities in hTRPA1, ranging from agonism, partial  
583 agonism, antagonism and desensitizing effects. The structurally related compound, ACA, an  
584 inhibitor of TRPM2, TRPM8, TRPC3, TRPC6 and TRPV1 [32] activates hTRPA1 with  
585 similar potency to those of other CADs evaluated in the study. CAD **13** (*p*-OH), with a strong  
586 EDG-substituent, and CAD **21** ( $\alpha$ -Ph), show promise as a selective hTRPA1 antagonist and  
587 agonist, respectively. Using the key SARs found in the study, the structures of the  
588 compounds could be optimized to make CADs more potent modulators of TRPA1. Moreover,  
589 F944 was found to be a key residue for many TRPA1 active modulators, including menthol,  
590 anthranilates including fenamates and CADs, and ASD. Activity for these modulators was  
591 much reduced in the F944A mutant, indicating that this and other residues (F909) within the  
592 TRPA1 S5-S6 putative pore region are crucial for chemosensation.

593

594 **Conflicts of Interest**

595 The authors declare no conflict of interest.

596

597 **Author Contributions**

598 A.C., L.R.S., A.N.B. and A.H.M. designed the study overall. A.C. performed the chemical  
599 and pharmacology experiments, and wrote the initial draft of the manuscript. L.R.S., A.N.B.,  
600 A.C. and A.H.M. contributed to the interpretation of the chemical and pharmacological data,  
601 and M.J.McP. performed the molecular modelling study and interpretation of the combined  
602 results therefrom. The final manuscript was reviewed and approved by all authors.

603

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612

### 613 **Supporting Information**

614 The following supplementary data to this article can be found online at: <http://xxxxxxxxxxxxx>

- 615 1. Chemical synthesis procedures. 2. Characterization data: NMR, MS, EA and m.p. for the  
616 compounds synthesized. 3. Table S1: Physicochemical properties of the CADs evaluated. 4.  
617  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of the compounds prepared.

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**Figure and Scheme Legends**

**Scheme 1.** Synthesis of *N*-cinnamoylanthranilate derivatives from cinnamic acids. The derivatives **1-8**, **13-17**, **20**, **21**, and their corresponding methyl *N*-cinnamoylanthranilate ester derivatives, including **22a**, were synthesized using this approach.

**Scheme 2.** Synthesis of *N*-cinnamoylanthranilic acid derivatives from aldehydes. The derivatives **9-12** were synthesized using this approach.

**Scheme 3.** Synthesis of *N*-cinnamoyl amino acids (*rac*-**18** and *S*-**19**).

**Figure 1.** Chemical structures of non-CAD TRPA1 modulators involved in this work.

**Figure 2.** Chemical structures of the *N*-cinnamoylanthranilate and related derivatives synthesized and evaluated in this study.

**Figure 3.** Dose-response curves of *N*-cinnamoylanthranilate derivatives in hTRPA1 (**a - f**) and hTRPM8 (**g**) HEK293 cells. Each data point on the curve represents the mean of three independent experiments ( $N = 3$ ) in duplicates ( $n = 6$ ) with their SEM.

**Figure 4.** Real-time spectrum recorded for 10 min (incubation period) for the compounds with bimodal activity (potent agonism and desensitization) in hTRPA1-HEK293 cells. (**a**) cinnamaldehyde (CA, 30  $\mu$ M), (**b**) flufenamic acid (FFA, 100  $\mu$ M), (**c**) *p*-Br CAD-**4** (10  $\mu$ M), (**d**) *p*-Cl CAD-**5** (10  $\mu$ M), (**e**) *o*-Cl CAD-**6** (10  $\mu$ M) and (**f**) *p*-F CAD-**7** (10  $\mu$ M). The concentration of CA in the second addition was 30  $\mu$ M.

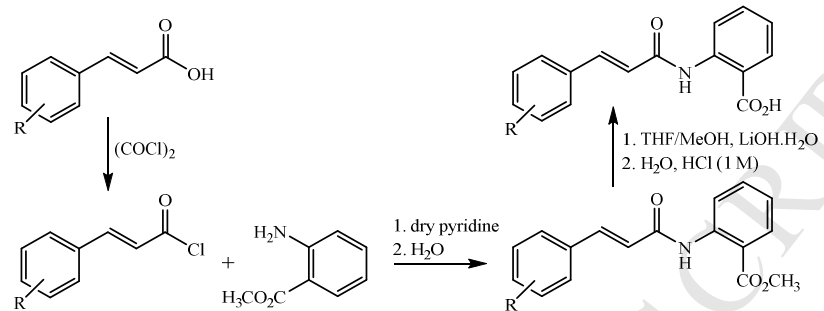
**Figure 5.** (a) Desensitizing effect and (b) reversibility of halogenated *N*-cinnamoylanthranilic acids **4-7** on hTRPA1-HEK293 cells. Each bar represents the mean  $\pm$  SEM ( $N = 3$ ,  $n = 6$ ), and the statistical significance was determined using one-way ANOVA at  $*p < 0.05$ .

