Synthesis and Characterization of High-Affinity 4, 4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene-Labeled Fluorescent Ligands for Human β-Adrenoceptors

Jillian G. Baker,[‡,||] Luke A. Adams,[†,||] Karolina Salchow,[‡] Shailesh N. Mistry,[†] Richard J. Middleton,[†,§] Stephen J. Hill,[*,‡] and Barrie Kellam[*,†]
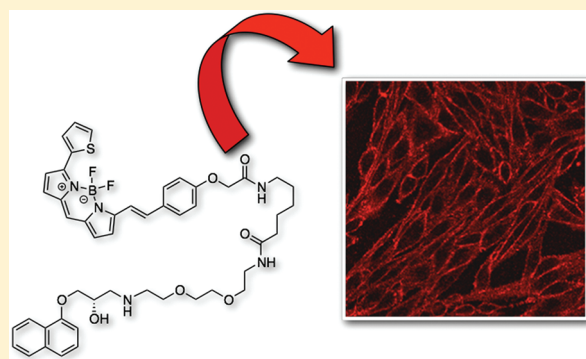
[†]School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

[‡]Institute of Cell Signalling, Medical School, University of Nottingham, Nottingham NG7 2UH, United Kingdom

[§]CellAura Technologies Ltd., BioCity, Pennyfoot Street, Nottingham NG1 1GF, United Kingdom

Ⓢ *Supporting Information*

ABSTRACT: The growing practice of exploiting noninvasive fluorescence-based techniques to study G protein-coupled receptor pharmacology at the single cell and single molecule level demands the availability of high-quality fluorescent ligands. To this end, this study evaluated a new series of red-emitting ligands for the human β-adrenoceptor family. Upon the basis of the orthosteric ligands propranolol, alprenolol, and pindolol, the synthesized linker-modified congeners were coupled to the commercially available fluorophore BODIPY 630/650-X. This yielded high-affinity β-adrenoceptor fluorescent ligands for both the propranolol and alprenolol derivatives; however, the pindolol-based products displayed lower affinity. A fluorescent diethylene glycol linked propranolol derivative (**18a**) had the highest affinity (log K_D of -9.53 and -8.46 as an antagonist of functional β2- and β1-mediated responses, respectively). Imaging studies with this compound further confirmed that it can be employed to selectively label the human β2-adrenoceptor in single living cells, with receptor-associated binding prevented by preincubation with the nonfluorescent β2-selective antagonist 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]-butan-2-ol (ICI 118551) (*J. Cardiovasc. Pharmacol.* **1983**, 5, 430−437.)

■ INTRODUCTION

G-protein coupled receptors (GPCRs) remain a prime target for potential disease modulation, and therefore, research focused upon them persists as a significant component of the modern day drug discovery arena.[2] The β-adrenoceptors (β-AR) represent just one subset of this broad receptor family and are themselves further classified into three specific subtypes, β1-, β2-, and β3-AR.[3] For several decades these subtypes have been the molecular target for numerous drug discovery projects, and the drugs resulting from these endeavors have established themselves as key therapeutic agents in the treatment of numerous chronic cardiovascular and respiratory diseases.[4] More recently, their potential involvement in metastatic cancer progression has become apparent and rekindled further the therapeutic importance of this receptor class.[5,6] Our current understanding of the molecular pharmacology of the β-AR has been derived primarily from the use of radioligand binding techniques for the elucidation of ligand−receptor interactions and the quantification of intracellular second messenger generation. As such, this type of experiment requires the use of large numbers of cells, and the

data generated represents an average for the entire cell population. Fluorescent techniques, however, offer a wider spectrum of more detailed pharmacological investigations to be undertaken, especially the quantification and visualization of specific ligand−receptor complexes in intact cells.[7]

