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1 Agonism of free fatty acid receptors 1 and 4 generate peptide YY-mediated inhibitory responses in  
2 mouse colon

3

4 **Short title:** FFA1 and FFA4 activities in mouse colon

5

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16

17 **Financial disclosures:** None.

18

19 **Statement of Conflicts of Interests:**

20 RM, IRT and HMC have nothing to disclose. DMS and MS are currently employed by and hold  
21 shares in AstraZeneca Plc.

22

23

24

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26

27 Background and purpose:

28 Free fatty acid receptor 1 (FFA1) and FFA4 are located on enteroendocrine L cells with the  
29 highest gastrointestinal (GI) expression in descending colon. Their activation causes the release of  
30 glucagon-like peptide 1 and peptide YY (PYY) from L cells. Additionally, FFA1 agonism  
31 releases insulin from pancreatic  $\beta$ -cells. As these receptors are modulators of nutrient-stimulated  
32 glucose regulation, this study's aim was to compare the pharmacology of commercially available  
33 agonists (TUG424, TUG891, GW9508) with proven selective agonists (JTT, TAK-875, AZ423,  
34 Metabolex-36), in the mouse.

35 Experimental approach:

36 Mouse (>10 weeks old; C57BL/6J) mucosa was mounted in Ussing chambers, voltage-clamped  
37 and the resultant short-circuit current ( $I_{sc}$ ) recorded continuously. The pretreatments included  
38 FFA1 antagonists or  $Y_1 \pm Y_2$  antagonists. Glucose-sensitivity was investigated by mannitol  
39 replacement apically and colonic and upper GI transit was assessed *in vitro* and *in vivo*.

40 Key Results:

41 FFA1 and FFA4 agonism required glucose and reduced  $I_{sc}$  in a PYY- $Y_1$ -dependent manner. The  
42 novel compounds were more potent than GW9508. The FFA1 antagonists (GW1100 and  
43 ANT825) blocked FFA1 activity only and revealed FFA1 tonic activity. The FFA4 agonist,  
44 Metabolex-36 slowed colonic transit *in vitro* but increased intestinal transit *in vivo*.

45 Conclusion and Implications:

46 The selective FFA1 and FFA4 agonists were more potent than GW9508, a dual FFA1 and FFA4  
47 agonist. We found that a PYY- $Y_1$  paracrine epithelial mechanism mediated their responses which  
48 were glucose-sensitive, potentially limiting hypoglycaemia. ANT825 revealed tonic activity and  
49 the possibility of endogenous FFA1 ligands causing PYY release. Finally, FFA4 agonism induced  
50 regional differences in transit.

51 **Keywords:** Free fatty acid receptors 1 and 4, peptide YY, glucagon-like peptide 1, mucosal ion  
52 transport, colonic transit

53

54

## 55 **Abbreviations**

- 56 ANOVA, analysis of variance;
- 57 ap, Apical;
- 58 AZ, AstraZeneca;
- 59 BIBO, BIBO3304;
- 60 BIIE, BIIE0246;
- 61 bl, Basolateral;
- 62 CCK, Cholecystokinin;
- 63 CNS, Central Nervous System;
- 64 DMSO, dimethyl sulphoxide;
- 65 FFA, free fatty acid;
- 66 FFA1, free fatty acid receptor 1;
- 67 FFA4, free fatty acid receptor 4;
- 68 GI, gastrointestinal;
- 69 GIP, Gastric inhibitory peptide;
- 70 GLP-1, glucagon-like peptide 1;
- 71 GLP-2, glucagon-like peptide 2;
- 72 GPCR, G protein-coupled receptor;
- 73 GSIS, Glucose stimulated insulin secretion;
- 74 HPMC, Hydroxypropyl methylcellulose;
- 75 i.p., Intraperitoneal;
- 76  $I_{sc}$ , Short-circuit-current;
- 77 KH, Krebs Henseleit;
- 78 KO, Knockout;
- 79 MC4 receptor, Melanocortin-4 receptor;
- 80 Met-36, Metabolex-36;
- 81 MMC, Myoelectric migrating complex;
- 82 OXM, oxyntomodulin;
- 83 Phlor, Phloridzin;
- 84 PLC, Phospholipase C;
- 85 PVP, polyvinylpyrrolidinone;
- 86 PYY, peptide YY;
- 87 PYY (3-36), peptide YY(3-36);

88 SDS, sodium dodecyl sulfate;  
89 SGLT1, Sodium-glucose co-transporter 1;  
90 SST, Somatostatin;  
91 T2DM, type 2 diabetes mellitus;  
92 UGIT, Upper gastrointestinal transit;  
93 VIP; vasoactive intestinal polypeptide;  
94 WT, wild type  
95

## 96 **Introduction**

97 Free fatty acid receptors 1 ([FFA1](#), previously known as GPR40) and 4 ([FFA4](#), previously known  
98 as GPR120) are modulators of nutrient-stimulated glucose regulation and therefore are therapeutic  
99 targets for the treatment of type 2 diabetes mellitus (T2DM). FFA1 selective agonists have been  
100 shown to cause glucose-sensitive insulin secretion (Tsujihata et al. 2011). FFA1 and FFA4 were  
101 deorphanised in 2003 and 2005 respectively, as receptors for medium to long chain unsaturated  
102 fatty acids (Hirasawa et al. 2005, Itoh et al. 2003). As endogenous free-fatty acids (FFAs) often  
103 activate more than one receptor type (Kostenis 2004) and are metabolised to act as intracellular  
104 signalling molecules (Warnotte et al. 1994), they have limited utility as selective tools to establish  
105 the pharmacology and functional significance of specific FFA receptors. The recent discovery of  
106 selective, small molecule agonists for FFA1 and FFA4 therefore provided a pharmacological  
107 advantage over non-selective endogenous FFAs and this study set out to utilise some of these new  
108 tool compounds to investigate FFA1 and FFA4 signalling specifically.

109 Apart from FFA1 and FFA4 there are alternative FFA receptors in the gastrointestinal (GI) tract  
110 including, FFA2 (formerly GPR43), FFA3 (GPR41) and the acylethanolamide receptor, GPR119,  
111 all of which are selectively expressed in L cells together with FFA1 and FFA4 (Overton et al.  
112 2006, Hirasawa et al. 2005, Edfalk et al. 2008, Karaki et al. 2006). Both FFA1 (Briscoe et al.  
113 2003) and FFA4 (Hirasawa et al. 2008) have the capacity to couple to  $G\alpha_{q/11}$ , activate  
114 phospholipase C (PLC) and elevate  $Ca^{2+}$  levels, leading to the release of L cell peptide hormones.  
115 Colonic L cells contain peptide YY (PYY), the incretin glucagon-like peptide 1 (GLP-1) plus  
116 GLP-2 and oxyntomodulin (OXM) (Habib et al. 2013, Egerod et al. 2012, Cho et al. 2014).  
117 Additionally, small intestine L cells express neurotensin and cholecystinin (CCK) (Egerod et al.  
118 2012, Habib et al. 2013). In this study, we utilised the descending colon because this colonic  
119 region exhibits a greater degree of PYY-[Y<sub>1</sub>](#) signalling compared with the ascending colon (Cox et

120 al. 2001) and L cell density is greater in the distal colon in mouse (Arantes and Nogueira 1997)  
121 and human (Adrian et al. 1985). GLP-1 slows gastric emptying and enhances satiety (Little et al.  
122 2006) while PYY is a well-known mediator of the ileal and colonic brake (Lin et al. 1996) and a  
123 major satiety-inducing agent together with its product, PYY<sub>(3-36)</sub> (Batterham et al. 2002). PYY is  
124 also a significant inhibitor of intestinal epithelial ion transport and we have previously utilised this  
125 activity to monitor the acute endogenous peptide functionality following L cell-specific  
126 stimulation with GPR119 agonists (Cox et al. 2010, Patel et al. 2014) or other L cell mediated  
127 mechanisms (Joshi et al. 2013, Panaro et al. 2014). In mouse and human distal colon, local  
128 mucosal PYY antisecretory actions are predominantly mediated via epithelial Y<sub>1</sub> receptors with a  
129 smaller component of submucosal neuron Y<sub>2</sub> activity (Tough et al. 2006, Tough et al. 2011), that  
130 together underpin endogenous PYY paracrine responses. FFA1 and FFA4 are also potential  
131 nutrient-sensing receptors in L cells and therefore are possible targets for the treatment of T2DM  
132 and obesity (Engelstoft et al. 2008).

133 The FFA1 agonist TAK-875 (Negoro et al. 2010), which in phase II trials lowered blood glucose  
134 without apparently increasing GLP-1 levels, was shown to signal via Gα<sub>q/11</sub> alone, in a manner  
135 similar to the proposed endogenous agonist, α-linolenic acid. In type II diabetic rats, TAK-875  
136 improved glucose tolerance and enhanced glucose-dependent insulin secretion (Tsujihata et al.  
137 2011). Despite the liver toxicity concerns of TAK-875, it significantly improved glycaemic  
138 control in diabetic Japanese patients in a phase III study (Kaku et al. 2015). JTT, a novel  
139 selective FFA1-targeted agonist (from patent number, WO 2009/054479), TAK-875 and the  
140 commercially available FFA1 agonist, TUG424 (Christiansen et al. 2008) were selected to assess  
141 the pharmacology of FFA1 in this study.

142  
143 FFA4 is also found abundantly in gastric-inhibitory peptide (GIP)-containing K cells of the small  
144 intestine, gastric somatostatin (SST)-containing D cells and gastric ghrelin-containing A cells (Ito  
145 et al. 2009, Parker et al. 2009, Engelstoft et al. 2013, Egerod et al. 2015). The functional roles of  
146 FFA4 include enhanced insulin secretion, GLP-1 secretion (Halder et al. 2013), reduced gastric  
147 emptying (Little et al. 2006) and anti-inflammatory effects (Oh et al. 2010). FFA4 knockout (KO)  
148 mice exhibited a significantly reduced L cell GLP-1 secretion (Xiong et al. 2013) and K cell GIP  
149 secretion (Iwasaki et al. 2015) indicating that signalling via FFA4 in wild type (WT) tissues can  
150 cause incretin peptide release. The novel selective agonists selected for this study, AZ423  
151 (Compound 34, McCoull et al. 2017) and Metabolex-36 (Ma et al. 2010), show 100-fold  
152 selectivity for murine FFA4 compared with FFA1 (Stone et al. 2014). Furthermore, AZ423 and

153 Metabolex-36 have exhibited a reduction in blood glucose excursion in an oral glucose tolerance  
154 test (Halder et al. 2013, McCoull et al. 2017). Additionally, the selected commercially available  
155 FFA4 agonist, [TUG891](#) is selective for murine and human FFA4 (Hudson et al. 2013). Whether  
156 intestinal FFA4 agonism resembles FFA1 activities and is also glucose-sensitive in  
157 enteroendocrine L cells has yet to be determined.