**Figure 6.** Screening results of TRPA1 ligands in the hTRPA1 mutants, F909A, F944A and C621A, and comparison of the responses against WT-hTRPA1 HEK293 cells, (a-e) agonism and (f) antagonism. Each bar represents the mean  $\pm$  SEM of  $N = 3$  ( $n = 6$ ), and statistical significance was compared relative to WT at  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  and  $****p < 0.0001$ , using one-way ANOVA; black stars indicate significant reduction and grey stars indicate significant increase in response relative to WT.

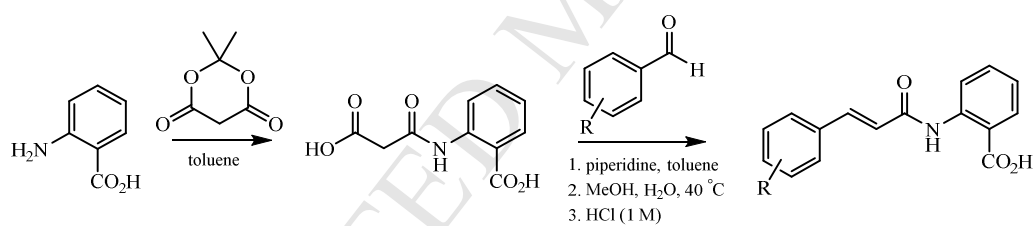
**Figure 7.** Effects of CADs **3**, **5** and **13** on isolated guinea pig vagus nerve preparations, (a - c) agonism and (d) antagonism against acrolein (ACR, 300  $\mu$ M) induced depolarization; each bar represents the mean  $\pm$  SEM ( $N = 4$ ). (e) Antagonism of CADs **5** and **13** against acrolein (300  $\mu$ M) in human TRPA1-HEK293 cells; each bar represents the mean  $\pm$  SEM ( $N = 3$ ,  $n = 9$ ).

**Figure 8.** **A.** Human TRPA1 atomic model 3J9P showing the location of residues F909 (red) and F944 (blue) as coloured spheres. These are located in the membrane portion of the channel. **B.** Top down view of the channel showing the central pore and the 'ring' of F909 and F944 residues. **C.** Close up of TRPA1 showing the F944 and F909 repeating residues. **D.** Close up with surface highlighting residues in red F909 (top) and T874 (bottom), and the location of the shallow pocket for putative antagonist binding. The location of F944 (blue) is buried and unavailable for ligand binding without significant structural movement.

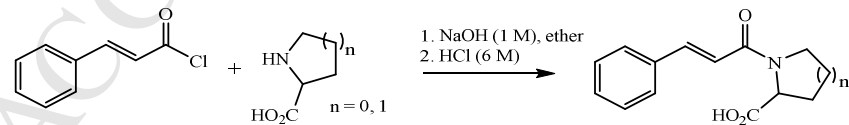
Scheme 1.



Scheme 2.



Scheme 3.



**Table 1.** Screening results of the synthesized *N*-cinnamoylanthranilic acids in hTRPA1 and hTRPM8-transfected HEK293 cells. The concentrations at which the compounds were tested are specified in the corresponding column title. Dose-response curves in hTRPA1 were carried out for the compounds that showed >25 % agonist activity at 30  $\mu$ M, and that had partial agonism and high antagonism, in the initial screening. Dose-response curves in hTRPM8 were carried out for the compounds which showed >60 % inhibitory effect in the initial screening. For compounds where an EC<sub>50</sub> or IC<sub>50</sub> could not be calculated, a dash (-) is given instead of a value. Each value represents the mean  $\pm$  SEM ( $N = 3$ ).