The recent discovery that GPCRs can regulate signaling pathways that are independent of G proteins has led to a realization that different signal transduction pathways can be selectively stimulated by agonists acting at a common cell surface receptor.[8−11] GPCRs are not uniformly distributed at the cell surface but instead are organized within membrane compartments and microdomains,[12,13] providing a mechanism by which intracellular signaling can be orchestrated in different areas of an individual cell and behave differently in specific cell types.[14] This new mechanistic information has focused attention on the need to develop fluorescent ligands to study the spatial and temporal aspects of ligand−receptor interactions.[15,16] With their established
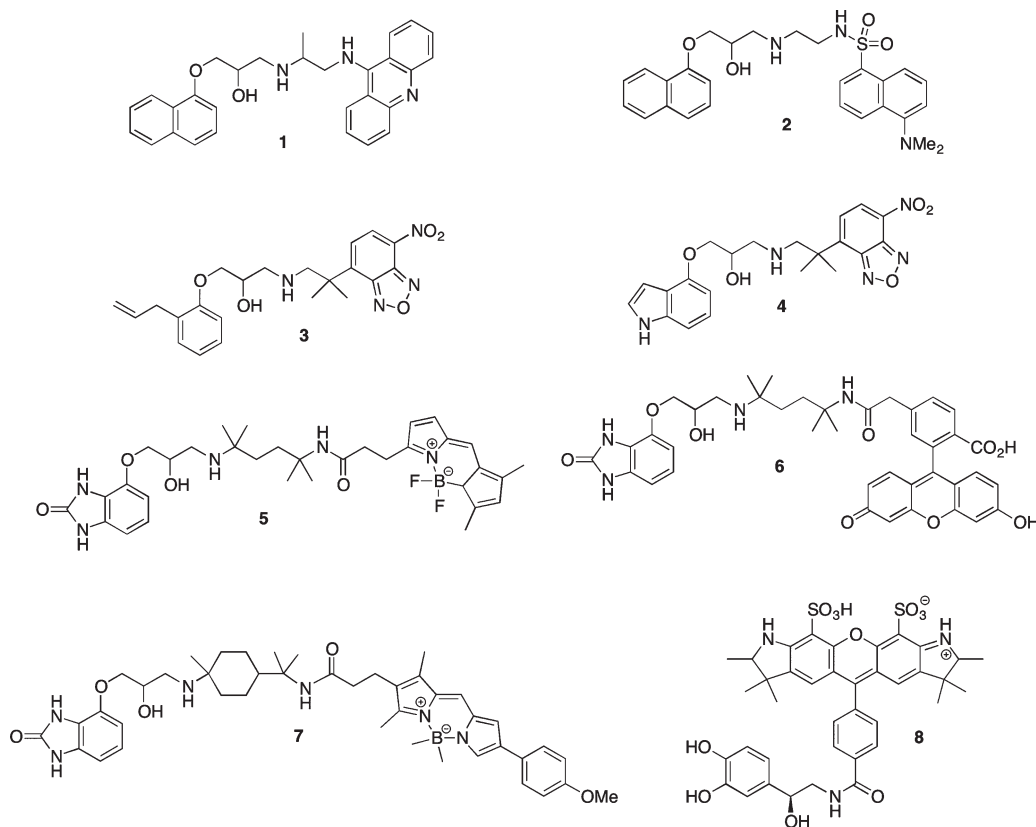
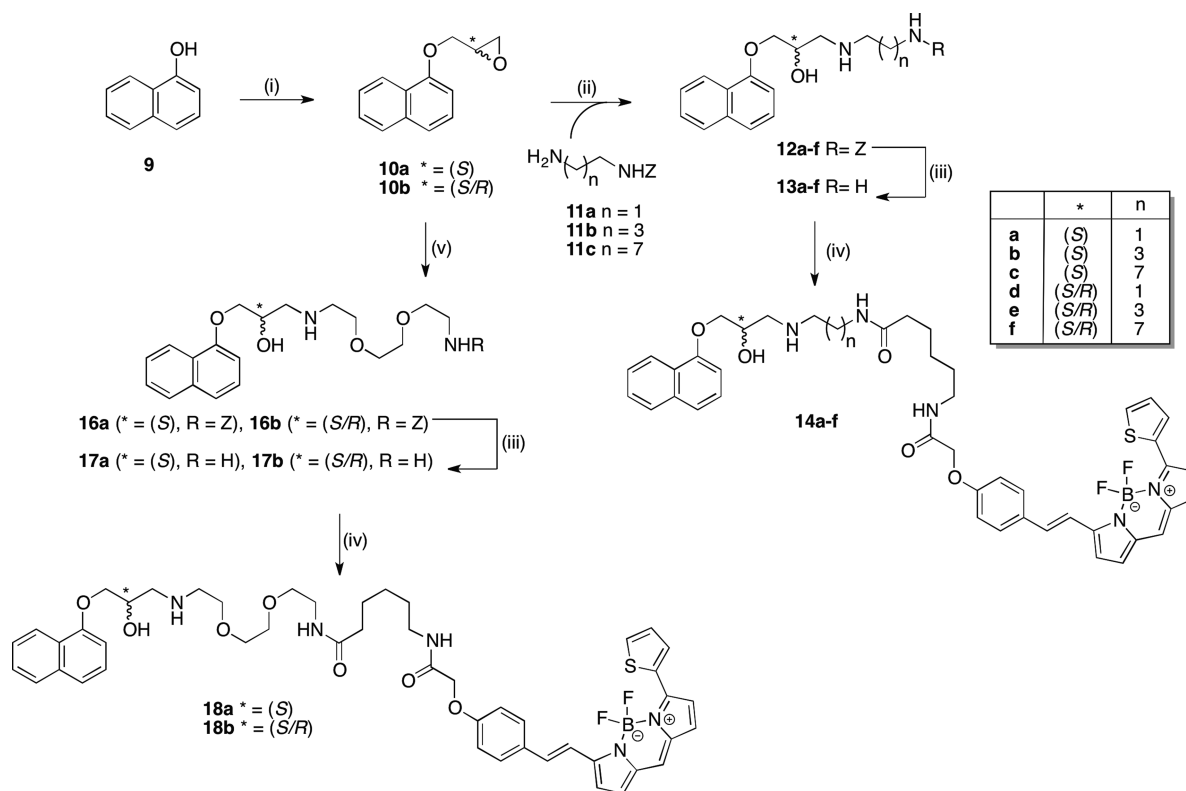**Figure 1.** Previously reported fluorescent β-AR ligands.

clinical and therapeutic importance,[17] it is therefore not surprising that β-AR have been investigated within this area of pharmacological imaging.