158  
159 As few functional studies have been performed in native preparations containing L cells, one aim  
160 of this study was to determine the potencies and efficacies of the commercially available agonists  
161 (TUG424 and TUG891) compared with the non-selective FFA1 and FFA4 agonist [GW9508](#), and  
162 importantly the novel selective agonists JTT, TAK-875, AZ423 and Metabolex-36 in mouse  
163 colon. We also assessed the selectivity of the FFA1-preferring antagonist [GW1100](#) (Briscoe et al.  
164 2006) comparing it with ANT825, a selective FFA1 antagonist (referred to as compound 39 in  
165 Waring, 2015) and determined their relative abilities to reveal tonic FFA1 activity. We also  
166 determined the involvement of endogenous PYY in these responses in colonic mucosae by  
167 blocking peptide activity pharmacologically. Finally, the effects of FFA1 and FFA4 agonism  
168 upon colonic motility *in vitro* and *in vivo* were established.

169

## 170 **Methods**

### 171 *Mucosal preparation and $I_{sc}$ measurement*

172 Mice (C57BL/6J, 12-20 weeks old, 20-30g, female and male) were procured from Charles River  
173 Laboratories (Margate, UK) had free access to standard chow (Rat and Mouse No 3 breeding diet,  
174 Special Diets Services, Baintree, UK) and water *ad libitum*. We used mice because the  $Y_1$ ,  $Y_2$  and  
175  $Y_4$  receptor localisation in the GI tract is the same in this species as in human colon (Cox and  
176 Tough. 2001, Tough et al. 2011). Mice were housed in open top conventional cages with Lignocel  
177 poplar bedding material along with the appropriate environmental enrichment. A maximum of 5  
178 mice were housed in a single cage. Housing rooms were maintained at 20-24 °C, humidity 55% ±  
179 10% and 12/12 h light and dark cycle. All animal care and experimental procedures complied with  
180 the Animals (Scientific procedures) Act 1986 and were approved by UK Home Office (licence  
181 number: PPL70/7887). Mice were killed by cervical dislocation and the whole colon was  
182 dissected (noting proximal and distal ends) and placed in fresh Krebs-Henseleit (KH), with the  
183 following composition (in mM); NaCl 118, KCl 4.7, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub>  
184 2.5, and D-glucose 11.1. Mucosae from the descending colon were prepared by removing the

185 overlying smooth muscle layers and associated myenteric plexi by micro-dissection. Mucosal  
186 lengths were cut in to 4-6 adjacent pieces, each of which was mounted between two halves of an  
187 Ussing chamber (WPI UK, Hitchin, Herts, UK) (exposed area, 0.14 cm<sup>2</sup>), voltage-clamped at 0  
188 mV (DVC1000 and amplifiers; WPI UK, Hitchin, Herts, UK) and equilibrated in oxygenated KH  
189 (95% O<sub>2</sub>/5% CO<sub>2</sub>, at 37°C) for 15 min. Drug additions were made to either the basolateral (bl) or  
190 apical (ap) reservoirs and changes in short-circuit current (I<sub>sc</sub>) were measured continuously.

191

#### 192 *Sidedness, potency and efficacy of the FFA1 and FFA4 compounds*

193 Mucosae were pretreated with vasoactive intestinal polypeptide (VIP, 30 nM, bl) to provide a  
194 degree of vectorial epithelial ion secretion upon which subsequent antisecretory signalling is more  
195 readily observed (as optimised previously; Tough et al. 2006; Cox et al. 2010). Once VIP  
196 responses had reached a maximum, a FFA1 or FFA4 agonist (TUG424 (300 nM), TUG891 (300  
197 nM), GW9508 (10 μM)) was added to either reservoir as indicated, to determine the approximate  
198 sidedness of the FFA1 and FFA4 responses. Single additions of apical FFA1 or FFA4 agonists  
199 were used to construct concentration-response curves and calculate single EC<sub>50</sub> values with  
200 GraphPad Prism v6.0. Changes in I<sub>sc</sub> to certain FFA1 agonists (TAK-875, JTT and GW9508) and  
201 FFA4 agonists (Metabolex-36, AZ423) were biphasic; the first component (1°) being a transient  
202 increase in I<sub>sc</sub> (within 5 min) followed by a longer-lasting reduction in I<sub>sc</sub> (the second component,  
203 2°) that was extrapolated from the waning VIP signal and reached its maximum within 10-15 min  
204 of FFA drug addition. When the biphasic changes in I<sub>sc</sub> were observed, the 1° and 2° components  
205 were analysed separately. To investigate whether there was a neuronal component to FFA1  
206 (TAK-875, 200 nM) and FFA4 (Metabolex-36, 100 nM) signalling, mucosae were pretreated with  
207 the neurotoxin, tetrodotoxin (TTX, 100 nM, bl) or vehicle (H<sub>2</sub>O) for 15 min, followed by VIP and  
208 subsequently the FFA1 or FFA4 agonist. The responses to TAK-875 and Metabolex-36 were  
209 monitored for 20 min and finally PYY (10 nM, bl) was added as an internal control. Changes in  
210 I<sub>sc</sub> to each drug or peptide were pooled and converted to μA.cm<sup>-2</sup>.

211

#### 212 *Establishing the selectivity of FFA1 and FFA4 agonism using antagonists*

213 In order to establish an IC<sub>50</sub> of the FFA1 antagonist, ANT825, basal I<sub>sc</sub> levels were allowed to  
214 stabilise before the addition of varying single concentrations (1 nM - 10 μM) of the FFA1  
215 antagonist. After 10-15 min, VIP was added, followed by the selected FFA1 agonist, JTT (300



216 nM) and consequent reductions in  $I_{sc}$  were recorded and converted to  $\mu A.cm^{-2}$ . PYY (10 nM) was  
217 added finally as an internal control. The antagonist concentration (10  $\mu M$ , ANT825) that  
218 abolished JTT responses was used in subsequent selectivity studies.

219 Blockade of the commercially available FFA1 agonists (TUG424, GW9508 and TAK-875) was  
220 confirmed using the antagonists, GW1100 (10  $\mu M$ ) (Briscoe et al. 2006) and ANT825 (10  $\mu M$ ).  
221 Here mucosae were pretreated apically for 10 min with the appropriate vehicle (0.1% DMSO) or  
222 the chosen FFA1 antagonist prior to VIP addition. TUG424 (100 nM), GW9508 (1  $\mu M$ ) were the  
223 FFA1 agonists tested initially, followed by TAK-875 (200 nM) and JTT (300 nM) while TUG891  
224 (100 nM), Metabolex-36 (100 nM) and AZ423 (100 nM) were used to preferentially activate  
225 FFA4 receptors unless otherwise quoted. Finally, PYY (10 nM) was added as an internal control.  
226 ANT825 was chosen as the preferred FFA1 antagonist in further studies.

227

#### 228 *Y<sub>1</sub> and Y<sub>2</sub> receptor antagonist (BIBO3304 and BIIE0246) studies*

229 Tissues were pretreated with previously optimised concentrations of the Y<sub>1</sub> antagonist, BIBO3304  
230 (BIBO, 300 nM, bl) or the Y<sub>2</sub> antagonist, BIIE0246 (BIIE, 1  $\mu M$ , bl) or both, versus their  
231 respective DMSO (0.003-0.1 %) controls. After 10-15 min VIP was added and once this response  
232 had stabilised, a single apical concentration of the FFA1 or FFA4 agonists was added and the  
233 consequent reduction in  $I_{sc}$  was converted to  $\mu A.cm^{-2}$ . PYY and the  $\alpha_2$ -adrenoceptor agonist,  
234 UK14,304 (1  $\mu M$ ) were used as internal controls.

235

#### 236 *Mucosal glucose-sensitivity studies*

237 Excised whole colon was placed in fresh glucose-free KH with the following composition (in  
238 mM); NaCl 118, KCl 4.7, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, and D-mannitol 11.1.  
239 Mucosae were dissected as described above, placed in Ussing chambers and were bathed in KH  
240 containing glucose on both sides or replacing glucose with mannitol (at 11.1 mM) on the ap side  
241 only. When investigating FFA1 and FFA4 agonism in the presence of lower or higher glucose;  
242 the glucose concentration was altered (either 5 mM or 25 mM in KH) on ap and bl sides  
243 simultaneously. Mucosal basal  $I_{sc}$  levels were allowed to stabilise before the addition of VIP and  
244 once stabilised, a single apical addition of the FFA1 or FFA4 agonists was added and consequent  
245 reductions in  $I_{sc}$  were recorded and converted to  $\mu A.cm^{-2}$ . Phloridzin (50  $\mu M$ , ap), the sodium-

246 glucose co-transporter 1 (SGLT1) inhibitor was used to block apically located SGLT1 and this  
247 electrogenic response should be lost when mannitol replaced apical glucose.

248

#### 249 *Faecal pellet propulsion in vitro*

250 The colon (from the caeco-colonic junction to the rectum) was excised, photographed (t = 0 min)  
251 and bathed in KH at 37°C with either vehicle (0.1% DMSO) or agonist (300 nM TUG424,  
252 TUG891, TAK-875 or Metabolex-36) as indicated. After 20 min (t = 20 min) each colon length  
253 was re-photographed and the distance travelled by the remaining pellets was measured from the  
254 rectum, as described previously (Tough et al. 2011). The effects on transit of the agonists were  
255 compared in the absence and presence of the FFA1 antagonist, GW1100 (10 µM). In these  
256 experiments, the colon was bathed in vehicle (0.1% DMSO) or antagonist, photographed at 20  
257 min, and then bathed in KH containing the agonist of choice for 20 min. The colon was re-  
258 photographed at t = 40 min. The pellet movement was measured as mean distance travelled  
259 relative to the total colonic length and was used to calculate the % colonic transit.

260

#### 261 *Colonic bead excretion in vivo*

262 Mice were acclimatised to handling 3 days prior to experimentation and were fasted 16 h prior to  
263 testing, although water was provided *ad libitum*. Plasma glucose before the fast was  $10.5 \pm 0.9$   
264 mmol/l (mean  $\pm$  1 SEM,  $n=9$ ) and  $4.8 \pm 0.3$  mmol/l after fasting ( $n=9$ ). Distal colonic propulsion  
265 was measured according to the methods described by Forbes et al (2012). One hour after  
266 administration of vehicle, or drug (FFA1 agonist, TAK-875; FFA4 agonist, Metabolex-36, FFA1  
267 antagonist, ANT825) or the positive control (loperamide hydrochloride (HCl) by oral gavage or  
268 intraperitoneal (i.p.) injection, mice were placed under 4-5% isoflurane anaesthesia (Isoflurane-  
269 VET, Merial Animal Health Ltd, Harlow, UK) and a 2 mm bead was inserted 2 cm intrarectally  
270 into the distal colon using blunt tubing (Portex,  $1.7 \times 0.4$  mm). The mice were subsequently  
271 placed into a grid bottom cage, monitored and the time to bead expulsion was measured. Once the  
272 bead was excreted, the mouse was killed by cervical dislocation. All drugs were suspended in  
273 their respective vehicles and sonicated for 30 min.

