The potentially beneficial CNS-activity profile of ivacaftor and its metabolites

Elena K. Schneider[1], Rachel M. McQuade[2], Vincenzo C. Carbone[3], Felisa Reyes-Ortega[4], John M. Wilson[5,6], Brenda Button[5,6], Ayame Saito[2], Daniel P. Poole[2], Daniel Hoyer[7,8,9], Jian Li[10†], Tony Velkov[1†*]

Supplementary Information

Results

Force contraction experiment

The muscarinic M_3 and β_3 receptor affinity observed in the radioligand binding assay was further assessed in an *ex vivo* rat colon contractile experiment. Individual colonic segments were exposed to increasing concentrations of either ivacaftor, iva-M1, iva-M6, lumacaftor, methacholine (M_3 agonist) (1 nM – 10 μM), or SR59230A (10 μM; selective β_3 antagonist) at 5 minute intervals to construct concentration-response curves (**Supplementary Figures 6-9**). Baths were washed to replace physiological saline solution every 20 - 30 minutes, in between drug additions throughout the experiment. Minimum and maximum amplitude of contractions were recorded following each drug application. Responses to drug application were also quantified as area under the curve (AUC) from minimum response. All values were normalised to the response given by the tissue at baseline. Application of the non-selective muscarinic receptor agonist methacholine significantly increased both the amplitude of contractions (**Supplementary Figure 6A**, **B,** $EC_{50} = 1.58 \times 10^{-6}$) and the area under the curve (**Supplementary Figure 6A**, $EC_{50} = 1.99 \times 10^{-6}$). Application of ivacaftor had no effect on the amplitude of drug-evoked contractions in the colon (**Supplementary Figure 7A**, $EC_{50} = 8.87 \times 10^{-8}$), neither did iva-M6 (**Supplementary Figure 7B,** $EC_{50} = 7.95 \times 10^{-9}$) or iva-M1 affect the amplitude of contraction in the colon (**Supplementary Figure 7C**, $EC_{50} = 9.99 \times$

10^{-7}). Application of lumacaftor had no effect on the amplitude of drug-evoked colonic contractions (**Supplementary Figure 7D**, $EC_{50} = 1.34 \times 10^{-8}$). No effect on AUC was found following application of ivacaftor in the colon (**Supplementary Figure 8A**, $EC_{50} = 2.49 \times 10^{-7}$), or iva-M6 (**Supplementary Figure 6B**, $EC_{50} = 2.78 \times 10^{-6}$) or iva-M1 (**Supplementary Figure 8C**, $EC_{50} = 2.29 \times 10^{-8}$). No drug-evoked effect on AUC was found following application of lumacaftor in the colon (**Supplementary Figure 8D**, $EC_{50} = 1.34 \times 10^{-8}$). There was no difference in basal tone when comparing drug treated vs sham treated colon (data not shown). Notably, both ivacaftor and iva-M6 evoked a 3- and 2-fold change in amplitude relative to the vehicle, respectively, in colon pre-treated with the β_3 selective antagonist SR59230A (**Supplementary Figure 9, Supplementary Table 4**). Collectively, these data suggest that ivacaftor and iva-M6 potentially bind to both to the M_3 and β_3 receptors in the rat colon.

Methods

Materials

Ivacaftor (VX-770), lumacaftor (VX-809) and tezacaftor (VX-661) was purchased from SelleckChem, TX, USA. Iva-M1 (Catalogue number 510242247CS) and iva-M6 (Catalogue number 510240849CS) were from Clearsynth (ON, Canada). Ketamine hydrochloride, fluoxetine hydrochloride, methacholine and the β3 receptor selective antagonist SR59230A were purchased from Sigma Aldrich (NSW, Australia). All of the chemicals were of the highest purity available.

Primary radioligand binding assays.

Primary radioligand binding assays were performed as described by Roth *et al (1, 2).* Briefly, compounds were initially dissolved in methanol and then serial diluted in buffer. Buffer composition was depending on the investigated receptor (**Supplementary Table 1**). A 50 µL aliquot of 10 µM ivacaftor, iva-M1, iva-M6, lumacaftor or tezacaftor or of a receptor selective reference compound was added in quadruplicate to the wells of a 96-well plate. Each well contained 50 µL of 5x radioligand (concentration depending on the receptor investigated e.g. 5x 1.57 nM for 5-HT_{2A}) (*1*) and 100 µL of buffer. The receptor or transporter containing crude membrane fractions were resuspended in buffer and dispensed into the 96-well plate. Radioligand binding was equilibrated for 1-2 h at room temperature. After equilibration, the bound radioactivity was isolated by filtration onto 0.3% (v/v) polyethyleneimine treated 96-well filter mats using a 96-well filter-mate harvester. The filter mats were dried, then scintillant was melted onto the filters and the retained radioactivity was counted on a Microbeta scintillation counter.