To-date, however, only a handful of reports have been published detailing limited success in the synthesis of fluorescent β-AR ligands (Figure 1). The earliest studies detailed the use of fluorescent propranolol derivatives 9-aminoacridinylpropranolol (9-AAP) (1)[18−22] and dansyl-propranolol (DAPN) (2)[23] to investigate receptor mapping and clustering. Following this, synthesis of an NBD−alprenolol derivative (3) was reported and utilized in further clustering studies.[24] However, it was later demonstrated that 3 and NBD−pindolol (4) were in fact both nonselective and noncompetitive.[25,26] Additionally, two reports also detailed that during their use of 1 and 2 they were unable to distinguish between the binding of these ligands to cellular receptors and autofluorescent granules, concluding that the fluorescent output from these ligands was therefore of too low an intensity to be detected by fluorescence microscopy.[27,28]

A decade later, a significant advance was achieved through the synthesis of bordifluoropyrromethene- (BODIPY) (5) and fluorescein-labeled (6) β-AR ligands.[29] On the basis of the hydrophilic β-AR ligand 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one (CGP 12177),[30] it was demonstrated that the BODIPY conjugate (5) displayed similar binding characteristics when compared with the standard ligand. The fluorescein conjugate's (6) receptor-specific signal was not sufficiently strong enough for subsequent pharmacological measurement and has indeed since been demonstrated to suffer from significant photobleaching during fluorescence microscopy experiments.[31] A variant of CGP 12177

(BODIPY TMR−CGP 12177) (7) has also been reported and shown to be a long-acting β2-AR partial agonist capable of labeling β2-AR in the plasma membrane of living cells.[32] More recently, the first description of a fluorescently labeled arterenol derivative (Alexa-NA) (8) and its use in fluorescence correlation spectroscopy (FCS) based studies to investigate β2-AR in living cells was published.[33] In addition, a europium-chelated fluorescent derivative of pindolol for use in time-resolved receptor−ligand binding assays has also been described, but its full structure was not revealed.[34]

Within this rapidly evolving arena of pharmacological imaging, we have previously reported the synthesis of red-shifted BOD-IPY-labeled fluorescent adenosine receptor agonists[16,35,36,38] and antagonists[37,38] and demonstrated their utility in measuring the diffusion of receptor species within defined membrane microdomains using the technique of fluorescence correlation spectroscopy (FCS).[39] Avoiding the issues of autofluorescence, highlighted as being severely problematic with many of the derivatives discussed above, was one of our primary objectives in designing pharmacologically useful fluorescent ligands. Clearly, fluorescent ligands that emit light in the red region of the electromagnetic spectrum meet this requirement and, in addition, often possess excellent photophysical properties suited for techniques such as LSCM and FCS. With the previously described fluorescent β-AR ligands either not meeting these requirements for fluorescence microscopy or, as in the case of BODIPY TMR−CGP 12177, no longer commercially available, we were keen to generate a new suite of red-shifted fluorescent β-AR ligands for use in LSCM- and FCS-based experiments. Three families of fluorescent ligands based on propranolol, alprenolol, and pindolol were synthesized,

Scheme 1. Synthetic Route to BODIPY 630/650-X-Labeled Propranolol Derivatives[a]

[a] Reagents and conditions: (i) for **10a**, (2S)-glycidyl-3-nitrobenzenesulfonate, NaH, DMF, 56%; for **10b**, epichlorohydrin, K_2CO_3, 2-butanone, Δ. (ii) **11a—c**, DMF/H_2O (9:1), 85 °C, 16 h, 35—70%. (iii) Pd/C, H_2, MeOH, rt, 3 h, quantitative. (iv) BODIPY-X-630/650-OSu, DMF, rt, 4 h. (v) Benzyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (**15**), DMF/H_2O (9:1), 85 °C, 16 h, 35—67%.