274

#### 275 *Upper GI transit in vivo*

276 Mice were acclimatized to handling 3 days prior to experimentation and were fasted for 16 h prior  
277 to testing. TAK875, Metabolex-36, ANT825, their respective vehicles or loperamide HCl was  
278 administered via i.p. injection, 60 min prior to testing. In the experiments that used the SST<sub>2</sub>  
279 antagonist, CYN 154806, mice were pretreated with the antagonist (3 mg/kg) or saline for 15 min,  
280 prior to Metabolex-36 (50 mg/kg, i.p.). A charcoal meal (10% plant charcoal in 5% gum acacia  
281 (Tough et al. 2011)) was given by intragastric gavage, and 30 min later the animal was killed by  
282 cervical dislocation and the small intestine was isolated from the pyloric to ileocecal junctions.  
283 Upper GI transit (UGIT), encompassing gastric emptying and small intestinal motility was  
284 determined as previously described (Forbes et al. 2012).

285

### 286 *Statistical analysis*

287 Pooled responses (as  $\mu\text{A}\cdot\text{cm}^{-2}$ ) are expressed as mean  $\pm$  SEM from the numbers of observations as  
288 shown, using GraphPad Prism version 6.0 (GraphPad Prism Inc., La Jolla, CA, USA). Single  
289 comparisons between groups of data were analysed by unpaired, two-tailed Student's *t*-tests or  
290 one-way ANOVA for multiple comparisons with *post hoc* Dunnett's test. *Post hoc* tests were run  
291 only when *F* achieved  $P < 0.05$  and there was no significant variance inhomogeneity.  $P < 0.05$   
292 was considered statistically significant. Mice (males and females) were randomised in this study.  
293 Additionally, drug treatments added to each mucosal preparation were randomised using the Latin  
294 square design technique. Furthermore, vehicle and drug treatments were alternated in the *in vivo*  
295 studies for each cohort. Explicit blinding was not performed however; each repeat was compared  
296 with one another and the drug-pretreated repeats. Any discrepancies between n number cohorts  
297 were due to the loss of a single mucosal preparation from a single animal *in vitro*, or a value was  
298 removed when it was more than two standard deviations from the mean *in vitro* and *in vivo*. The  
299 data and statistical analyses comply with the recommendations on experimental design and  
300 analysis in pharmacology (Curtis et al. 2015).

301

### 302 *Materials*

303 FFA1 (JTT and TAK-875) and FFA4 agonists (AZ423 and Metabolex-36) and the FFA1  
304 antagonist (ANT825) were obtained from AstraZeneca, Gothenburg, Sweden. Stock solutions of  
305 drugs were dissolved in neat DMSO (at  $10^{-2}$  M or  $10^{-3}$  M). Initial 1:10 dilutions were in neat  
306 DMSO and subsequent serial dilutions were in distilled water (excluding the FFA1 agonist, TAK-

307 875 and the FFA4 agonist, AZ423, where all serial dilutions were performed in distilled water).  
308 BIBO3304, (*N*-[(1*R*)-1-[[[4-[[[4-(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-  
309 [(aminoiminomethyl)amino]butyl]- $\alpha$ -phenyl-benzeneacetamide ditrifluoroacetate), BIIE0246, (*N*-  
310 [(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-  
311 yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b,e*]azepin-11-yl)-1-  
312 piperazinyl]-2-oxoethyl]-cyclopentaneacetamide), GW9508 (4-[[[3-  
313 Phenoxyphenyl)methyl]amino]benzenepropanoic acid), TUG424, (3-(4-(*o*-  
314 Tolylethynyl)phenyl)propanoic acid), TUG891 (4-[(4-Fluoro-4'-methyl[1,1'-biphenyl]-2-  
315 yl)methoxy]-benzenepropanoic acid) and CYN 154806 (Ac-(4-NO<sub>2</sub>-Phe)-*cyc*(D-Cys-Tyr-D-Trp-  
316 Lys-Thr-Cys)-D-Tyr-NH<sub>2</sub>) were purchased from Tocris Bioscience (Bristol, UK). GW1100 (4-[5-  
317 [(2-ethoxy-5-pyrimidinyl)methyl]-2-[[[4-fluorophenyl)methyl]thio]-4-oxo-1(4*H*)-pyrimidinyl]-  
318 benzoic acid, ethyl ester) and loperamide HCl was from Cambridge Bioscience (Cambridge, UK).  
319 VIP and PYY were purchased from Cambridge Bioscience (Cambridge, UK) and TTX from  
320 Abcam, (Cambridge, UK). VIP, PYY and TTX were dissolved in distilled water. All peptide  
321 stocks were stored at -20° C until required and underwent a single freeze-thaw cycle. Phloridzin  
322 (1-[2-( $\beta$ -D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone) was  
323 purchased from Sigma-Aldrich (Dorset, UK) and it too was dissolved in distilled water. The  
324 following *in vivo* vehicles were all suspended in warm saline, methylcellulose and  
325 polyvinylpyrrolidone (PVP) from Sigma-Aldrich (Dorset, UK), sodium dodecyl sulfate (SDS)  
326 from VWR International (Leicestershire, UK), hydroxypropyl methylcellulose (HPMC) from  
327 Alfa Aesar (Lancashire, UK) and Tween-80 from Boston BioProducts (Ashland, USA).

328

### 329 *Nomenclature of Targets and Ligands*

330 Key protein targets and ligands in this article are hyperlinked to corresponding entries in  
331 <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide  
332 to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide  
333 to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

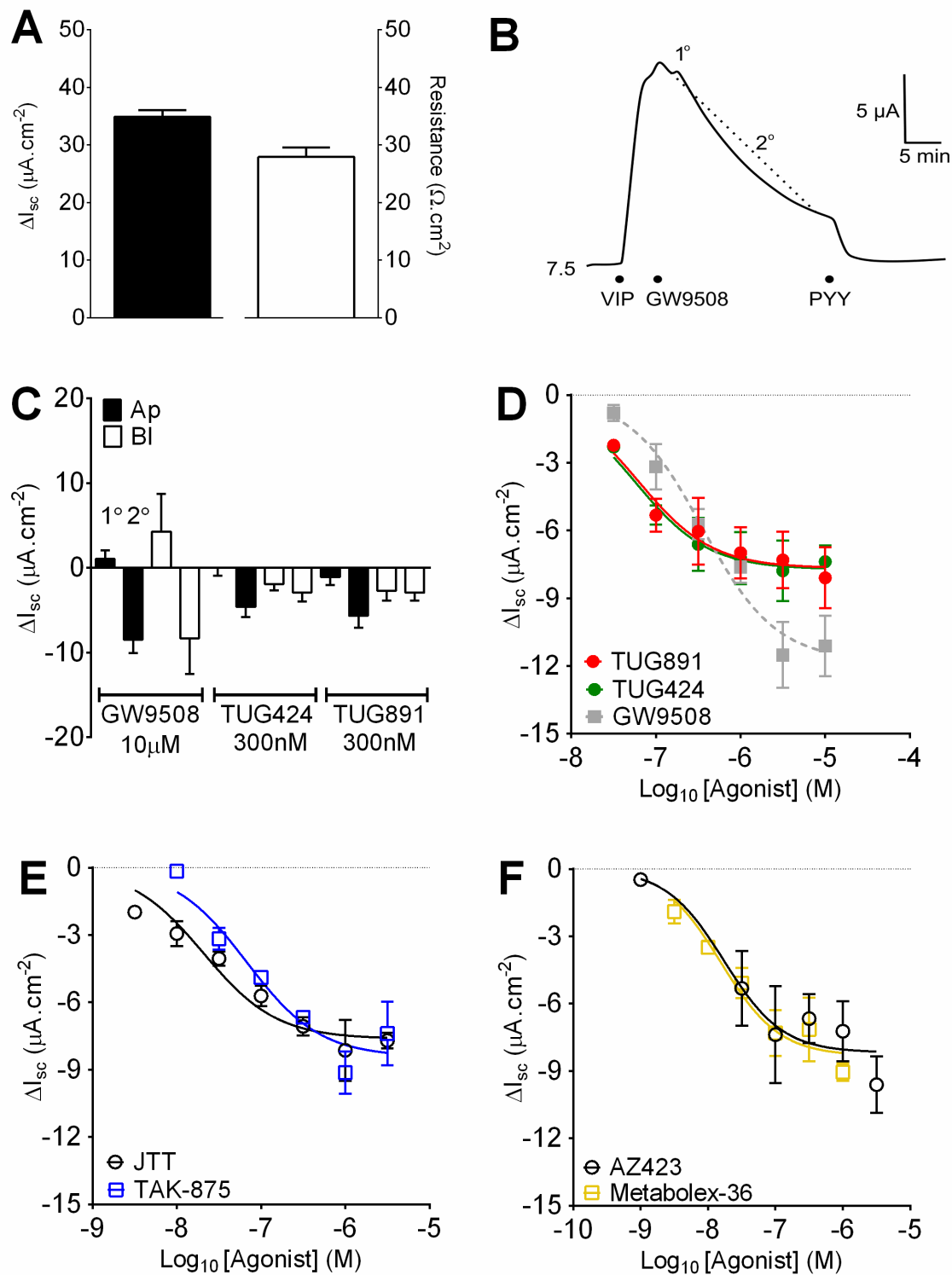
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## 335 **Results**

336 *The sidedness, potency and efficacy of FFA1 and FFA4 agonists in descending colon mucosa*

337 First, basal resistance and  $I_{sc}$  levels after stabilisation of the colonic mucosae were within ranges  
338 published previously (Figure 1A) (Tough et al. 2011). The activity of the non-selective FFA1 and  
339 FFA4 agonist, GW9508 (Figure 1B), as well as the TUG compounds added apically or  
340 basolaterally induced a biphasic change in  $I_{sc}$ . When the reductions in  $I_{sc}$  were compared, there  
341 were no significant differences between the response sizes to each agonist added to either side  
342 (Figure 1C). The more selective FFA1 agonists, TAK-875 and JTT and FFA4 agonists,  
343 Metabolex-36 and AZ423 were added apically and induced a biphasic  $I_{sc}$  response. The rapid first  
344  $I_{sc}$  component was attributed to the vehicle, 0.1% DMSO ( $3.0 \pm 1.1 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=12$ ) when  
345 compared with the same component for JTT ( $1.7 \pm 0.9 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=5$ ) and Metabolex-36 ( $3.2 \pm 1.7$   
346  $\mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=4$ ) or 0.01% DMSO ( $3.0 \pm 1.1 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=6$ ) when compared with TAK-875 ( $2.5 \pm$   
347  $1.3 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=6$ ) and AZ423 ( $4.6 \pm 1.4 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=6$ ). Only the later reductions in  $I_{sc}$  to varying  
348 concentrations of each FFA1 and FFA4 agonists are shown in Figure 1 D - F. To determine  
349 whether the FFA1 and FFA4 responses were neuronal, we pretreated the mucosae with TTX (100  
350 nM, bl). The responses to TAK-875 ( $-11.2 \pm 2.0 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=5$ ) and Metabolex-36 ( $-8.3 \pm 2.3$   
351  $\mu\text{A}\cdot\text{cm}^{-2}$ ,  $n=5$ ) were unaffected by TTX compared to their DMSO controls ( $-7.5 \pm 1.8 \mu\text{A}\cdot\text{cm}^{-2}$  and  
352  $-8.1 \pm 2.2 \mu\text{A}\cdot\text{cm}^{-2}$  respectively,  $n=5$ ), indicating FFA1 and FFA4 responses are most likely  
353 epithelial in origin and their responses are not mediated by TTX-sensitive neurons.