The total bound radioactivity of the radioligand receptor probe was measured from the negative control wells containing neither the test nor the reference compound, and taken as 100% binding. Non-specifically bound radioactivity was measured using wells containing the radioligand probe and 10 µM of a reference compound and adjusted to 0%. The average bound radioactivity in the presence of ivacaftor, iva-M1, iva-M6, lumacaftor or tezacaftor or a receptor selective reference compound at 10 µM was then expressed on a percent scale calculated as follows:

$$\% \text{ inhibition} = 100\ \% - \%\ \text{Radioactivity bound test compound}$$

Secondary radioligand binding assays

A stock solution of the test compounds ivacaftor, iva-M1, iva-M6, lumacaftor or tezacaftor and the receptor specific reference compounds were prepared in DMSO and serially diluted (**Supplementary Table 3**). An aliquot of 50 µL was pipetted into wells containing 100 µL of buffer, and 50 µL of respective radioligand. Crude membrane fractions of cells expressing recombinant target receptor were resuspended in chilled buffer, homogenized and 50 µL was dispensed per well. The plates were equilibrated for 1.5 h in the dark at room temperature and then harvested by rapid filtration onto Whatman GF/B glass filters pre-soaked with 0.3% polyethylenimine using a 96-well Brandel harvester. Plates were washed four times with washing buffer. The filter mats were dried, then scintillant was melted onto the filters and the retained radioactivity was counted on a Microbeta scintillation counter. Non-specific binding was measured as described above.

The data were fitted by non-linear regression to the built in three parameter logistic model describing ligand binding to radio-ligand labeled sites using the GraphPad Software (GraphPad Software, CA, USA):

$$Y = \text{bottom} + [(\text{top-bottom}) / (1+ 10^{x-\log IC_{50}})]$$

Where bottom equals the non-specific radioligand binding; and top equals the total radioligand binding observed in the absence of the competing test compound. The log IC_{50} is calculated and the K_i is determined using the Cheng-Prusoff equation:

$$K_i = IC_{50}/ (1 + [\text{ligand}]/K_D)$$

Where [ligand] is the radioligand concentration and K_D represents the radioligand affinity (dissociation constant) for the target receptor.

Molecular modelling of the ivacaftor - 5-HT_{2C} and Iva-M6 - 5-HT_{2A} receptor complexes

Models of human $5HT_{2A}$ and $5HT_{2C}$ receptor molecules were produced using the online structural prediction software I-Tasser *(3)* and Swiss-Model (*4*). To produce a model of ligand binding, ivacaftor was docked in the putative active site of the modelled $5HT_{2C}$ and iva-M6 into the orthosteric site of $5HT_{2A}$ receptor. Molecular docking experiments were carried out using the programme *GOLD* (Genetic Optimization for Ligand Docking) version 5.1 (*5*) and favouring a Piecewise Linear Potential (CHMPLP) Fitness function. Given the inherent flexibility in the receptor site ensemble docking on several prepared models was carried out to obtain the best potential binding pose. The presumptive active site was defined as residues that fell within 10 Å of the superimposed (*S*)-carazolol molecule from the crystal structure of human β2-adrenergic G protein-coupled receptor (*6*) (pdb identifier 2RH1) for $5HT_{2A}$ and eticlopride from the crystal structure of human dopamine D_3 receptor (*7*) (pdb identifier 3PBL). Both active site molecules were also used for similarity constraints calculations during the docking procedure. A 200 % search efficiency was employed generating ten GA runs for each docked molecule and all side-chain residues were rigid

excluding Trp151, Asp155 and Ser159. The generated binding poses were inspected and conformations were chosen for further analysis taking into account their ranking and interactions with the active site residues. Additional molecular visualizations and figures were prepared using the software package PYMOL (Delano Scientific, San Carlos, CA, USA).