Compound	CAD variant	hTRPA1-HEK293						hTRPM8-HEK293			
		Agonism / (% calcimycin) 30 $\mu$ M $N = 3 \pm$ SEM	Antagonism, CA response /(% CA) 100 $\mu$ M $N = 3 \pm$ SEM	LogEC <sub>50</sub> / (M) $n = 6$ , $N = 3 \pm$ SEM	EC <sub>50</sub> /( $\mu$ M)	LogIC <sub>50</sub> / (M) $n = 6$ , $N = 3 \pm$ SEM	IC <sub>50</sub> /( $\mu$ M)	Agonism / (% calcimycin) 30 and 100 $\mu$ M $N = 3 \pm$ SEM	Antagonism, WS5 response /(% WS5) 100 $\mu$ M $N = 3 \pm$ SEM	LogIC <sub>50</sub> / (M) $n = 6$ , $N = 3 \pm$ SEM	IC <sub>50</sub> /( $\mu$ M)
1	-	24 $\pm$ 1	42 $\pm$ 3	-	-	-	-	48 $\pm$ 6	-	-	
2	4-NO <sub>2</sub>	25 $\pm$ 1	33 $\pm$ 6	-3.7 $\pm$ 0.2	219	-	-	39 $\pm$ 7	-	-	
3	4-CF <sub>3</sub>	51 $\pm$ 1	28 $\pm$ 2	-4.57 $\pm$ 0.06	27	-	-	17 $\pm$ 3	-4.5 $\pm$ 0.2	34	
4	4-Br	60 $\pm$ 3	6 $\pm$ 2	-4.94 $\pm$ 0.09	12	-5.0 $\pm$ 0.2	9	35 $\pm$ 2	-4.2 $\pm$ 0.4	59	
5	4-Cl	62 $\pm$ 1	7 $\pm$ 1	-5.00 $\pm$ 0.07	10	-5.4 $\pm$ 0.1	4	40 $\pm$ 5	-	-	
6	2-Cl	44 $\pm$ 2	5 $\pm$ 2	-4.37 $\pm$ 0.07	42	-5.0 $\pm$ 0.1	11	25 $\pm$ 4	-4.7 $\pm$ 0.5	21	
7	4-F	37 $\pm$ 1	13 $\pm$ 4	-4.5 $\pm$ 0.1	30	-4.6 $\pm$ 0.1	26	61 $\pm$ 8	-	-	
8	4-CH <sub>3</sub>	30 $\pm$ 1	40 $\pm$ 1	-3.8 $\pm$ 0.1	149	-	-	48 $\pm$ 3	-	-	
9	2-OCH <sub>3</sub>	33 $\pm$ 1	14 $\pm$ 2	-4.1 $\pm$ 0.1	75	-4.3 $\pm$ 0.2	51	32 $\pm$ 8	-4.4 $\pm$ 0.3	41	
10	4-OCH <sub>3</sub>	27 $\pm$ 1	19 $\pm$ 6	-4.3 $\pm$ 0.2	49	-4.9 $\pm$ 0.1	12	68 $\pm$ 8	-	-	
11	3,4-(OCH <sub>3</sub> ) <sub>2</sub>	6 $\pm$ 3	56 $\pm$ 13	-	-	-3.9 $\pm$ 0.2	126	65 $\pm$ 6	-	-	
12	3-OEt-4-OH	5 $\pm$ 1	34 $\pm$ 6	-	-	-4.0 $\pm$ 0.3	106	73 $\pm$ 6	-	-	
13	4-OH	1 $\pm$ 1	35 $\pm$ 11	-	-	-4.4 $\pm$ 0.1	43	70 $\pm$ 5	-	-	
14	3,4-(OH) <sub>2</sub>	Fluorescing compounds						Fluorescing compounds			
15	4-N(CH <sub>3</sub> ) <sub>2</sub>										
16	$\alpha,\beta$ -saturated	10 $\pm$ 3	81 $\pm$ 11	-	-	-	-	48 $\pm$ 4	-	-	
17	$\alpha,\beta$ -saturated, 4-OCH <sub>3</sub>	6 $\pm$ 1	74 $\pm$ 14	-	-	-	-	57 $\pm$ 5	-	-	
18	Pipecolinic acid	7 $\pm$ 1	71 $\pm$ 9	-	-	-	-	72 $\pm$ 6	-	-	
19	Proline	5 $\pm$ 2	79 $\pm$ 4	-	-	-	-	65 $\pm$ 5	-	-	
20	$\alpha$ -CH <sub>3</sub>	33 $\pm$ 2	29 $\pm$ 2	-3.9 $\pm$ 0.1	133	-	-	28 $\pm$ 7	-4.6 $\pm$ 0.2	27	
21	$\alpha$ -Ph	66 $\pm$ 2	22 $\pm$ 1	-4.95 $\pm$ 0.04	11	-	-	14 $\pm$ 1, 34 $\pm$ 3	not measured	-	

**Table 2.** Screening results of the methyl *N*-cinnamoylanthranilates (3  $\mu$ M) in hTRPA1 and hTRPM8-HEK293 cells. As **2a** (4-NO<sub>2</sub>) was insoluble in DMSO at room temperature, it was not evaluated. Each value represents the mean  $\pm$  SEM ( $N = 3$ ).

Compound	CAD variant	hTRPA1-HEK293		hTRPM8-HEK293			
		Agonism / / (% calcimycin) 3 $\mu$ M $N = 3 \pm$ SEM	Antagonism, CA response / (% CA) 3 $\mu$ M $N = 3 \pm$ SEM	Agonism / (% calcimycin) 3 $\mu$ M $N = 3 \pm$ SEM	Antagonism, WS5 response / (% WS5) 3 $\mu$ M $N = 3 \pm$ SEM		
<b>1a</b>	-	22 $\pm$ 1	No inhibitory effect.	No agonism.	61 $\pm$ 1		
<b>3a</b>	4-CF <sub>3</sub>	29 $\pm$ 2			81 $\pm$ 11		
<b>4a</b>	4-Br	41 $\pm$ 2			79 $\pm$ 4		
<b>5a</b>	4-Cl	45 $\pm$ 2			70 $\pm$ 4		
<b>6a</b>	2-Cl	29 $\pm$ 2			53 $\pm$ 1		
<b>7a</b>	4-F	34 $\pm$ 3			85 $\pm$ 9		
<b>8a</b>	4-CH <sub>3</sub>	28 $\pm$ 3			88 $\pm$ 3		
<b>13a</b>	4-OAc	10 $\pm$ 1			84 $\pm$ 2		
<b>14a</b>	3,4-(OAc) <sub>2</sub>	12 $\pm$ 1			102 $\pm$ 2		
<b>15a</b>	4-N(CH <sub>3</sub> ) <sub>2</sub>	27 $\pm$ 1			86 $\pm$ 22		
<b>20a</b>	$\alpha$ -CH <sub>3</sub>	49 $\pm$ 2			73 $\pm$ 7	30 $\pm$ 4	
<b>21a</b>	$\alpha$ -Ph	63 $\pm$ 2			51 $\pm$ 2	23 $\pm$ 4	48 $\pm$ 7
<b>22a</b>	Phenoxy	45 $\pm$ 2 (at 30 $\mu$ M)			No inhibitory effect.	No agonism	12 $\pm$ 2 (at 30 $\mu$ M)