354 The reductions in  $I_{sc}$  to each agonist were concentration-dependent, with TUG424 and TUG891  
355 being similarly potent, approximate  $EC_{50}$  values were 57.1 nM (24.7 – 131.8 nM) and 62.5 nM  
356 (24.1 – 162.3 nM) respectively (Figure 1D). GW9508 was less potent with an  $EC_{50}$  value of 354.8  
357 nM (191.6 – 656.8 nM) and appeared to be slightly more efficacious; however, this was not  
358 significantly different from the TUG agonists (Figure 1D) or the selective FFA1 or FFA4 agonists.  
359 JTT was more potent ( $EC_{50}$  of 20.7 nM (12.7 – 34.0 nM)) than TAK-875 ( $EC_{50}$  of 67.6 nM (30.6 –  
360 149.4 nM)) and they exhibit similar efficacy (Figure 1E). Furthermore, the FFA4 agonist  
361 Metabolex-36 ( $EC_{50}$  of 15.4 nM (7.9 – 30.4 nM)) and AZ423 ( $EC_{50}$  of 17.3 nM (3.6 – 83.8 nM))  
362 were similarly potent and efficacious (Figure 1F). In comparison to the selective FFA1 and FFA4  
363 agonists, GW9508 appeared to be a dual agonist.



364

365 **Figure 1. FFA1 and FFA4 responses in descending colon mucosa.** In A: basal I<sub>sc</sub> and  
 366 resistance values after stabilisation of mucosal preparations (*n*=20). In B: representative trace  
 367 showing the biphasic nature of apical GW9508 (10  $\mu M$ ) response in descending colon mucosa  
 368 after VIP (30 nM) and followed by PYY (10 nM). In C: pooled data showing the primary (1°) and

369 secondary (2°) responses to apical (ap) or basolateral (bl) additions of GW9508 (*n*=5), TUG424  
370 (*n*=5) or TUG891 (*n*=5). The 1° responses were recorded within 0-5 min of agonist addition (and  
371 were due to vehicle), whereas 2° reductions in *I*<sub>sc</sub> occurred within 10-15 min. Concentration-  
372 response curves for the 2° effects of TUG424 (*n*=5 for all concentrations, except 300 nM (*n*=6))  
373 and TUG891 (*n*=5) compared with GW9508 (*n*=5) shown in grey in D; for JTT (*n*=5) and TAK-  
374 875 (10 nM (*n*=5), 30 nM (*n*=5), 100 nM (*n*=6), 300 nM (*n*=5), 1 μM (*n*=7) and 3 μM (*n*=8)) in E;  
375 and AZ423 (*n*=5) and Metabolex-36 (*n*=5) in F. Bars and points are the means ± 1SEM.

376

377 *GW1100 and ANT825 each inhibit agonist-induced FFA1 responses in the descending colon*

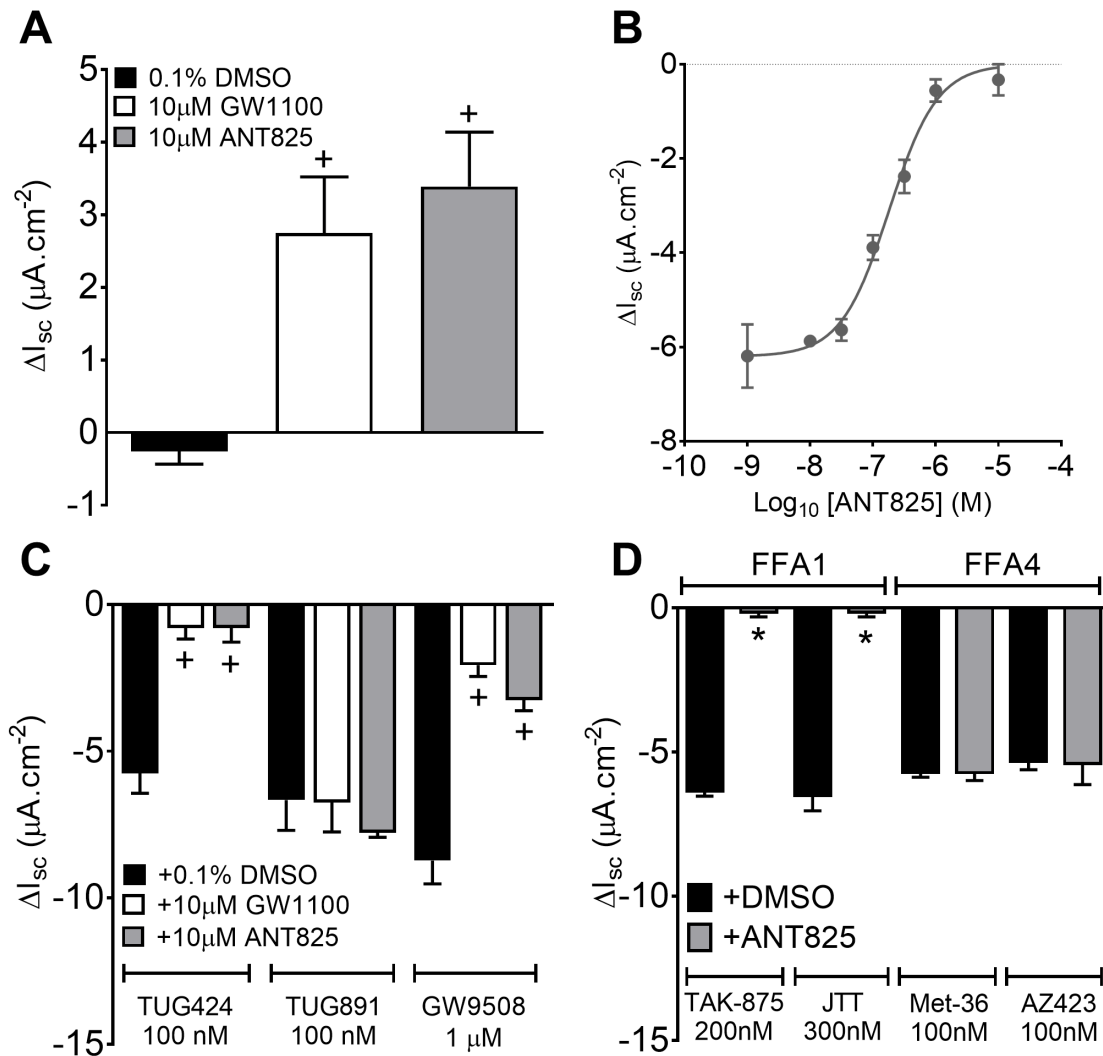
378 The FFA1 antagonists, GW1100 and ANT825 alone revealed a similar degree of FFA1 tonic  
379 activity under basal conditions in the distal colon (Figure 2A). ANT825 inhibited JTT responses  
380 competitively (Figure 2B), with an IC<sub>50</sub> of 219 nM. Optimal blocking concentrations of each  
381 FFA1 antagonist, GW1100 or ANT825 revealed the selectivity of TUG424 and GW9508 for the  
382 FFA1 receptor. TUG891 responses were not affected by either FFA1 antagonist. Additionally,  
383 only part of the GW9508 response was reduced in the presence of ANT825 or GW1100 (Figure  
384 2C). The FFA1 selective agonists (TAK-875 and JTT) were significantly inhibited by ANT825  
385 whereas the FFA4 selective agonists, Metabolex-36 and AZ423 responses were unaffected (Figure  
386 2D).

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393 **Figure 2. Tonic FFA1 activity and inhibition of FFA1 responses by GW1100 or ANT825.** In  
394 A: changes in  $I_{sc}$  to vehicle, DMSO or the FFA1 antagonists, GW1100 or ANT825 alone. In B:  
395 competitive inhibition of the FFA1 agonist, JTT responses by pretreatment with the FFA1  
396 antagonist, ANT825. Each point is the mean  $\pm 1$ SEM ( $n=5$  for all concentrations of JTT except 10  
397  $\mu M$  ( $n=6$ )). In C: pooled data shows the selective inhibition of apical TUG424 and GW9508  
398 responses, but not TUG891 responses following apical GW1100 or ANT825 treatment compared  
399 with respective vehicle controls (+DMSO controls for TUG424 ( $n=6$ ), for TUG891 ( $n=5$ ) and for  
400 GW9508 ( $n=5$ )). In D: selective inhibition of the FFA1 agonists TAK-875 and JTT responses but  
401 not the FFA4 agonist, AZ423 and Metabolex-36 (Met-36) responses following apical ANT825  
402 treatment (10  $\mu M$ ,  $n=5$ ). Statistical differences from respective vehicle controls are shown as



403 follows; \*  $P < 0.05$  (Student's  $t$  test) and <sup>+</sup>  $P < 0.05$  (one-way ANOVA with Dunnett's *post hoc*  
404 test).