characterized, and pharmacologically assessed across the human forms of the $\beta1$-, $\beta2$-, and $\beta3$-ARs. Our choices of β-AR ligands were based primarily upon synthetic tractability, a desire to improve upon those ligands previously described, and to address three key questions surrounding ligand design: (i) Was the effect of linker length and composition similar to what we had observed for the corresponding adenosine ligands?[36] (ii) With structural extension of the pharmacophore to include both a linker and fluorophore, did the stereochemistry of the crucial carbinol center exert the same effect on affinity as that previously established for β-AR antagonists? (iii) Could the best fluorophore—linker combination from the initial "propranolol" library be transposed onto alternative orthosteric head-groups (alprenolol and pindolol) without deleterious effects on pharmacology.
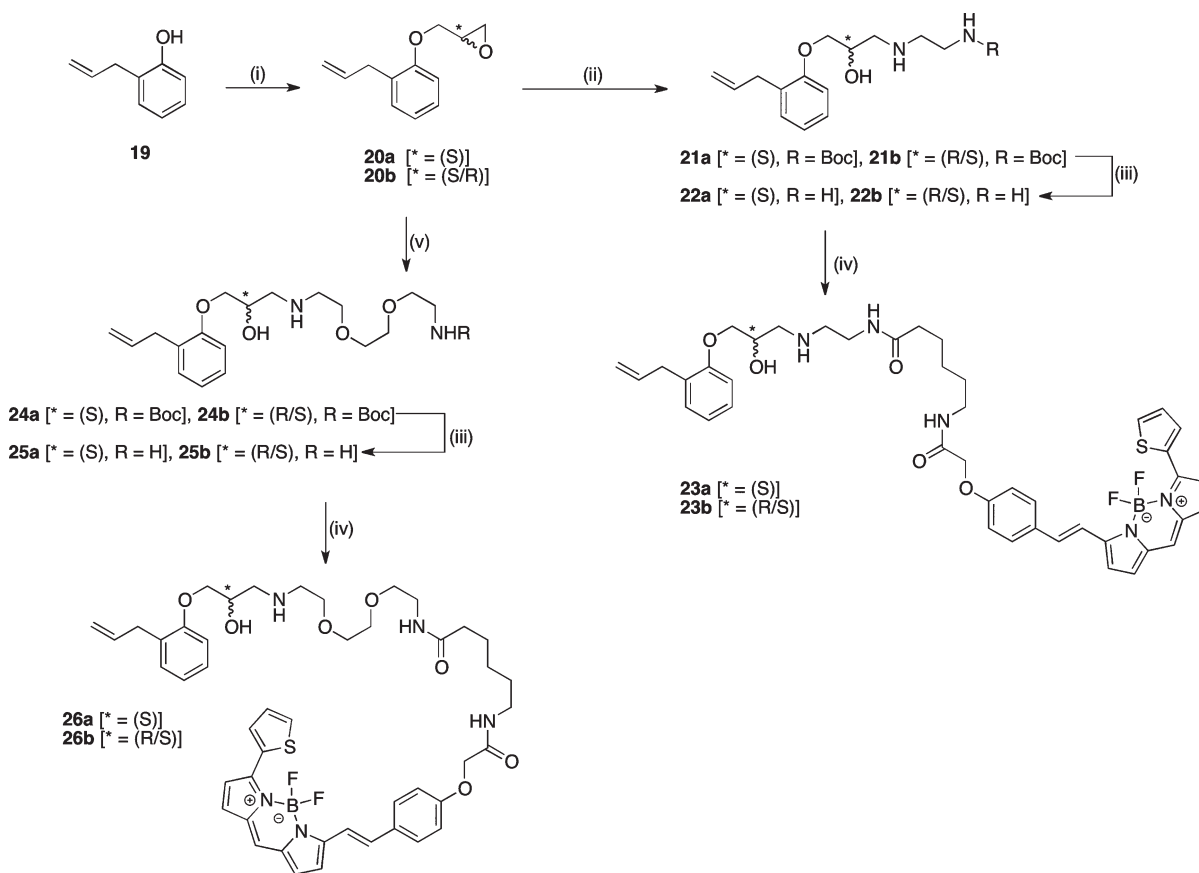
RESULTS AND DISCUSSION

Synthesis. We initially embarked upon the synthesis of a focused library of fluorophore-conjugated single enantiomer and racemic propranolol derivatives (Scheme 1). Commercially available 1-napthol (**9**) was therefore alkylated with either (2S)-glycidyl-3-nitrobenzenesulfonate or racemic epichlorohydrin as previously described to afford epoxides **10a** and **10b**, respectively.[40]

Nucleophilic ring-opening of the epoxides was effected using a range of appropriately mono-Cbz-protected diamines (**11a—c** and **15**) to afford carbinolamines **12a—f**, **16a**, and **16b**. Subsequent hydrogenolysis of the Cbz protecting groups afforded the linker-modified congeners **13a—f**, **17a**, and **17b** primed for

chemoselective acylation of the terminal primary amine with commercially available 6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid succinimidyl ester (BODIPY 630/650-X, SE; Invitrogen). Aminolysis of the fluorophore active ester proceeded smoothly and the fluorescently labeled ligands **14a—f**, **18a**, and **18b** were isolated and purified using preparative thin-layer chromatography (PTLC); their purity was confirmed using RP-HPLC with photodiode array detection between 190 and 800 nm. Evaluation of these ligands highlighted that linker length exerted minimal overall influence on receptor affinity, and for expeditious reasons, the alprenolol and pindolol conjugates were therefore synthesized using only the ethylamino (short hydrocarbon) and ethoxyethoxyethylamino (long polyether) linker variants.

The S-enantiomer (**23a** and **26a**) and racemic (**23b** and **26b**) alprenolol-BODIPY fluorescent ligands were therefore synthesized using an identical strategy to that of the propranolol derivatives, but starting from commercially available 2-allylphenol and replacing the Cbz-protected diamines with their tert-butoxycarbonyl (Boc) equivalents (Scheme 2).

Finally, having also established with these initial two series that the (S)-enantiomer exerted the expected shift in log K_D values, we generated the final pindolol-based molecules in their inexpensive racemic form. Therefore, ($\pm$)-4-[(oxiran-2-yloxy)-methyl]-1H-indole (**28**) was ring-opened using either **11a** or **15** to afford the Cbz-protected carbinolamine congeners **29** and **32**, respectively. Cbz hydrogenolysis and acylation of the relinquished amines with BODIPY 630/650-X, SE ultimately

Scheme 2. Synthetic Route to BODIPY 630/650-X-Labeled Alprenolol Derivatives[a]

[a] Reagents and conditions: (i) for **20a**, (2S)-glycidyl-3-nitrobenzenesulfonate, NaH, DMF, 56%; for **20b**, epichlorohydrin, K_2CO_3, 2-butanone, Δ, 54%. (ii) *tert*-Butyl 2-aminoethylcarbamate, DMF/H_2O (9:1), 85 °C, 16 h, 35−70%. (iii) 2 M HCl in dioxane, 89−94%. (iv) BODIPY-X-630/650-OSu, DMF, rt, 4 h, 68−98%. (v) *tert*-Butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate, DMF/H_2O (9:1), 85 °C, 16 h, 77%.

afforded the racemic fluorescent pindolol derivatives **31** and **33** (Scheme 3).