**Table 3.** The EC<sub>50</sub> and IC<sub>50</sub> values of the known ligands obtained in hTRPA1-HEK293 cells using a FlexStation, compared with the literature values of the original findings. Abbreviations: FLIPR, fluorometric imaging plate reader assay; EP, electrophysiology; FS, FlexStation; ND, not determined; NE, not estimable.

Ligand	Obtained value		Literature value
	LogEC <sub>50</sub> (M) ± SEM	EC <sub>50</sub>	EC <sub>50</sub> , species, assay [ref.]
CA	-5.07 ± 0.09	9 μM	61 ± 9 μM, mouse TRPA1-CHO cells, FLIPR.[29]
ACR	-4.57 ± 0.07	27 μM	5 ± 1 μM, human TRPA1- <i>Xenopus oocytes</i> , EP.[7]
AITC	-5.36 ± 0.04	4 μM	22 ± 3 μM, mouse TRPA1-CHO cells, FLIPR.[29]
Menthol	-4.5 ± 0.1	30 μM	95 ± 15 μM, mouse TRPA1-CHO cells, EP.[58]
Thymol	-4.22 ± 0.06	60 μM	20 μM, human TRPA1-HEK293 cells, FLIPR.[60]
Carvacrol	-4.78 ± 0.09	17 μM	ND
Eugenol	-3.77 ± 0.09	168 μM	261 ± 9 μM, human TRPA1-HEK293 cells, EP.[71]
FFA	-5.16 ± 0.06	7 μM	24 ± 3 μM, WI-38 cells (human fibroblast), FS.[30] 57 ± 5 μM, human TRPA1-HEK293 cells, FS.[30]
MFA	-4.78 ± 0.09	16 μM	61 ± 5 μM, WI-38 cells (human fibroblast), FS.[30]
DCF	-4.2 ± 0.1	56 μM	210 ± 22 μM, WI-38 cells (human fibroblast), FS.[30]
NPPB	-6.22 ± 0.09	0.6 μM	0.32 μM, human TRPA1-HEK293 cells, FLIPR.[35]
ACA	-4.55 ± 0.06	28 μM	ND
SB366791	inactive	-	ND
NDGA	-4.45 ± 0.08	35 μM	4.9 ± 1.7 μM, human TRPA1-HEK293 cells, FS.[72]
FTS	-6.08 ± 0.07	0.8 μM	7 ± 4 μM, human TRPA1-HEK293 cells, FLIPR.[73]
URB597	-0.87	0.1 μM	24.5 ± 3.2 μM, human TRPA1-HEK293F cells, FLIPR.[74]
Nicotine	0.34	2 M	~10 μM, mouse TRPA1-CHO cells, EP.[75]
Probenecid	NE	-	4.2 mM, human TRPA1-CHO cells, FS.[39]
Ligand	LogIC <sub>50</sub> ± SEM	IC <sub>50</sub>	IC <sub>50</sub> , species, assay
A967079	-7.10 ± 0.09	79 nM	67 nM against AITC (30 μM), human TRPA1-HEK293F cells, FLIPR[76]
AP18	-5.35 ± 0.08	4 μM	3.1 μM against CA (50 μM), human TRPA1-CHO cells, FLIPR.[77]
HC030031	-4.75 ± 0.09	18 μM	6.2 ± 0.2 μM against AITC (5 μM), human TRPA1-HEK293 cells, fluorescence-based plate reader.[78]
ASD	-5.0 ± 0.1	10 μM	12.5 μM against AITC (5 μM), human TRPA1-HEK293 cells, FS.[79, 80]

Figure 1.