405

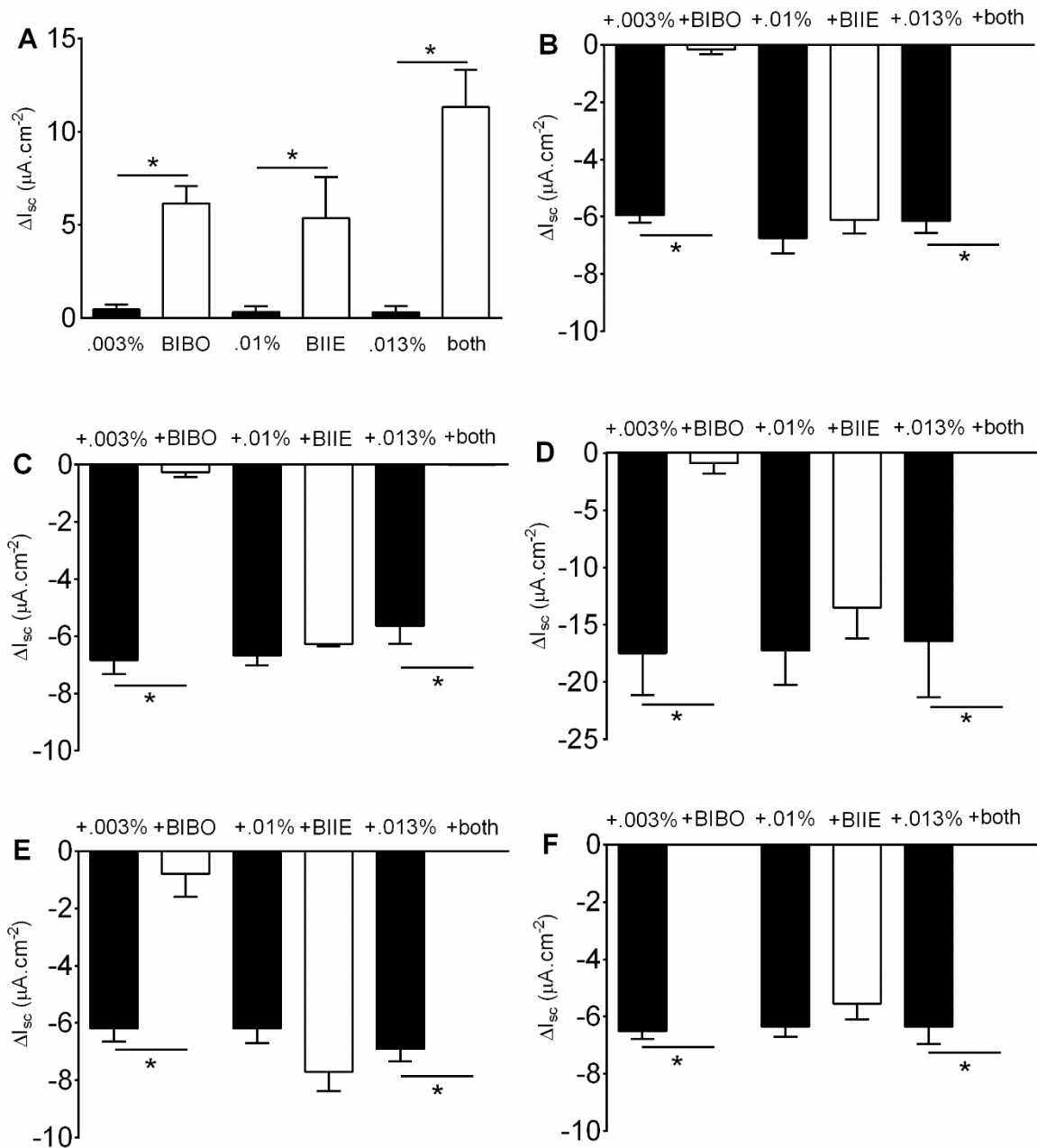
406 *FFA1 and FFA4 colonic responses are BIBO3304 but not BIIE0246 sensitive*

407 Endogenous PYY mediation of the FFA1 (JTT and TAK-875) and FFA4 (AZ423 and  
408 Metabolex-36) agonist responses were determined by blocking with the  $Y_1$  antagonist,  
409 BIBO3304, the  $Y_2$  antagonist, BIIE0246 or both antagonists together. Each antagonist  
410 revealed endogenous PYY- $Y_1$  and  $Y_2$  tonic activity under basal conditions (Figure 3A)  
411 similar to that observed in the mouse colon previously (Tough et al. 2011, Hyland et al.  
412 2003). FFA1 responses to JTT (Figure 3B) and TAK-875 (Figure 3C) were abolished by the  
413  $Y_1$  antagonist, indicating PYY- $Y_1$  signalling predominantly mediates FFA1 responses. The  
414 FFA1 responses in the presence of the  $Y_2$  antagonist were slightly reduced, suggestive of a  
415 minor role for  $Y_2$  receptors as seen previously (Tough et al. 2011). Notably, there was no  
416 influence of the  $I_{sc}$  level at the time of adding the FFA1 agonist on subsequent FFA1  
417 efficacy. Taken together these observations confirm selective  $Y_1$ -mediation of FFA1  
418 responses. Subsequent exogenous PYY responses were also abolished by the  $Y_1$ , but not by  
419 the  $Y_2$  antagonist alone, while the combination abolished PYY activity (Figure 3D). The  
420 responses to FFA4 agonists, AZ423 (Figure 3E) and Metabolex-36 (Figure 3F) were also  
421 abolished by the  $Y_1$  but not  $Y_2$  antagonist and thus both FFA1 and FFA4 responses in the  
422 mouse colon are  $Y_1$  receptor mediated.

423

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426

427 **Figure 3.  $Y_1$ - but not  $Y_2$ -receptor sensitivity of FFA1 and FFA4 agonism in the descending**  
 428 **colon.** The effects of the  $Y_1$  (BIBO3304, BIBO, 300 nM)  $\pm$  the  $Y_2$  (BIIE0246, BIIE, 1  $\mu$ M)  
 429 antagonist and corresponding DMSO controls (+.003 %, +.01 %, +.013 %) on baseline  $I_{sc}$  levels  
 430 are shown in A. The effect of the FFA1 agonists, JTT ( $n=5$  for all data groups except +.003% and  
 431 +.013% ( $n=6$ )) and TAK-875 ( $n=5$  for all data groups except +BIBO ( $n=6$ )) in the absence or  
 432 presence of Y antagonists are shown in B and C respectively. Exogenous PYY (10 nM) responses  
 433 ( $n=5$  for all data groups except +.003% and +.013% ( $n=6$ )) after JTT treatment, in the presence of  
 434 DMSO or  $Y_1 \pm Y_2$  antagonists are shown in D. The FFA4 agonist's responses, AZ423 ( $n=5$ ) and

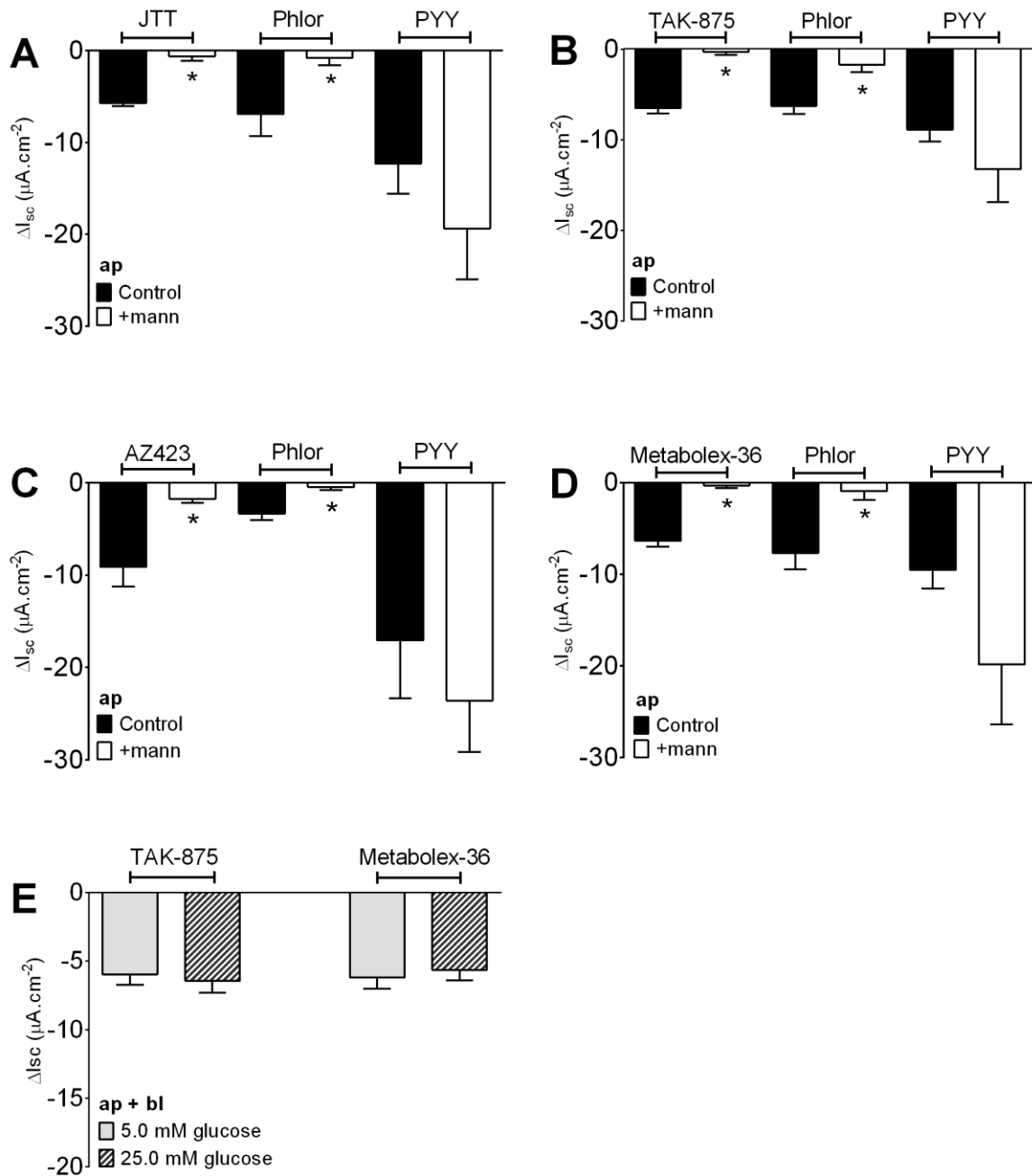
435 Metabolex-36 ( $n=5$  for all data groups except +.01% ( $n=6$ )) are shown in E and F respectively.  
436 Bars represent the mean  $\pm$  1 SEM. Statistical differences between vehicle controls (0.003 %, 0.01  
437 % and 0.013 % DMSO) and respective antagonists BIBO, BIIE or both are shown as follows; \*  $P$   
438  $< 0.05$  (Student's  $t$  test).

439

440 *FFA1 and FFA4 responses are glucose-sensitive in the descending colon mucosa*

441 In order to establish the glucose-sensitivity of FFA signalling in mucosal preparations, the  
442 responses to the FFA1 and FFA4 agonists were compared in the presence or absence of apical  
443 glucose. Mannitol replacement of apical glucose abolished the FFA1 responses (Figure 4A & B)  
444 and inhibited FFA4 activity (Figure 4C & D) compared to vehicle. Internal controls using  
445 phloridzin (Phlor, added ap) decreased the  $I_{sc}$ , but as expected only in the presence of glucose  
446 (because SGLT1 requires glucose to function and is targeted apically). Thus, FFA1 and FFA4  
447 receptors can be activated in a glucose-sensitive manner. In contrast, PYY responses were not  
448 glucose-sensitive (Figure 4A-D). Furthermore, TAK-875 and Metabolex-36 responses were not  
449 affected when the glucose concentration (ap + bl) was varied to represent a hypoglycaemic (5.0  
450 mM) or hyperglycaemic (25.0 mM) environment (Figure 4E).