Animals

For the forced swim test, adult male C57BL/6 mice (6 weeks old, 20 - 26 g) were purchased from Janvier Labs, France and experiments were conducted at Pronexus, Sweden. The mice were housed in Eurostandard Type III cages (42.5 × 26.6 × 15.5 cm; Tecniplast, Italy) with eight mice per cage. Mice were maintained in a controlled environment (22 ± 1°C; 50 - 55% relative humidity) on a 12 h dark/12 h light (40 Lux) cycle. The mice had free access to standard lab chow (RM1A (P), SDS, Scanbur, Sweden) and tap water under the entire experimental period. After arrival, mice were allowed a minimum of 7 days of acclimatization. The mice were examined and weighed prior the initiation of the study to assure adequate health and suitability. Each mouse was randomly assigned across the treatment groups. For the M_3 agonist force contraction experiment, male Sprague Dawley rats aged 8 - 9 weeks (230 - 300g) were purchased from the Monash Animal Research Platform (Melbourne, Australia). Rats had free access to food and water and were kept under a 12 h light/dark cycle in a well-ventilated room at an approximate temperature of 22°C.

All testing procedures were carried out in accordance with the directives of the Swedish Animal Welfare Act 1988:534 and complying with the Directive 2010/ 63/ EU (Council of the European parliament) "The Guide for the Care and Use of Laboratory Animals" and the "Principles of Laboratory Animal Care" (NIH publication No. 85-23). All efforts were made to minimize animal suffering and the number of animals used for the study.

The results were reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (*8*).

Statistical analysis of the animal models

Statistical analysis was performed using GraphPad software. The values are presented as mean ± standard error of mean (SEM), and differences were considered to be statistically significant at the $p < 0.05$. The means in the vehicle and treated groups were compared by use of one-way analysis of variance (ANOVA) followed by Dunnett´s multiple comparison test.

Force contraction experiment

The entire colon was removed from each rat following asphyxiation by carbon dioxide (CO_2) and placed into oxygenated physiological saline (composition mM: 118.1 mM NaCl, 4.69 mM KCl, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 11.7 mM glucose, 0.5 mM $MgSO_4$, and 2.5 mM $CaCl_2$; bubbled with 95% O_2: 5% CO_2). The distal colon was separated and cut into segments approximately 3 cm in length, cleared of luminal contents as well as surrounding mesentery, fat and blood vessels. Cotton was used to mount colonic segments between a static platinum hook embedded in the tissue holder and an FT03 force displacement transducer to measure force displacement caused by muscle contractility (Grass instruments, MA, USA). Force transducers were connected to a PowerLab (ADInstruments, NSW, Australia) and changes in force displacement were recorded using LabChart software (V5.0.2, ADInstruments, Australia). Throughout the experiment colonic tissue was suspended in a 10 mL jacketed organ bath filled with physiological saline maintained at 37°C and bubbled with 95% O_2: 5% CO_2. Individual colonic segments were exposed to increasing concentrations of either ivacaftor, iva-M1, iva-M6, lumacaftor, methacholine (1 nM – 10 µM) or SR59230A (10 µM) at 5 minute intervals construct a concentration-response curve. Baths were washed to replace physiological saline solution every 20~30 minutes, in between drug additions

throughout the experiment. Minimum and maximum amplitude of contractions were recorded following each drug application. Responses to drug application were also quantified as area under the curve (AUC) from minimum response. All values were normalised to the response given by the tissue at baseline. Response to electrical field stimulus (EFS) colonic tissue segments was determined (3 pulse per sec; pulse duration of 0.5 milliseconds; train duration 1 sec; 60 volts) prior to and following drug application SR59230A (10 μM). Data from tissues that did not produce response to baseline EFS in a robust and reproducible manner were omitted from data analysis. Stimulus was administered a minimum of 3 times at each given concentration, with a minimum of 5 minutes between each stimulus. GraphPad Prism was used to assess the data using one-way ANOVA followed by Dunnet's or Tukey's post-hoc test. Data are presented as mean ± SEM. Differences in mean values were considered statistically significant at $p < 0.05$.