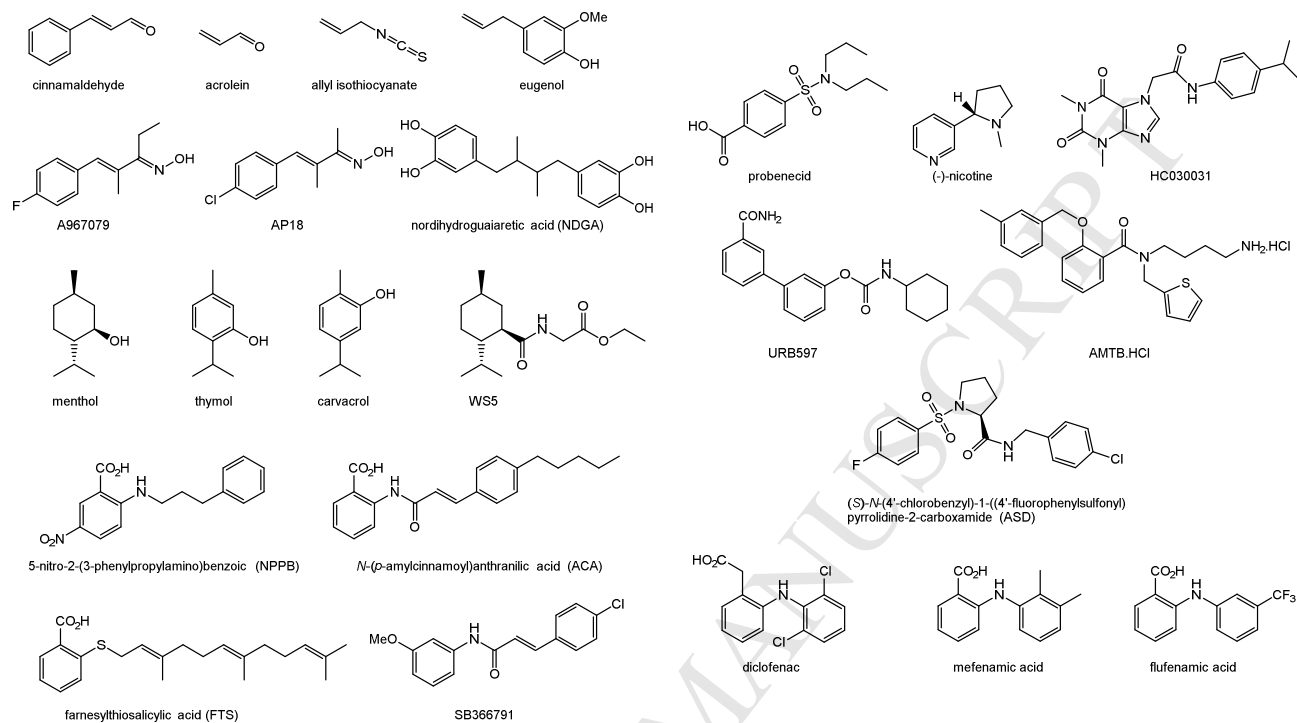




Figure 2

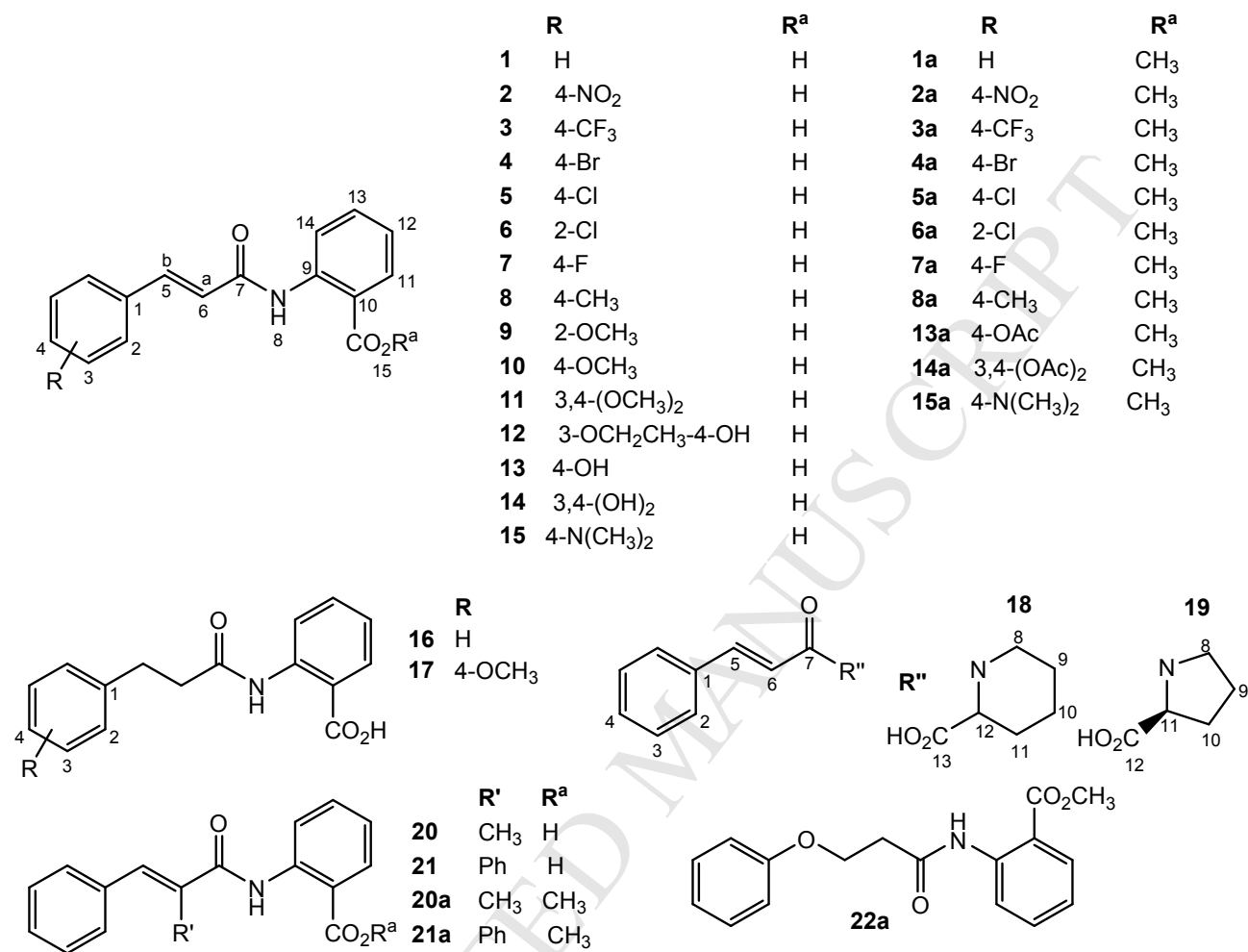