Pharmacology. *[3]H-CGP 12177 Whole Cell Binding Studies.* Measurement of ^{3}H-CGP 12177 binding to CHO cells expressing the human β1-, β2-, or β3-AR indicated that all compounds displayed the highest affinity for the human β2-AR (Table 1). The lowest affinity compounds were the pindolol derivatives **31** and **34**. Increasing the linker chain length from C-2 to C-8 for the propranolol series caused a modest increase in affinity from C-2 (**14a**, **14d**) to C4 (**14b**, **14e**) but then a 10-fold reduction in affinity with a further doubling in linker chain length to C-8 (**14c**, **14f**) (Table 1). It was notable that the C-8 derivative **14f** did not completely displace the specific binding of ^{3}H-CGP 12177 to the human β1-AR (maximal displacement of specific binding =65.9 ± 2.0%; Table 1; Figure 2a).

This was seen to a lesser extent with the pure *S*-enantiomer **14c** (85.3 ± 2.4% maximal inhibition; Figure 2a), suggesting that the *R*-enantiomer may be contributing an allosteric influence on the orthosteric binding site. It was also striking that **14f** did not produce an effect to the same degree in cells expressing the human β2-AR (Figure 2b).
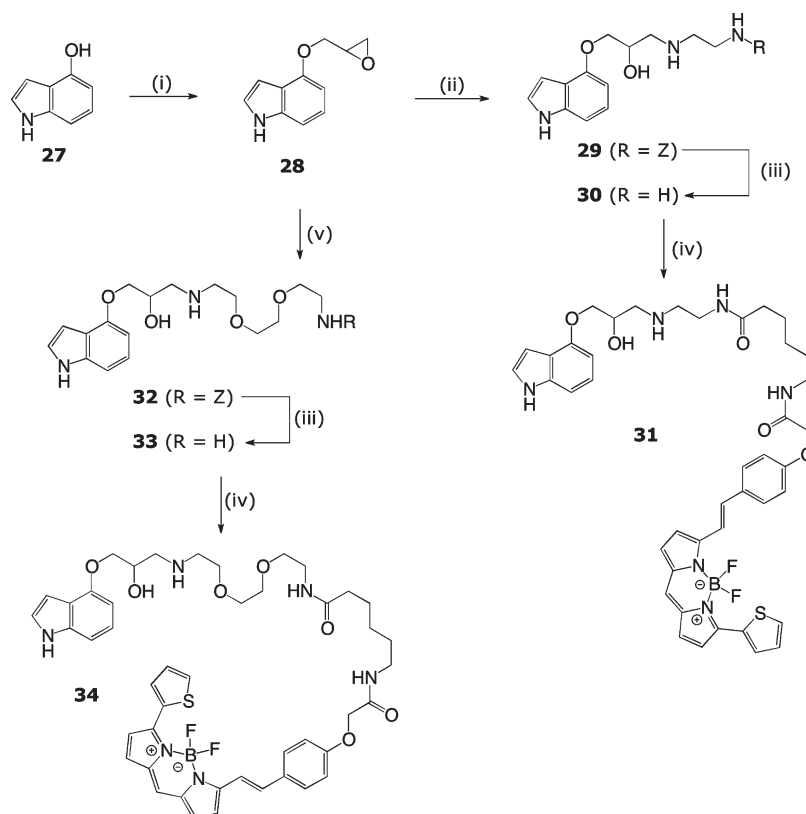
Substitution of a polyethylene glycol linker in place of the C-8 hydrocarbon chain in **14c** provided the highest affinity ligand **18a** at the human β2-AR (log K_D = −9.21; Table 1). This compound also had lower but respectable affinities at the human β1-AR (log

K_D = −7.76) and β3-AR (log K_D = −7.09). The equivalent molecule from the alprenolol series **26a** also displayed high affinity for the β1-AR (log K_D = −7.65) and β2-AR (log K_D = −9.03; Figure 3). However, **34**, from the pindolol series, was an order of magnitude less potent compared to its parent molecule (Table 1).

It was notable that the addition of fluorophore and linker did not markedly change the affinities of the parent compounds for the β3-AR. In the case of both propranolol and alprenolol, the C-2 linker derivatives had the highest affinity for this particular receptor (Table 1).

Functional Reporter Gene Studies. To gain some insight into the ability of these ligands to antagonize agonist-stimulated responses at each receptor, we evaluated their ability to attenuate functional activity in the CHO cells as they also expressed the target receptor and a six cyclic AMP response element (6 × CRE) reporter gene driving the expression of a human-secreted placental alkaline phosphatase reporter gene.[41,42] Agonist responses were elicited by cimaterol (β1, site 1), salbutamol (β2), and fenoterol (β3). For the β1-AR site 2, agonist responses were elicited by CGP 12177. All of the propranolol derivatives were potent competitive antagonists of this response, with **18a** once again displaying the highest affinity (Table 2; Figure 4).