451



452

453 **Figure 4. Glucose-sensitivity of FFA1 and FFA4 agonism in the descending colon.** Glucose-  
 454 sensitivity of apical FFA1 agonists, JTT (300 nM, *n*=5) in A, TAK-875 (200 nM, *n*=5) in B and  
 455 apical FFA4 agonists, AZ423 (100 nM, *n*=5) in C and Metabolex-36 (100 nM, *n*=5) in D, in the  
 456 presence (black bars) and absence (white bars) of 11.1 mM glucose. Control mucosae were  
 457 bathed in glucose both sides whereas, mannitol (+ mann, 11.1 mM) replaced glucose apically  
 458 only. Phloridzin (Phlor, 50μM, apically only) and PYY (10 nM) responses are also shown. In E:  
 459 TAK-875 (200 nM, *n*=5) and Metabolex-36 (100 nM, *n*=5) antisecretory responses in the presence  
 460 of apical and basolateral (ap + bl) 5.0 mM glucose or 25.0 mM glucose. Bars represent the mean -

461 1SEM. Statistical differences between agonist or phloridzin responses in the presence or absence  
462 of glucose, are shown as follows; \*  $P < 0.05$  (Student's  $t$  test).

463

464 *Caeco-colonic transit is inhibited by FFA1 and FFA4 agonists*

465 Since FFA1 and FFA4 mucosal responses in the colon were mediated by PYY and this  
466 endogenous peptide is known to slow colonic transit (Tough et al. 2011), we next assessed  
467 whether the FFA1 and FFA4 agonists reduced transit at single optimal concentrations (300 nM;  
468 Figure 5A). In isolated colons, TUG424, TUG891, TAK-875 and Metabolex-36 significantly  
469 decreased transit in comparison to vehicle-treated (DMSO, 0.1%) controls.

470 Since GW1100 (10  $\mu$ M) inhibited FFA1 responses in colonic mucosa, we set out to determine  
471 whether GW1100 alone could increase basal faecal transit and reverse the effect of the FFA1  
472 agonist on colonic transit, *in vitro*. Figure 5B shows that GW1100 alone significantly increased  
473 basal colonic transit, again indicating endogenous FFA1 inhibitory tone. After 20 min  
474 pretreatment with the FFA1 antagonist, GW1100 the effect of TUG424 and TUG891 was assessed  
475 in the presence and absence of GW1100. In the presence of GW1100, the inhibitory effect on  
476 colonic transit of TUG424 was reversed, but GW1100 had no effect on TUG891 (FFA4) activity,  
477 showing FFA1 selectivity (Figure 5B).

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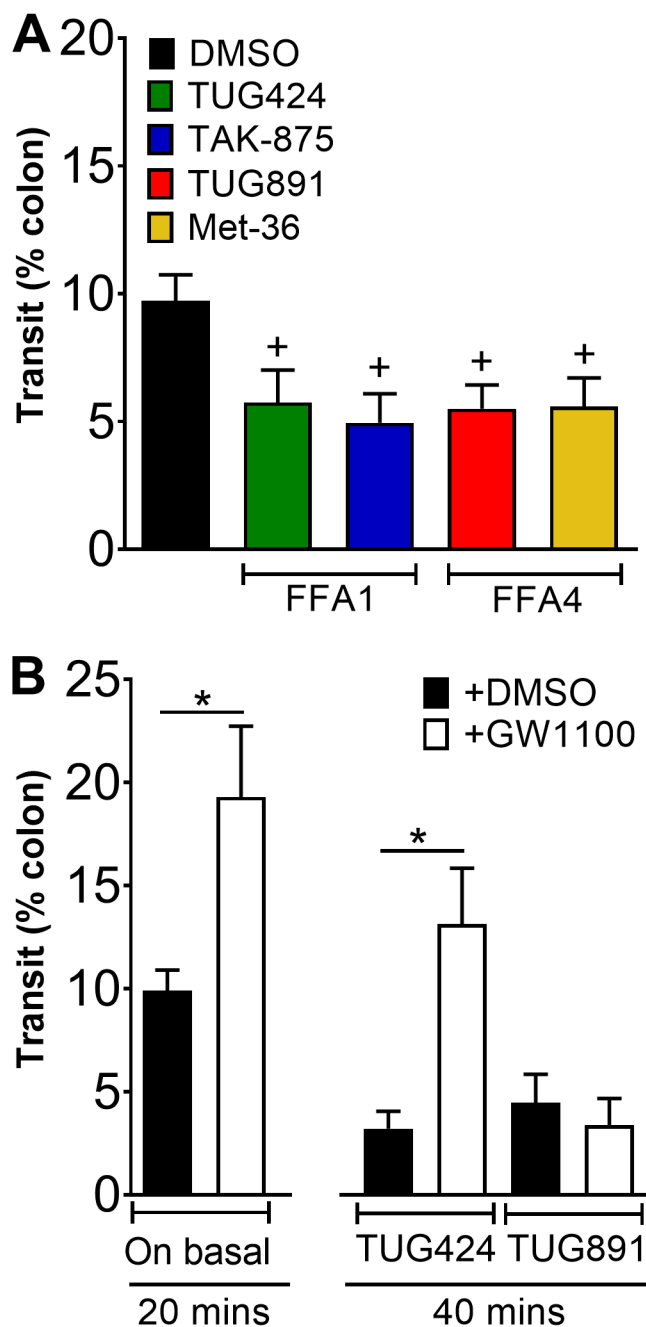
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506 **Figure 5. Colonic transit is slowed by FFA1 and FFA4 agonists in isolated colons *in vitro*.**  
 507 Colonic transit is slowed by 300 nM of TUG424 ( $n=6$ ), TAK-875 ( $n=6$ ), TUG891 ( $n=6$ ) and  
 508 Metabolex-36 (Met-36,  $n=6$ ) in A at  $t=20$  min. In B: the effects of FFA1 antagonist GW1100 (10  
 509  $\mu\text{M}$ ) are compared on basal faecal transit at  $t=20$  min and on subsequent inhibition of transit  
 510 following 20 min treatment with TUG424 or TUG891 at  $t=40$  min ( $n=5$ ). Each bar represents  
 511 the mean + 1SEM. Statistically significant differences from controls were; \*  $P < 0.05$  (Student's  $t$   
 512 test) and <sup>+</sup>  $P < 0.05$  (one-way ANOVA with Dunnett's *post hoc* test).

513

514 *Colonic bead propulsion is slowed by Metabolex-36 not TAK875 or ANT825 in vivo*

515 As the FFA1 and FFA4 agonists, (TAK-875 and Metabolex-36 respectively) slowed transit in  
516 isolated colons *in vitro*; we assessed the ability of these agonists and loperamide HCl to slow  
517 colonic transit *in vivo*, comparing oral administration versus i.p. injection. Additionally, we  
518 investigated whether ANT825 had the potential to reveal FFA1 tone *in vivo*. Oral administration  
519 of the positive control, loperamide HCl significantly slowed transit compared to its vehicle  
520 control. Two doses of Metabolex-36 were assessed orally; the lower dose had no effect on transit  
521 whereas the higher dose significantly slowed transit. Furthermore, oral gavage of TAK-875 and  
522 ANT825 had no effect on colonic transit (Figure 6A). Both loperamide HCl and Metabolex-36  
523 significantly slowed colonic transit when given via i.p. injection whereas TAK-875 and ANT825  
524 had no effect (Figure 6B). As i.p. injection of loperamide HCl slowed transit more efficiently in  
525 comparison to oral gavage, we used this route of administration for each drug in the subsequent *in*  
526 *vivo* studies.

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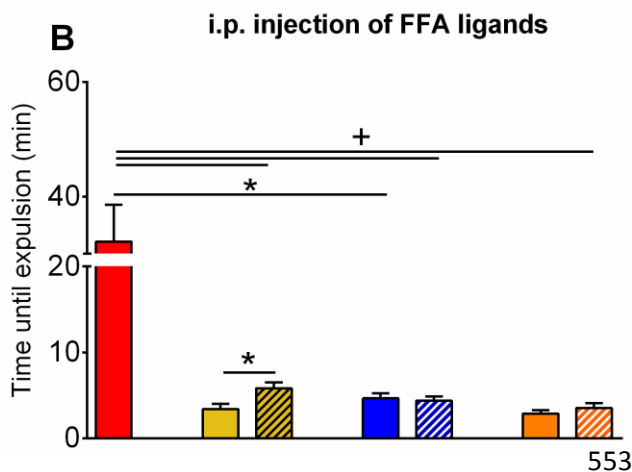
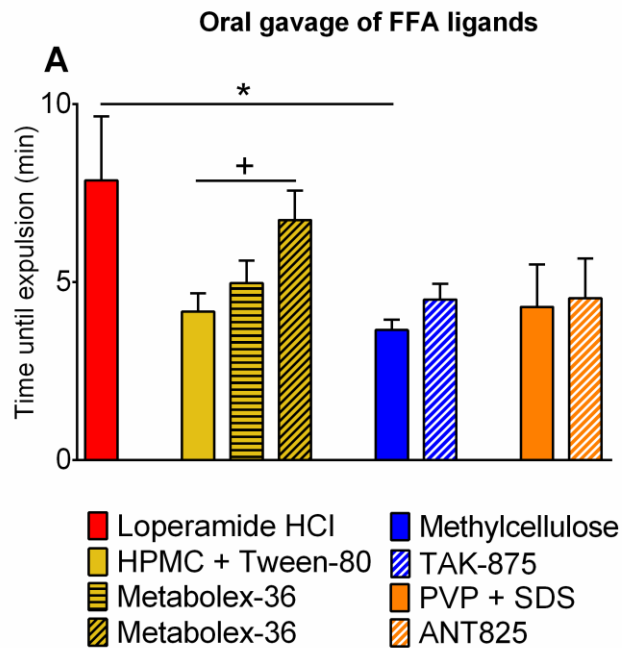
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554 **Figure 6. The effect of oral gavage versus i.p. injection of FFA ligands and loperamide HCl**  
 555 **on colonic bead expulsion *in vivo*.** The effect on colonic bead expulsion (expressed as time until  
 556 bead expelled (min)) after oral gavage or i.p. injection of loperamide HCl (10 mg/kg, red bar,  
 557  $n=11$ ) compared with its vehicle control, 0.5% methylcellulose (blue bar,  $n=11$ ); the FFA4  
 558 agonist, Metabolex-36 at two doses: 6 mg/kg (yellow bar with black horizontal stripes,  $n=6$ ) and  
 559 50 mg/kg (yellow bar with black diagonal stripes,  $n=10$ ) compared with their vehicle control,  
 560 0.5% hydroxylpropyl methylcellulose (HPMC) + 0.1% Tween-80 (yellow bar,  $n=10$ ); the FFA1  
 561 agonist, TAK-875 (27 mg/kg, blue bar with white diagonal stripes,  $n=11$ ) compared with its  
 562 vehicle control (0.5% methylcellulose, blue bar) and finally the FFA1 antagonist, ANT825 (29  
 563 mg/kg, orange bar with white diagonal stripes,  $n=7$ ) compared with its vehicle (0.25%  
 564 polyvinylpyrrolidone (PVP) + 0.05% sodium dodecyl sulfate (SDS), orange bar,  $n=7$ ). Each bar