Figure 3

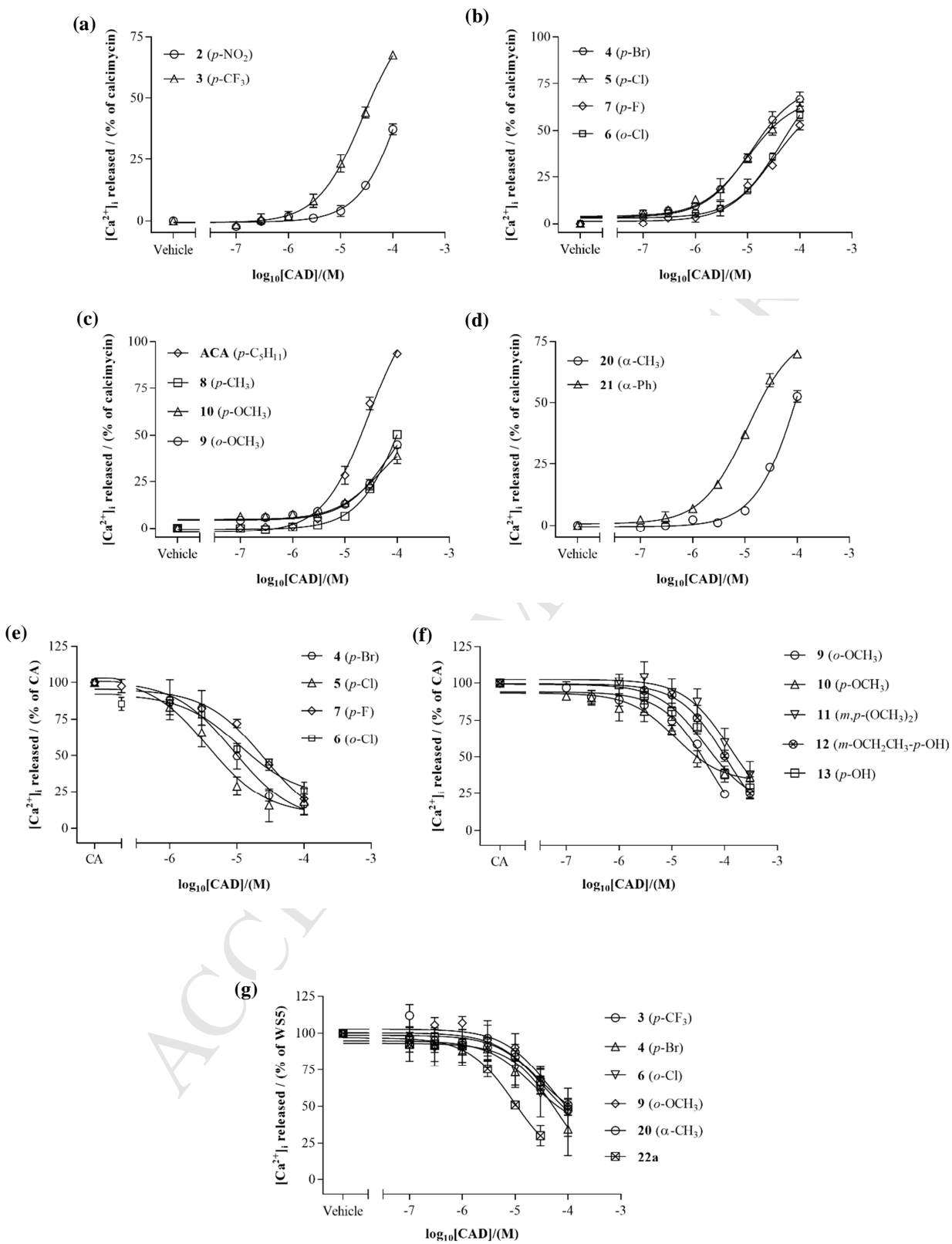


Figure 4

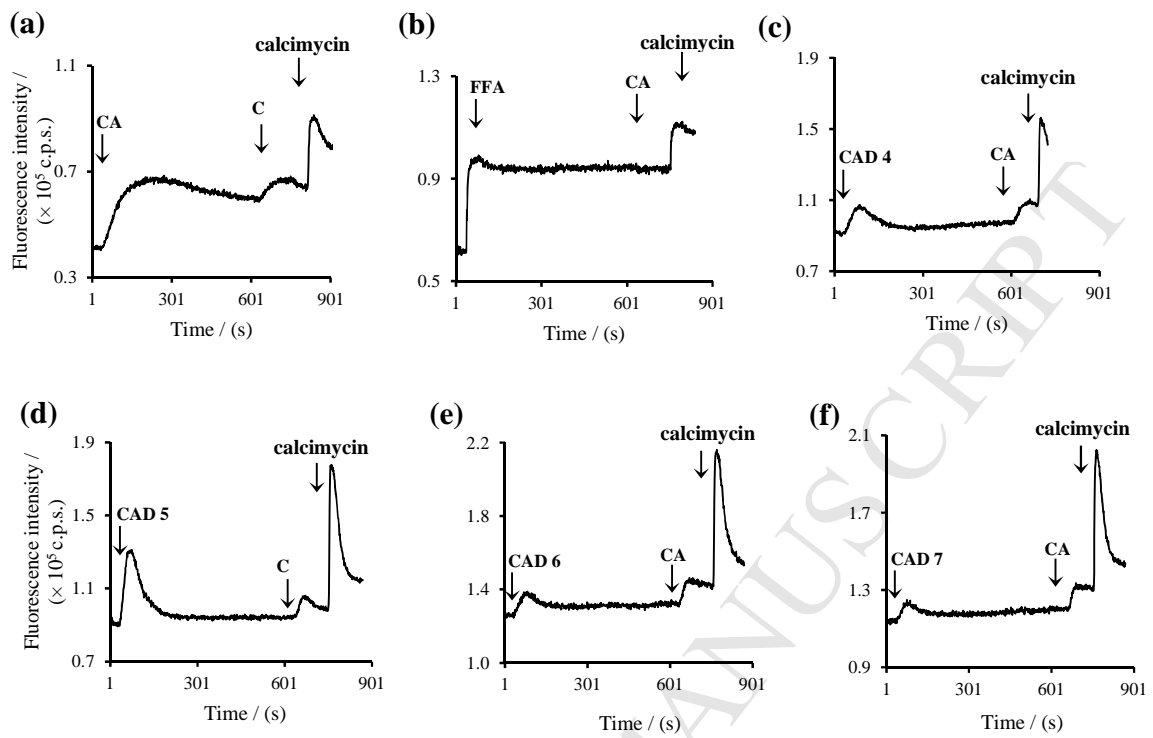