Supplementary Materials:

- Supplementary Figure 1 A-F
- Supplementary Figure 2 A-D
- Supplementary Figure 3 A-C
- Supplementary Figure 4 A&B
- Supplementary Figure 5 A-C
- Supplementary Figure 6 A-C
- Supplementary Figure 7 A-D
- Supplementary Figure 8 A-D
- Supplementary Figure 9
- Supplementary Table 1
- Supplementary Table 2
- Supplementary Table 3
- Supplementary Table 4

Supplementary Figures:

Supplementary Figure 1. Secondary binding data for Ivacaftor. **(A)** [^{3}H]mesulergine displacement data (K_i = 866 nM) binding to the 5-HT_{2C} receptor. The reference compound is ritanserin (K_i = 0.57 nM); **(B)** [^{3}H]prazosin displacement data (K_i > 10,000 nM) binding to the α_{1A} receptor. The reference compound is prazosin (K_i =0.42 nM); **(C)** [^{3}H]WIN35428 displacement data (K_i = 2,935 nM) binding to the DA transporter. The reference compound is GBR12909 (K_i = 3.1 nM) **(D)** [^{3}H]citalopram displacement data (K_i > 10,000 nM) binding to the 5-HT transporter. The reference compound is amitripyline (K_i = 5.4 nM); **(E)** [^{125}I]pindolol displacement data (K_i = 1,934 nM) binding to the β_3 receptor. The reference compound is alprenolol (K_i = 18 nM). **(F)** [^{3}H]DADLE displacement data (K_i = 2,589 nM) binding to the DOR receptor. The reference compound is naltrindole (K_i = 0.25 nM).

Supplementary Figure 2. Secondary binding data for iva-M1. **(A)** [^{3}H]citalopram displacement data (K_i = 10,000 nM) binding to SERT. The reference compound is amitripyline (K_i = 15 nM); **(B)** [^{3}H]QNB displacement data (K_i > 1,832 nM) binding to the M_3 receptor. The reference compound is atropine (K_i =0.54 nM); **(C)** [^{3}H]prazosin displacement data (K_i > 10,000 nM) binding to the α_{1A} receptor. The reference compound is prazosin (K_i =0.42 nM); **(D)** [^{3}H]mesulergine displacement data (K_i = 1,546 nM) binding to the 5-HT_{2C} receptor. The reference compound is ritanserin (K_i = 0.57 nM).

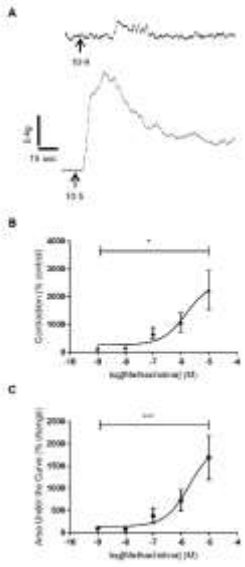
Supplementary Figure 3. Secondary binding data for iva-M6. **(A)** [^{3}H]ketanserin displacement data (K_i = 147 nM) binding to the 5-HT_{2A} receptor. The reference compound is clozapine (K_i = 2.4 nM). **(B)** [^{3}H]prazosin displacement data (K_i > 10,000 nM) binding to the α_{1A} receptor. The reference compound is prazosin (K_i =0.42 nM); **(C)** [^{3}H]citalopram

displacement data (K_i > 10,000 nM) binding to the 5-HT transporter. The reference compound is amitripyline (K_i = 5.4 nM).

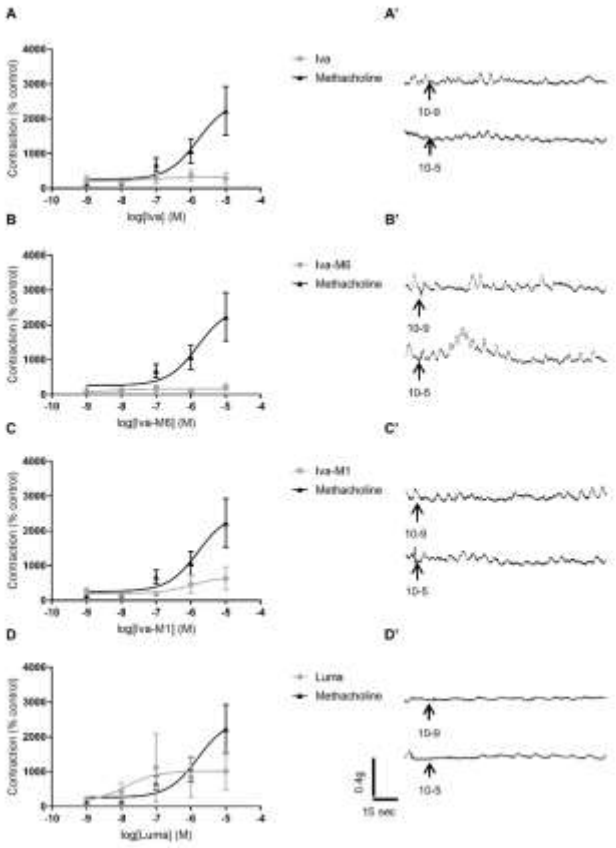
Supplementary Figure 4. Secondary binding data for tezacaftor. **(A)** [^{3}H]prazosin displacement data (K_i = 5,451 nM) binding to the α_{1D} receptor. The reference compound is prazosine (K_i = 0.76 nM). **(B)** Tezacaftor displacement data (K_i = 1,015 nM) binding to the PBR receptor. The reference compound is 4-chlorodiazepam (K_i = 12 nM).