The alprenolol and pindolol series showed partial agonist activity (Figure 5; Table 3) on all three β-AR subtypes. These

Scheme 3. Synthetic Route to BODIPY 630/650-X-Labeled Pindolol Derivatives[a]

[a] Reagents and conditions: (i) epichlorohydrin, Cs_2CO_3, microwave (MW) 120 °C, 30 min, 97%. (ii) **11a**, DIPEA, [i]PrOH/MeCN/H_2O (7:2:1), MW 90 °C, 60 min, 48%. (iii) Pd/C, H_2, MeOH, rt, 3 h, 88%. (iv) BODIPY-X-630/650-OSu, DMF, rt, 4 h. (v) Benzyl 2-(2-(2-aminoethoxy)-ethoxy)ethylcarbamate (**15**), [i]PrOH/MeCN/H_2O (7:2:1), MW 90 °C, 60 min, 40%.

Table 1. Ligand-Binding Parameters for Fluorescent Antagonists Acting at the Human β-AR[a]

	chirality	linker[b]	$\beta1$ log K_D	n	$\beta2$ log K_D	n	$\beta3$ log K_D	n
propranolol			-8.22 ± 0.04	9	-9.22 ± 0.03	10	-6.67 ± 0.10	4
14a	S-enantiomer	C-2	-7.44 ± 0.05	8	-8.86 ± 0.03	8	-7.35 ± 0.06	4
14b	S-enantiomer	C-4	-7.48 ± 0.07	9	-9.14 ± 0.06	9	-6.91 ± 0.08	7
14c	S-enantiomer	C-8	-6.73 ± 0.09^c	9	-8.15 ± 0.06	9	-7.17 ± 0.05	4
14d	racemate	C-2	-7.05 ± 0.07	6	-8.56 ± 0.04	6	-7.03 ± 0.07	6
14e	racemate	C-4	-7.25 ± 0.07	6	-8.87 ± 0.04	6	-6.98 ± 0.13	6
14f	racemate	C-8	-6.72 ± 0.05^d	6	-8.24 ± 0.05	6	-7.11 ± 0.09	6
18a	S-enantiomer	PEG-8	-7.76 ± 0.06	8	-9.21 ± 0.06	8	-7.09 ± 0.08	4
18b	racemate	PEG-8	-7.14 ± 0.08	6	-8.48 ± 0.06	6	-6.53 ± 0.07	6
alprenolol			-7.95 ± 0.03	6	-9.30 ± 0.02	6	-6.86 ± 0.08	6
23a	S-enantiomer	C-2	-7.04 ± 0.05	7	-8.54 ± 0.07	7	-7.16 ± 0.09	7
23b	racemate	C-2	-7.26 ± 0.07	6	-8.63 ± 0.05	6	-7.33 ± 0.12	6
26a	S-enantiomer	PEG-8	-7.65 ± 0.07	6	-9.03 ± 0.06	6	-7.00 ± 0.06	5
26b	racemate	PEG-8	-7.50 ± 0.05	12	-8.91 ± 0.06	12	-7.09 ± 0.04	10
pindolol			-8.58 ± 0.04	7	-9.27 ± 0.07	7	-6.78 ± 0.10	7
31	racemate	C2	-6.06 ± 0.07	7	-7.33 ± 0.09	7	-6.48 ± 0.06	7
34	racemate	PEG-8	-7.01 ± 0.09	6	-7.96 ± 0.10	6	-6.42 ± 0.12	6

[a] log K_D values obtained from ^{3}H-CGP 12177 binding in whole CHO cells expressing either the human $\beta1$-, $\beta2$-, or $\beta3$-AR. Values are mean $\pm$ SE from n separate experiments. [b] The linker composition describes the atoms between the amino group of the carbinolamine and the nearest amide nitrogen: C-2, ethyl; C-4, butyl, C-8, octyl; PEG-8, ethoxyethoxyethyl. [c] Apparent log K_D value (85.3 $\pm$ 2.4% maximal inhibition of specific binding). [d] Apparent log K_D value (65.9 $\pm$ 2.0% maximal inhibition of specific binding).
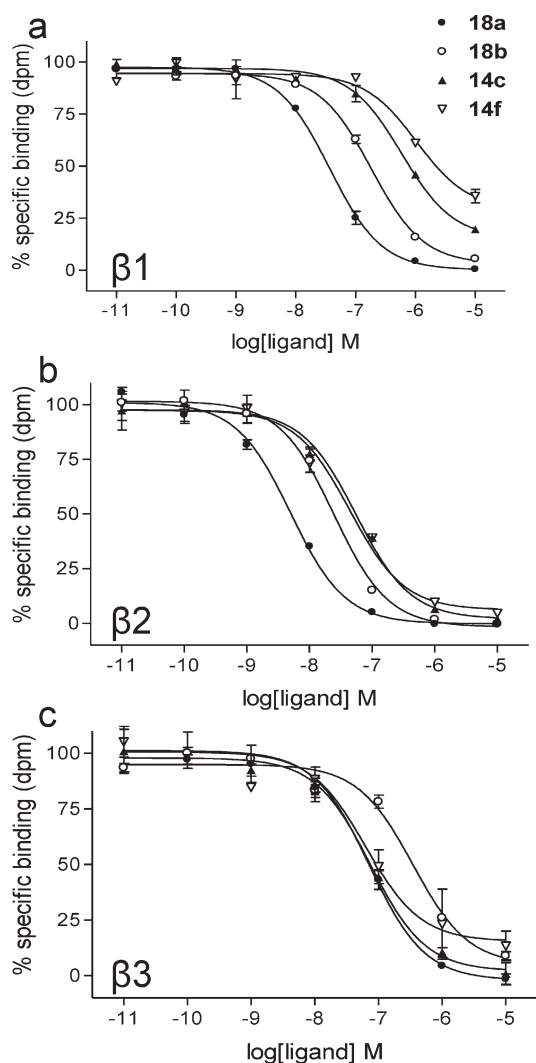
Figure 2. Fluorescent ligand displacement of ^{3}H-CGP 12177 specific binding at the human β-AR in whole cells. Inhibition of ^{3}H-CGP 12177 specific binding to whole cells by compounds **18a**, **18b**, **14c**, and **14f** in (a) β1 cells, (b) β2 cells, and (c) β3 cells. Nonspecific binding was determined by (a) 10 μM CGP 20712A and (b and c) 10 μM ICI 118551.[1] The concentrations of ^{3}H-CGP 12177 present in each case are (a and b) 1.3 nM and (c) 18 nM. Data points are mean $\pm$ SE of triplicate determinations. These single experiments are representative of (a and b) six and (c) four separate experiments.