Figure 5

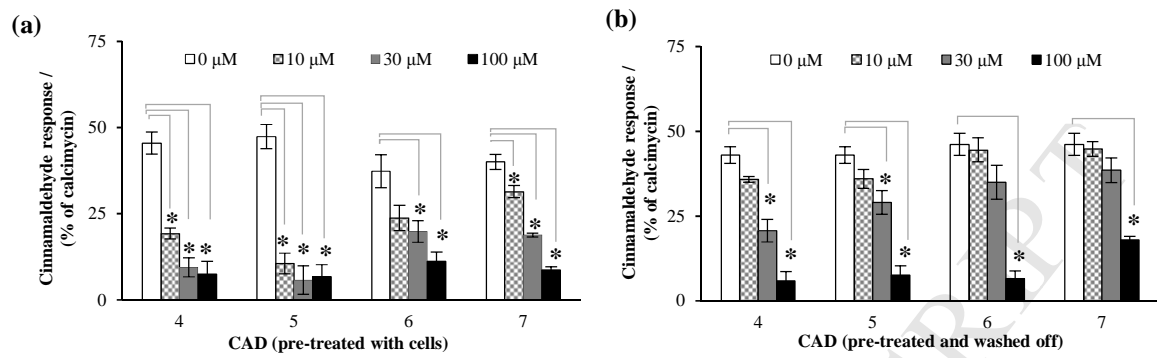


Figure 6