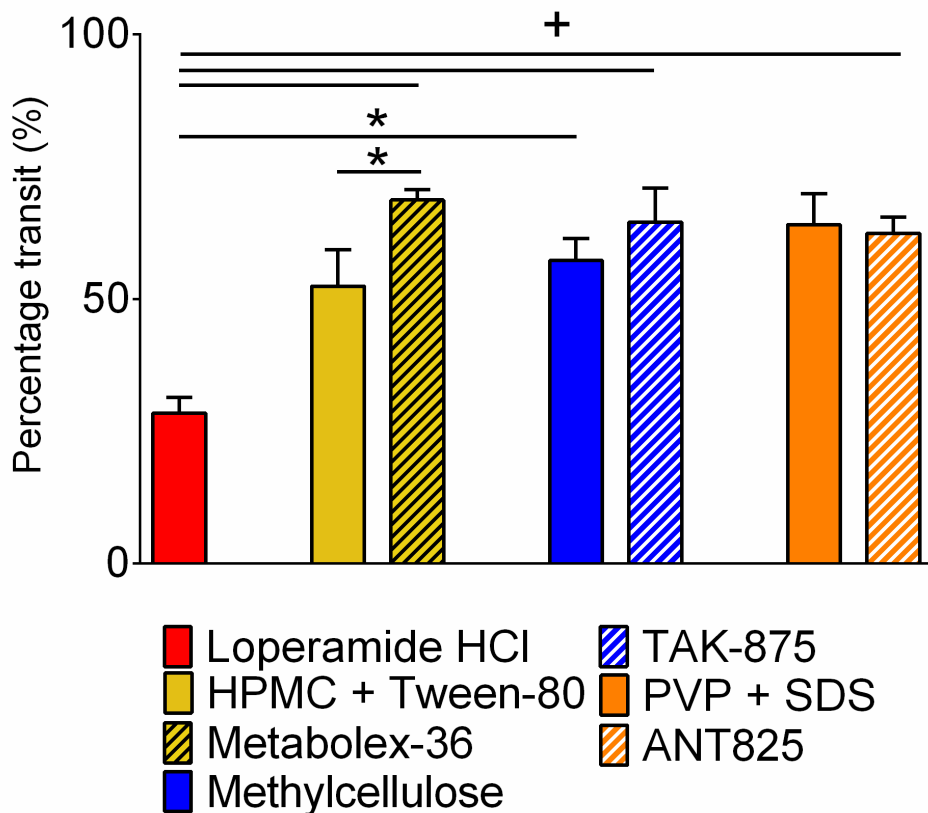
565 represents the mean + 1SEM. Statistically differences from controls were; \*  $P < 0.05$  (Student's  $t$ -  
566 test). Differences between the oral doses of Metabolex-36 from its control and additionally  
567 between loperamide HCl (i.p.) and the FFA drugs were; +  $P < 0.05$  (one-way ANOVA with  
568 Dunnett's *post hoc* test).

569

570 *Upper GI transit is increased by Metabolex-36 in vivo*

571 Here we investigated whether the FFA agonists (i.p.) and loperamide HCl could slow intestinal  
572 transit and additionally whether ANT825 could increase intestinal transit (revealing FFA1 tone),  
573 after mice were given a charcoal meal (as described in the methods). The positive control,  
574 loperamide HCl significantly slowed GI transit, as expected compared to its vehicle control.  
575 Unexpectedly, Metabolex-36 significantly increased intestinal transit whereas TAK-875 and  
576 ANT825 had no effect (Figure 7).

577



589

590 **Figure 7. The effect of an i.p. injection of loperamide HCl and FFA ligands on UGIT *in vivo*.**

591 The intestinal transit of a charcoal meal (expressed as a percentage of the small intestine length)  
592 after i.p. injection of the positive control, loperamide HCl (10 mg/kg, red bar,  $n=6$ ) compared with  
593 its vehicle control, 0.5% methylcellulose (blue bar,  $n=6$ ); the FFA4 agonist, Metabolex-36 (50  
594 mg/kg, yellow bar with black diagonal stripes,  $n=6$ ) compared with its vehicle control, 0.5%  
595 hydroxypropyl methylcellulose (HPMC) + 0.1% Tween-80 (yellow bar,  $n=6$ ); the FFA1 agonist,  
596 TAK875 (27 mg/kg, blue bar with white diagonal stripes,  $n=5$ ) compared with its vehicle control  
597 (0.5% methylcellulose, blue bar) and the FFA1 antagonist, ANT825 (29 mg/kg, orange bar with  
598 white diagonal stripes,  $n=5$ ) compared with its vehicle (0.25% polyvinylpyrrolidone (PVP) +  
599 0.05% sodium dodecyl sulfate (SDS), orange bar,  $n=5$ ). Each bar represents the mean + 1SEM.  
600 Statistically differences from controls were; \*  $P < 0.05$  (Student *t*-test) whereas differences  
601 between loperamide HCl and FFA drugs were; +  $P < 0.05$  (one-way ANOVA with Dunnett's *post*  
602 *hoc* test).

603

604 *SST<sub>2</sub> inhibition has no effect on Metabolex-36 induced UGIT effect in vivo*

605 As Metabolex-36 increased UGIT *in vivo*, we investigated whether this was due to an inhibitory  
606 FFA4 effect (via a  $G\alpha_i$ -mediated mechanism) on somatostatin-containing D cells in the GI tract.  
607 This inhibition would result in disinhibition and ultimately a pro-motile effect, as somatostatin is a  
608 known inhibitor of GI motility. Mice were pretreated with the  $SST_2$  antagonist, CYN 154806 or  
609 saline before the effect of Metabolex-36 was tested. CYN 154806 + Metabolex-36 had no effect  
610 on the UGIT of the charcoal meal, in comparison to mice pretreated with saline (Figure 8).

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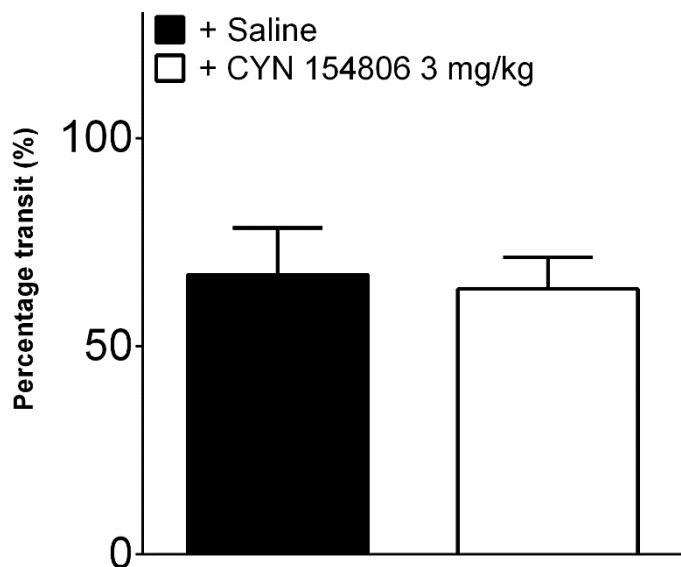
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626 **Figure 8. CYN 154806 had no effect on the Metabolex-36 induced increase in UGIT *in vivo*.**  
 627 The effect of Metabolex-36 (50 mg/kg, i.p. injection) on UGIT in the absence (saline,  $n=5$ ) or  
 628 presence ( $n=5$ ) of the SST<sub>2</sub> antagonist, CYN 154806. Bars represent mean + 1SEM.

629

### 630 Discussion

631

632 *The improved selectivity of FFA1 and FFA4 agonists versus commercially available agonists*

633 FFA1 and FFA4 agonism was independent of the surface of administration and the time-  
 634 dependence of  $I_{sc}$  changes was similar, suggesting that these receptors are located on both the  
 635 apical and basolateral surfaces. However, it should be noted that all the drugs used were lipid  
 636 soluble requiring DMSO as a vehicle therefore, drugs added on the apical surface could activate  
 637 FFA receptors on the basolateral side and vice versa. Interestingly, Christensen et al. (2015)  
 638 showed preferential but not exclusive vascular (basolateral) FFA1-induced GLP-1 secretion using  
 639 endogenous and synthetic agonists, in rat small intestine. We utilised apical administration and  
 640 the reductions in  $I_{sc}$  we observed occurred within the same time frame as other apically located L  
 641 cell  $G\alpha_q$ -coupled receptors, some activated by water-soluble compounds (Joshi et al. 2013, Forbes  
 642 et al. 2015, Alamshah et al. 2016) thus indicating apical receptors.

643 GW9508 is reported to act via FFA1 and FFA4 with 100x higher affinity for FFA1 than FFA4  
 644 (Briscoe et al. 2006). GW9508 is a dual agonist at the FFA1 and FFA4 and this may explain its

645 slightly greater efficacy than more selective agonists we used. We found that the selective FFA1  
646 and FFA4 agonists exhibited a similar potency which was more potent than the potency of  
647 GW9508. Both FFA4 agonists, Metabolex-36 and AZ423 have >100-fold higher selectivity for  
648 FFA4 in comparison to the FFA1. This phenomenon of higher potency but lower efficacy has  
649 been observed previously with a GPR119 agonist, PSN-GPR119 (Patel et al. 2014) and may  
650 indicate acute receptor desensitisation.

651 FFA1 agonists are preferentially  $G\alpha_{q/11}$ -linked and recent evidence suggests that 'G $\alpha_{q/11}$ -only'  
652 FFA1 agonists (e.g. TAK-875) stimulate the release of incretin hormones, GLP-1 and GIP, with  
653 reduced efficacy compared to FFA1 agonists that signal via  $G\alpha_{q/11}$  and  $G\alpha_s$  pathways; to cause a  
654 more robust release of GLP-1 and GIP (Hauge et al. 2015). Our FFA1 agonists appear to be  
655  $G\alpha_{q/11}$ -coupled preferentially as mucosal responses were transient in comparison with  $G\alpha_s$ -coupled  
656 L cell signalling e.g. GPR119 or MC4 (Cox et al. 2010, Panaro et al. 2014).