observations are consistent with the agonist nature of the parent compounds on all three receptors (Table 3).[41−43] Interestingly, the presence of the fluorophore/linker combination reduced the agonist efficacy of both alprenolol and pindolol at the β2-AR, particularly, but had a much smaller influence on agonist efficacy at the other two β-ARs (Table 3). This suggests that the presence of the BODIPY unit or the associated linker chemistry can modify agonist efficacy in a receptor subtype specific manner and implies that the design of ligands with receptor selectivity based on efficacy rather than ligand-binding affinity may be possible.[44] As a consequence of the partial agonist activity, the log K_D values for alprenolol, pindolol, and their derivatives shown in Table 2 were calculated according to the partial agonist method of Stephenson.[45]

Previous work on the human β1-AR has shown that this receptor can exist in two conformations that can both elicit functional responses. The first site has high affinity for the majority of β-antagonists and is the site at which isoprenaline and other catecholamines elicit their agonist responses. The second site can be activated by high concentrations of CGP 12177 and is generally relatively resistant to antagonism by standard β-blockers.[46] In order to evaluate the ability of fluorescent ligands to antagonize functional responses via these two sites, we used cimaterol as an agonist of site 1 and CGP 12177 as an agonist of site 2.[47,10] All fluorescent ligands were effective antagonists of site 1 of the β1-AR with the PEG-8 derivatives **18a** (Table 2), **26a**, and **34** being the most potent. The inhibition of agonist responses to CGP 12177 provided the first indication of the affinity of these fluorescent ligands for site 2 of the β1-AR. All compounds tested had a lower affinity for site 2 than for site 1 (Table 2). However, it was striking that linker length and the presence of the fluorophore did not markedly change the affinity of the parent ligand for site 2 (Table 2). As noted from binding studies, this was also true of the β3-AR, and the functional studies confirmed the higher affinity of C2-linked propranolol and alprenolol derivatives for this receptor (Table 2).

Confocal Imaging. An essential requirement of a fluorescent ligand for any GPCR is that its binding to cell surface receptors can be visualized and that this binding displays the pharmacology of the target receptor. We have previously shown from our work on adenosine A1 and A3 receptors that the BODIPY 630/650 fluorophore is heavily quenched in aqueous environments but markedly increases its quantum yield when bound to cell surface receptors because of its location in a more lipid environment.[38] This property has proved highly beneficial in studying ligand-binding kinetics at the single cell level.[15] Figure 6 shows the binding of 3 nM **18a** to CHO cells expressing the β2-AR. Bright labeling of cell surface β2-AR can be detected even in the continued presence of **18a**.

Quenching of the ligand present in the extracellular fluid is evident from the lack of fluorescence between the individual cells. Cell surface binding of **18a** can be markedly inhibited by increasing concentrations (1−100 nM) of the β2-selective antagonist ICI 118551, consistent with its high affinity for the β2-AR. In contrast, the selective β1-AR antagonist 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol (CGP 20712A)[48] only began to displace at concentrations above 10 μM, which is consistent with its low affinity for this receptor.[49] Finally, 3nM **18a** produced only a low level of binding to the cell surface of control CHO cells not expressing the β2-AR (Figure 6). This low level of binding was entirely consistent with the nonspecific binding observed in receptor-expressing cells incubated with 100 nM ICI 118551 (Figure 6).