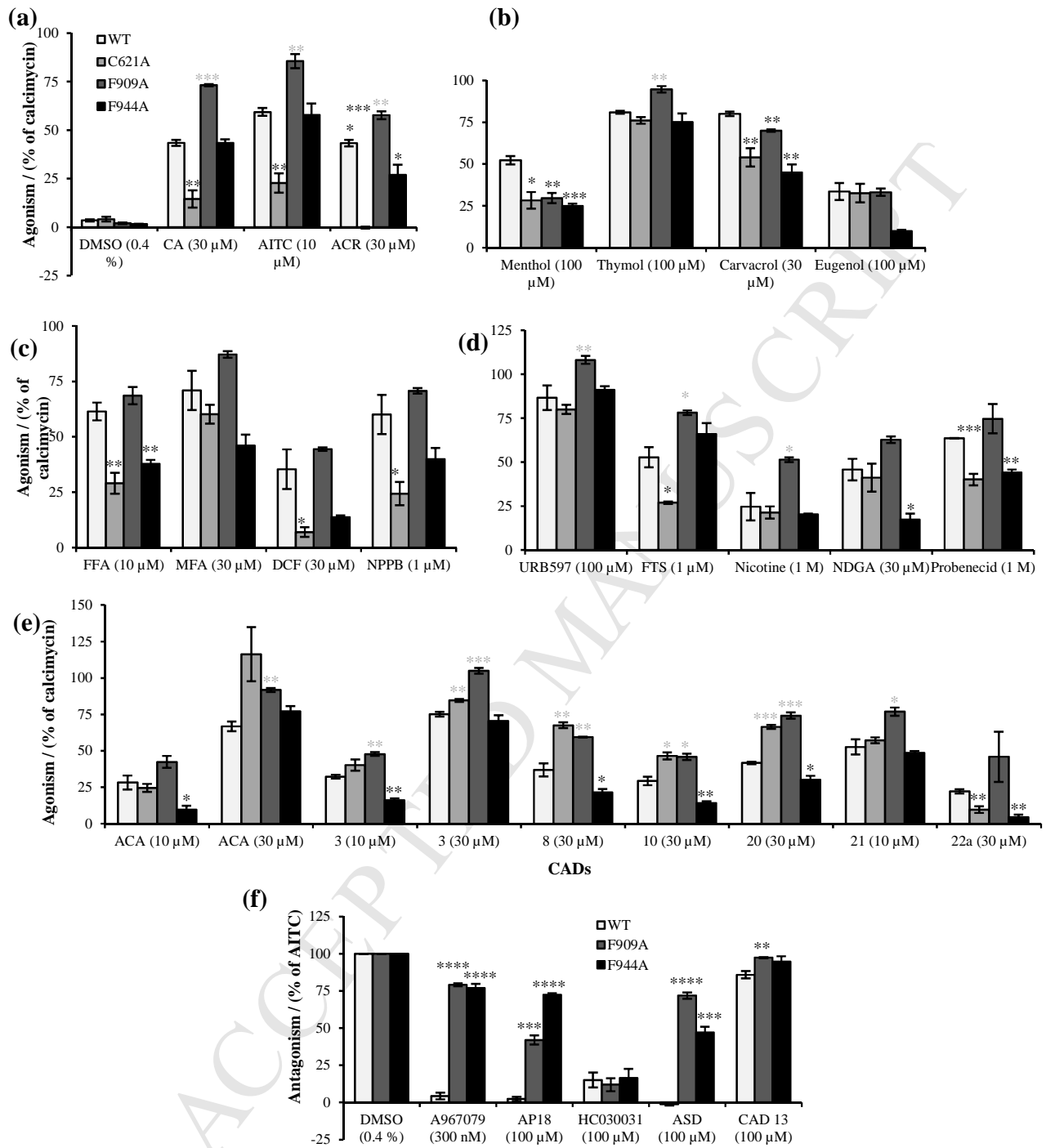


Figure 7

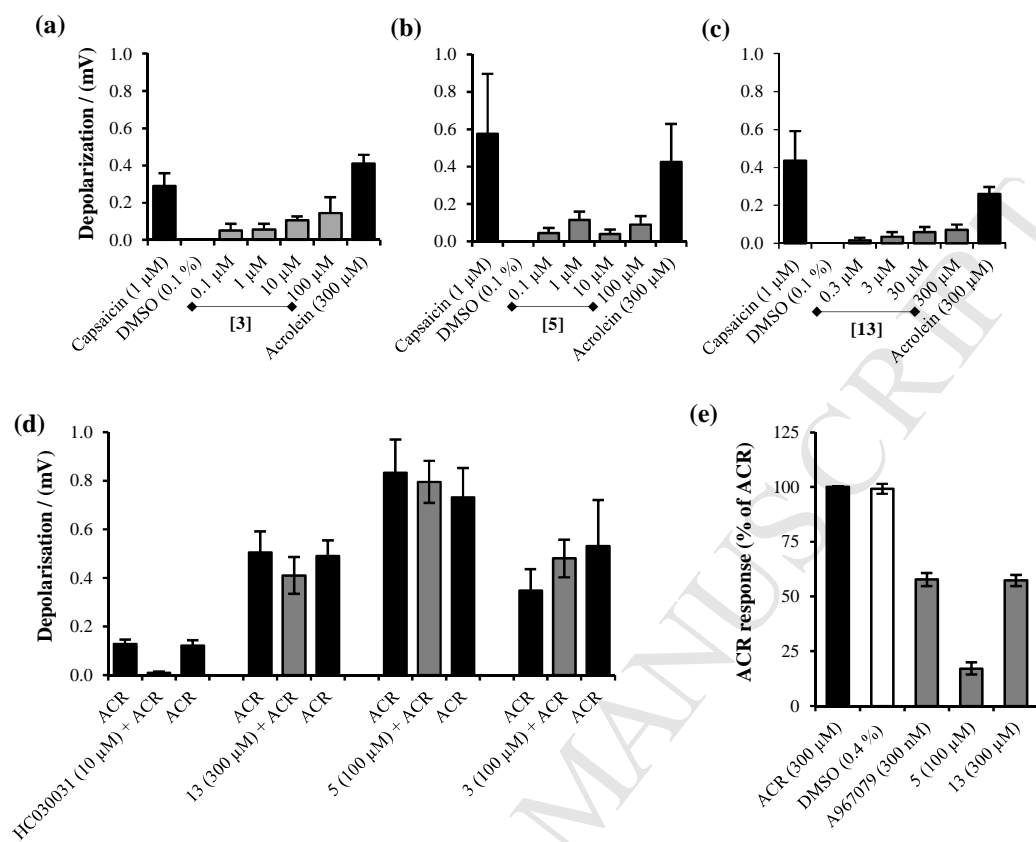


Figure 8