Supplementary Figure 5. Secondary binding data for lumacaftor. **(A)** [^{3}H]prazosin displacement data (K_i > 10,000 nM) binding to the α_{1B} receptor. The reference compound is prazosine (K_i = 0.092 nM). **(B)** [^{3}H]prazosin displacement data (K_i = 4,838 nM) binding to the α_{1D} receptor. The reference compound is prazosine (K_i = 0.76 nM). **(C)** Lumacaftor displacement data (K_i = 79 nM) binding to the PBR receptor. The reference compound is 4-chlorodiazepam (K_i = 12 nM).

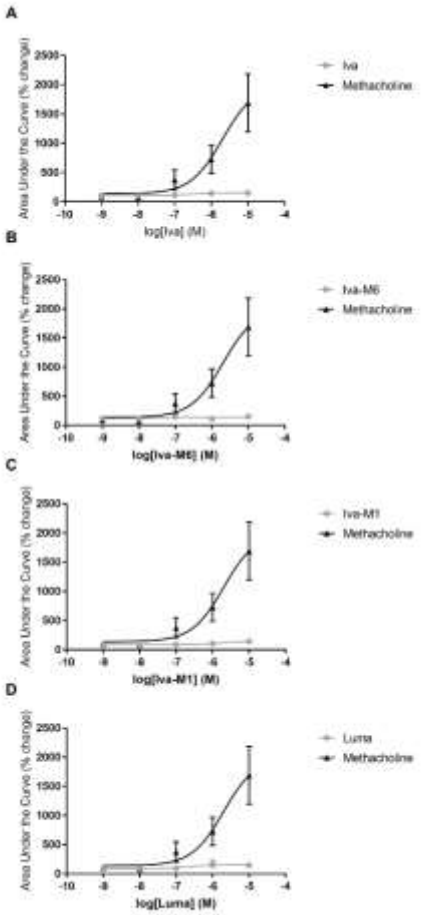
Supplementary Figure 6. Representative traces of colonic contractile activity following application of 1 nM and 10 µM methacholine (**A**). Effect of methacholine application on the amplitude of colonic contractile activity (**B**). Effect of methacholine application on area under the curve. Arrows indicate drug application. Data are presented as the mean ± SEM, *$p < 0.05$, **$p < 0.001$.



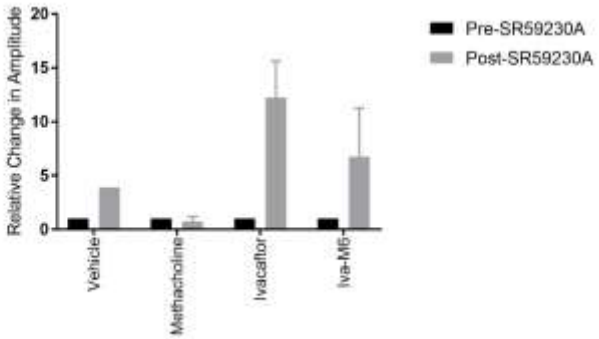
Supplementary Figure 7. Effect of ivacaftor on the amplitude of colonic contractile activity (**A**). Representative traces of colonic contractile activity following application of 1 nM and 10 µM ivacaftor (**A'**). Effect of iva-M6 on the amplitude of colonic contractile activity (**B**). Representative traces of colonic contractile activity following application of 1 nM and 10 µM Iva-M6 (**B'**). Effect of iva-M1 on the amplitude of colonic contractile activity (**C**). Representative traces of colonic contractile activity following application of 1 nM and 10 µM iva-M1 (**C'**). Effect of lumacaftor on the amplitude of colonic contractile activity (**D**). Representative traces of colonic contractile activity following application of 1 nM and 10 µM lumacaftor (**D'**). Arrows indicate drug application. Data presented as mean ± SEM.