657

658 *FFA1 antagonism reveals FFA1 mucosal and anti-motility tone and agonist-specificity.*

659 The FFA1 antagonists, ANT825 and GW1100 revealed, for the first time, a degree of endogenous  
660 FFA1 tonic activity in colonic mucosa. The degree of tonic activity was similar to that observed  
661 with the MC4 receptor antagonist, HS014 (3.6  $\mu\text{A}\cdot\text{cm}^{-2}$ ) (Panaro et al. 2014). Since endogenous  
662 FFA1 and MC4 agonism stimulates PYY release from L cells, blockade of these receptors inhibits  
663 endogenous PYY release. Crivellato et al (2002) demonstrated that enteroendocrine cells in  
664 mouse colon undergo piecemeal degranulation, hormone release that may be related to the tonic  
665 activity we observe in mucosa. Endogenous lipids, natural ligands of the FFA1 (Itoh et al. 2003)  
666 may be responsible for the observed tonic activity via FFA1.

667 The potency of the FFA1 competitive antagonist, ANT825 was not dissimilar from its potency  
668 ( $\text{pEC}_{50}$  of 6.8) obtained in a human embryonic kidney 293 cell line expressing human FFA1,  
669 measuring inositol monophosphate (Waring 2015). We found GW1100 ( $\text{pIC}_{50}$  of 5.99; (Briscoe et  
670 al. 2006)) abolished TUG424 responses but not TUG891 responses, showing FFA1 selectivity as  
671 seen previously (Briscoe et al. 2006). Like GW1100, ANT825 abolished FFA1 responses but  
672 both FFA1 antagonists' also partially inhibited GW9508 responses, indicating that at this  
673 concentration (1  $\mu\text{M}$ ); GW9508 exerts a dual agonism via FFA1 and most likely FFA4. Briscoe et  
674 al (2006) revealed that GW1100 had no effect on the ability of GW9508 to activate FFA4. We  
675 conclude that GW9508 is a dual FFA1 and FFA4 agonist.

676 FFA1 (TUG424, TAK-875) and FFA4 (TUG891, Metabolex-36) agonists attenuated colonic  
677 motility *in vitro* to a similar degree as seen previously with MC4 receptor activation and GPR119  
678 agonism (Panaro et al. 2014). They decreased colonic motility presumably via the release of  
679 PYY, which mediates the colonic brake. Pretreatment with the FFA1 antagonist, GW1100  
680 revealed basal colonic mucosal tone (as seen with Ussing chamber studies) indicative of  
681 endogenous FFA1 activity. In the presence of the FFA1 antagonist GW1100, the colonic brake  
682 induced by the FFA1 agonist (TUG424) was disinhibited and therefore colonic transit increased  
683 whereas transit induced by FFA4 agonist (TUG891) remained unaffected, showing selectivity of  
684 the FFA1 agonist.

685 As the FFA1 agonist, TAK875 and the FFA4 agonist, Metabolex-36 slowed transit *in vitro*; we  
686 investigated their ability to slow transit *in vivo*. Following the overnight fast, plasma glucose  
687 levels were reduced to 5 mM. However, we established that FFA1 and FFA4 signalling is  
688 unchanged at 5 mM compared with 11.1 mM glucose as seen previously for GPR119 agonism  
689 (Patel et al. 2014). Metabolex-36 slowed colonic transit significantly presumably via PYY,  
690 whereas TAK-875 failed to show significant inhibition of bead colonic transit when administered  
691 orally or via i.p. injection. This apparent lack of efficacy may be due to TAK-875's partial  
692 agonism or its administration as a suspension. In our *in vitro* transit studies, the isolated colon is  
693 severed from the central nervous system (CNS) and TAK-875 acts directly upon colonic FFA1  
694 receptors. In the *in vivo* model, it's possible that the ability of TAK-875 to release PYY is not  
695 sufficient to alter colonic motility, with the added complication of modulatory CNS pathways. In  
696 addition, there are concerns in the literature that isoflurane interferes with GI transit. In 2005,  
697 Torjman and colleagues reported that 6 min exposure to isoflurane in rats slows UGIT by 50%  
698 (Torjman et al. 2005). In our study, mice received a 2 min exposure of isoflurane 1 h after the  
699 drug, vehicle or positive control were administered, followed by bead insertion and excretion.  
700 Therefore, we conclude that this brief exposure of isoflurane in our protocol (at the time of  
701 maximal drug exposure) should have minimal effect on colonic motility. Furthermore, we note  
702 that there are no differences in colonic transit rates between different vehicle controls (oral gavage  
703 vs i.p.) in this study, compared with those in a previous investigation (Forbes et al. 2012).

704 Unexpectedly, Metabolex-36 was pro-motile in the upper intestine. FFA4 is expressed on I cells,  
705 K cells and L cells causing release of CCK, GIP and PYY (and GLP-1) respectively, via  $G\alpha_q$ -  
706 signalling (Iwasaki et al. 2015, Hirasawa et al. 2005, Tanaka et al. 2008). These peptides inhibit  
707 gastric emptying and small intestinal transit and additionally, PYY is responsible for both ileal and  
708 colonic brakes (Spiller et al. 1984, Spiller et al. 1988). However, recently FFA4 has been

709 identified in gastric ghrelin and somatostatin cells, signalling via  $G\alpha_i$  proteins to inhibit peptide  
710 release (Engelstoft et al. 2013, Egerod et al. 2015). Somatostatin is known to inhibit motility and  
711 increase the interval between migrating myoelectric complexes (MMC), therefore slowing transit.  
712 We hypothesised that Metabolex-36 increased intestinal motility by inhibiting somatostatin release  
713 and tested this possibility with the SST<sub>2</sub> antagonist, CYN 154806 at an effective dose used  
714 previously in rat small intestine, *in vivo* (Booth et al. 2001). However, CYN 154806 was  
715 ineffective (at 3 mg/kg) and we conclude that either the antagonist dose used was too low in the  
716 mouse, or Metabolex-36 produces a pro-motile effect via an alternative FFA4 mechanism.

717

#### 718 *PYY-Y<sub>1</sub> but not PYY-Y<sub>2</sub> mediation of FFA1 and FFA4 signalling in mouse colonic mucosa*

719 FFA1 and FFA4 agonist responses in the mouse colon were Y<sub>1</sub> receptor mediated. Additionally,  
720 these responses were unaffected by TTX indicating a predominantly epithelial origin for FFA1  
721 and FFA4 activity, not involving TTX-sensitive submucosal neurons. The degree of antiseecretory  
722 tone mediated by the Y<sub>1</sub> and Y<sub>2</sub> receptors was similar to that shown previously in mouse colon  
723 (Hyland et al. 2003, Tough et al. 2011). The FFA1 and FFA4 signalling in colonic mucosa are  
724 mediated by endogenous PYY acting on local epithelial Y<sub>1</sub> receptors. The epithelial Y<sub>1</sub> mediation  
725 and lack of any Y<sub>2</sub> (neuronal) involvement is consistent with our previous findings for other L  
726 cell-enriched receptors e.g. MC4 (Panaro et al. 2014), GPR119 (Patel et al. 2014), FFA2 (Forbes  
727 et al. 2015) and the calcium-sensing receptor (Joshi et al. 2013). There appears to be no GLP-1  
728 mediation of FFA1 and FFA4 responses in this study. GLP-1 has a half-life of 1.5 min which  
729 limits the ability to detect its response, if any. Additionally, we have discovered that the GLP-1  
730 response can vary across the colon, with largest GLP-1 responses observed in the ascending colon  
731 and smallest in the descending colon (Tough et al. 2017). Here we have used descending colon  
732 and in combination with the short half-life of GLP-1, this may explain why we do not see a GLP-1  
733 mediated response.

734

#### 735 *FFA1 and FFA4 responses in mouse colon mucosa are glucose-sensitive*

736 Our studies demonstrate for the first time that activation of FFA1 and FFA4 responses in native  
737 tissues are glucose-sensitive as seen in previous investigations of L cell-specific activation by  
738 GPR119 (Cox et al. 2010), MC4 receptor (Panaro et al. 2014), the activation of the calcium-  
739 sensing receptor by L-glutamine (Joshi et al. 2013) and FFA2 agonism (Forbes et al. 2015).

740 Theoretically these agonists should not therefore cause hypoglycaemia *in vivo*, a disadvantage of  
741 certain anti-diabetic drugs currently on the market. FFA1 and FFA4 receptors are currently  
742 clinically favourable targets as they are not only co-expressed in GI L cells but differentially  
743 expressed in the pancreas. FFA1 is expressed in pancreatic  $\beta$ -cells (Itoh et al. 2003) and FFA4 has  
744 recently been discovered in somatostatin-containing delta cells (Stone et al. 2014). PYY and  
745 GLP-1 released from the L cells should enhance satiety (an advantage for patients with associated  
746 obesity) and elevate insulin release respectively via activation of GLP-1 receptors on pancreatic  $\beta$   
747 cells. Furthermore, an FFA1 agonist could simultaneously directly elevate insulin release after a  
748 meal. This combination of indirect and direct FFA1 effects should improve glucose tolerance  
749 without hypoglycaemia.

750

#### 751 *Final conclusions*

752 We have shown that selective FFA1 and FFA4 agonism is PYY- $Y_1$  receptor mediated and  
753 glucose-sensitive in mouse descending colon mucosa. These compounds would limit potential  
754 hypoglycaemia, a side effect of certain commercially available anti-diabetic drugs. ANT825 and  
755 GW1100 revealed tonic FFA1 activity and the possibility of FFA1 endogenous ligands causing  
756 PYY release. Furthermore, we have shown for the first time that agonism of FFA1 and FFA4  
757 inhibits colonic transit *in vitro* and FFA4 agonism induces regional differences in transit *in vivo*.  
758 In colonic L cells agonism of FFA4 is presumably  $G\alpha_q$ -mediated and leads to antisecretory  
759 mucosal responses that slows colonic transit, but leads to upper GI pro-motile activity *in vivo* in  
760 the mouse, which remains unresolved. FFA1 and FFA4 co-agonism may be beneficial  
761 therapeutically and would more closely mimic the combinatorial activity of long chain fatty acids  
762 in the diet.

763

#### 764 *Author contributions*

765 RM and IRT performed and analysed the experiments and contributed with HMC to the design  
766 and interpretation of the data, and writing of the manuscript. DMS and MS provided AstraZeneca  
767 in-house data and contributed to the design and interpretation of the work. MS and HMC had the  
768 project idea and got funding. RM, IRT, DMS, MS and HMC reviewed and approved the final  
769 manuscript version and agreed to be accountable for all aspects of the work.

770

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