Supplementary Figure 8. Effect of ivacaftor on area under the curve following drug addition. (**A**). Effect of iva-M6 on area under the curve (**B**). Effect of iva-M1 on area under the curve (**C**). Effect of lumacaftor on area under the curve (**D**). Data presented as mean ± SEM.

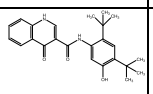
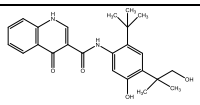
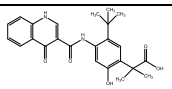
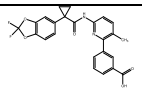
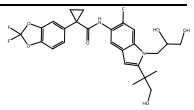


Supplementary Figure 9. Effect of ivacaftor and iva-M6 as the relative change in amplitude pre- and posttreatment with the selective β_3 antagonist SR59230A compared to treatment with the vesicle DMSO or methacholine.



Supplementary Tables:

Supplementary Table 1. Primary neuroreceptor binding screen for ivacaftor, iva-M1 and iva-M6, lumacaftor and tezacaftor.

Receptor	**Ivacaftor**	**Hydroxymethylivacaftor (M1)**	**Ivacaftorcarboxylate (M6)**	**Tezacaftor**	**Lumacaftor**
					
$5HT_{1A}$	3.4	29	-11	-16.4	2.1
$5HT_{1B}$	-5.8	-13	-8.3	-	-
$5HT_{1D}$	6.4	7.7	-8.2	39.2	8.2
$5HT_{1E}$	-9.2	-30	-19	-	-
$5HT_{2A}$	-1.2	-18	-44	36.3	-4.4
$5HT_{2B}$	18	6.1	-8.1	-18.1	2.9
$5HT_{2C}$	85	78	2.4	-16.9	-15.1
$5HT_{3}$	-8.6	-4.9	5.5	-19.3	-13.7
$5HT_{5A}$	31	29	15	1.1	8.8
$5HT_{6}$	24	16	21.8	1.2	14.3
$5HT_{7}$	23	50	-5.9	27.5	1.5
α_{1A}	58	80	92	-13.8	-18.1
α_{1B}	-6.7	-1.6	5.3	34.5	57.4
α_{1D}	28	20	-0.2	-48	-42.7
α_{2A}	-7.9	27	50	18.1	34.9
α_{2B}	-0.4	3.4	20	35	38.4
α_{2C}	21	20	25	48.2	35.3
β_{1}	-4.2	-7.1	-8.2	-10.6	3.6
β_{2}		2	-7.7	3.7	4.2
β_{3}	76	36	-12	0.7	-12.1
BZP rat brain	-12	39	-0.0	17.1	8.5

site					
D_1	47	29	-2.0	8	-3.1
D_2	3.1	4.9	1.6	-11.7	1.2
D_3	30	32	34	1.1	76
D_4	1.0	-4.7	-14	15.2	0.3
D_5	50	45	17	15.3	5.5
DAT	55	46	32	-10.1	-4.9
DOR	51	37	24	23.5	30.6
$GABA_A$	0.6	4.3	8.1	31	40.1
H_1	25	30	18	53.3	71.2
H_2	16	4.9	2.0	9.9	18.8
H_3	-13	-17	-7.3	-13.9	4.8
H_4	-0.7	-6	-4.1	1.2	3.4
KOR	44	10	15	16.8	15.9
M_1	-1.5	2.0	-16	-	-
M_2	18	-8.5	-8.1	10.5	-16.6
M_3	9.8	-29	-15	28.1	34.4
M_4	7.0	7.0	-16.3	38.3	11.9
M_5	3.0	-8.8	-15	29.7	29.8
MOR	33	-0.5	-0.1	-6.8	-0.1
NET	28	30	40	-1.6	-4.3
PBR	13	19	4.2	63.7	82.9
SERT	61	57	51	28.6	14
$Sigma_1$	-22	-21	-23	9.4	19.6
$Sigma_2$	-8.3	-0.2	-33	33.9	20.1

*The data in red represents the % displacement, with significant displacement considered as >50% (n = 4). Negative binding (-) represents stimulation of radioligand binding.

Supplementary Table 2. List of cell lines, media, binding and washing buffers used for the targets identified in the secondary binding assays according to Roth *et al* (*1*).

Receptor	Radioligand	Parental Cell Line	Cell Media Composition	Binding Buffer	Washing Buffer
$5HT_{2A}$ $5HT_{2C}$	[^{3}H]Ketanserin [^{3}H]Mesulergine	HEKT Flp-IN HEK	COS/HEK DMEM 100µgmL Hygromycin B	50mM Tris HCl, 10mM $MgCl_2$, 0.1mM EDTA, pH 7.4, RT*	50mM Tris HCl, pH 7.4, cold
α_{1A} α_{1B} α_{1D}	[^{3}H]Prazosine	stable HEKT	500G418	20 mM Tris HCl, 145mM NaCl, pH 7.4, RT	50mM Tris HCl, pH 7.4, cold
β_3	[^{125}I]Pindolol	HEK Flp-IN	DMEM 100µgmL Hygromycin B	50mM Tris HCl, 3 mM $MgCl_2$, pH 7.7 RT	50mM Tris HCl, pH 7.4, cold
DAT	[^{3}H]WIN35428	stable HEK	350G418	10mM HEPES, 135 mM NaCl, 5mM KCl, 0.8mM $MgCl_2$, 1mM ETGA, pH 7.4, RT	10mM HEPES, 135 mM NaCl, 5mM KCl, 0.8mM $MgCl_2$, 1mM ETGA, pH 7.4, cold
DOR	[^{3}H]DADLE	stable HEK	200G418	50mM Tris HCl, 10mM $MgCl_2$, 0.1mM EDTA, pH 7.4, RT	50mM Tris HCl, pH 7.4, 4-8°C
M_3	[^{3}H]QNB	stable CHO	500G418	#1: 50mM Tris HCl, pH 7.7, RT + SWB #2: 25mM Sodium phosphate, 5mM $MgCl_2$, pH 7.4, RT	25mM Sodium phosphate, 5mM $MgCl_2$, pH 7.4, cold
SERT	[^{3}H]Citalopram	stable HEK	500G418	10mM HEPES, 135 mM NaCl, 5mM KCl, 0.8mM $MgCl_2$, 1mM ETGA, pH 7.4, RT	10mM HEPES, 135 mM NaCl, 5mM KCl, 0.8mM $MgCl_2$, 1mM ETGA, pH 7.4, cold

* Abbreviation: RT = room temperature.

Supplementary Table 3. Secondary neuroreceptor binding screen for ivacaftor, iva-M1 and iva-M6, tezacaftor, lumacaftor.

	K_i (nM)*				
Receptor	**Ivacaftor**	**Hydroxymethylivacaftor (Iva-M1)**	**Ivacaftorcarboxylate (Iva-M6)**	**Tezacaftor**	**Lumacaftor**
$5HT_{2A}$			47		
$5HT_{2C}$	866	1546			
α_{1A}	>10,000	>10,000	>10,000		
α_{1B}					>10,000
α_{1D}				5,451	4,838
β_3	1,934				
PBR				1,015	79
DAT	2,935				
DOR	2,589				
M_3		1,832			
SERT	>10,000	>10,000	>10,000		

* The inhibition constant represents the mean of 4 independent measurements.

Supplementary Table 4. EC50 values following application of ivacaftor, iva-M1, iva-M6, lumacaftor and methacholine as the normalised data to both the baseline (pre-drug) reading as well as the minimum drug concentration (1 nM).

	Without Stimulus	
Compound	**% Maximum Amplitude normalised to baseline control**	**Area Under the Curve normalised to baseline**
Ivacaftor	8.866×10^{-8}	2.492×10^{-7}
Ivacaftor-M1	9.993×10^{-7}	2.775×10^{-6}
Ivacaftor-M6	7.951×10^{-9}	2.294×10^{-8}
Lumacaftor	1.344×10^{-8}	2.269×10^{-7}
Methacholine	1.576×10^{-6}*	1.993×10^{-6}***

*P<0.05 significantly different to pre-treated (baseline) control

***P<0.001 significantly different to pre-treated (baseline) control

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