■ CONCLUSIONS

A key feature of the current state of research into GPCRs is a growing awareness of the need to investigate their cellular location and involvement in protein−protein interactions. This requires the development of methodologies to investigate the allosteric regulation of the ligand−receptor binding by small molecules or associated signaling proteins. We have shown previously that fluorescent ligands can be used to provide insights into the membrane organization of GPCRs using fluorescence correlation spectroscopy[37,16] and also to reveal novel allosteric interactions using kinetic analysis of ligand association and dissociation in single living cells.[15,50] A key prerequisite for these
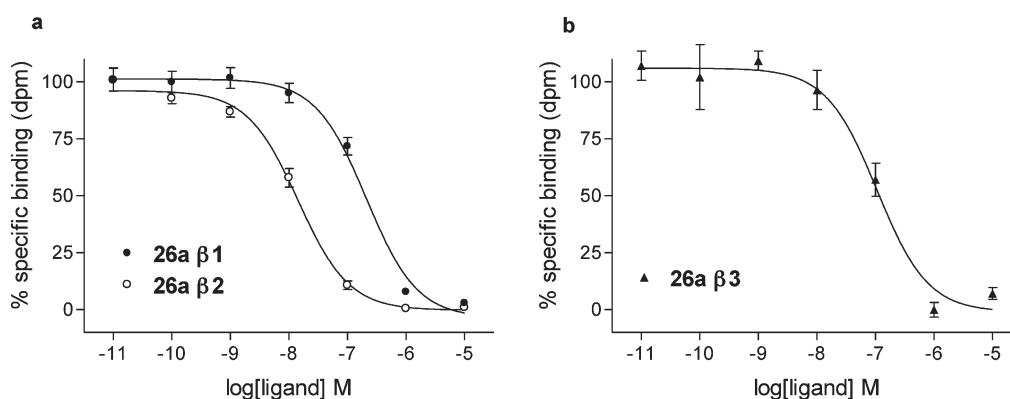
Figure 3. Fluorescent ligand **26a** displacement of ^{3}H-CGP 12177 specific binding at the human β-adrenoceptors in whole cells. Inhibition of ^{3}H-CGP 12177 specific binding to whole cells by compound **26a** in (a) $\beta1$ and $\beta2$ cells and (b) $\beta3$ cells. Nonspecific binding was determined by (a) 10 μM CGP 20712A ($\beta1$) and (b) 10 μM ICI 118551 ($\beta2$ and $\beta3$). The concentrations of ^{3}H-CGP 12177 present in each case are (a) 1.7 nM and (b) 6.4 nM. Data points are mean $\pm$ SE of triplicate determinations. These single experiments are representative of (a) six and (b) five separate experiments.

Table 2. Fluorescent Ligand log K_D Values from Antagonism of Agonist-Mediated Enhancement of CRE Reporter Gene Responses at the Human β-AR[a]

	$\beta1$ (site 1) log K_D	n	$\beta1$ (site 2) log K_D	n	$\beta2$ log K_D	n	$\beta3$ log K_D	n
propranolol	-8.74 ± 0.03	10	-6.70 ± 0.10	21	-9.62 ± 0.05	13	-6.79 ± 0.02	6
14a	-8.08 ± 0.06	8	-7.04 ± 0.09	9	-9.37 ± 0.04	15	-7.58 ± 0.07	13
14b	-7.87 ± 0.10	11	-6.74 ± 0.09	7	-9.56 ± 0.05	13	-6.82 ± 0.09	6
14c	-7.28 ± 0.08	9	-6.10 ± 0.10	13	-8.76 ± 0.10	16	-6.85 ± 0.05	7
14d	-7.12 ± 0.08	10	-6.45 ± 0.10	10	-8.59 ± 0.08	15	-7.20 ± 0.09	12
14e	-7.51 ± 0.06	9	-6.62 ± 0.08	8	-9.13 ± 0.06	18	-7.10 ± 0.05	10
14f	-6.66 ± 0.05	10	>-6	5	-8.60 ± 0.05	9	-6.94 ± 0.09	12
18a	-8.46 ± 0.07	7	-7.17 ± 0.06	7	-9.53 ± 0.11	17	-6.97 ± 0.08	6
18b	-7.38 ± 0.08	8	-6.95 ± 0.10	5	-9.36 ± 0.11	18	-7.24 ± 0.09	9
alprenolol	-8.37 ± 0.07	12	-6.75 ± 0.12	7	-9.53 ± 0.07	13	-7.12 ± 0.08	12
23a	-7.33 ± 0.08	9	-7.13 ± 0.11	9	-8.74 ± 0.06	12	-7.38 ± 0.05	8
23b	-7.36 ± 0.06	4	-6.92 ± 0.09	4	-8.80 ± 0.08	9	-7.63 ± 0.19	9
26a	-8.12 ± 0.09	7	-7.38 ± 0.08	3	-9.37 ± 0.08	6	-7.21 ± 0.07	7
26b	-7.76 ± 0.08	13	-7.11 ± 0.11	11	-9.21 ± 0.09	15	-7.24 ± 0.10	17
pindolol	-8.62 ± 0.07	9	>-5	8	-9.18 ± 0.04	6	>-6	7
31	-6.52 ± 0.06	4	>-6	4	-7.52 ± 0.05	8	-6.75 ± 0.02	4
34	-7.42 ± 0.05	8	>-6	4	-8.14 ± 0.07	12	-6.54 ± 0.11	6

[a] log K_D values were obtained from inhibition of functional CRE-SPAP responses to cimaterol ($\beta1$, site 1), CGP 12177 ($\beta1$, site 2), salbutamol ($\beta2$), and fenoterol ($\beta3$) in CHO cells expressing either the human $\beta1$-, $\beta2$- or $\beta3$-AR. Values are the mean $\pm$ SE from n separate experiments.

approaches is the design and synthesis of novel fluorescent ligands with the correct pharmacological and biophysical properties. In the present study, we have designed, synthesized, and characterized 14 new red-emitting fluorescent ligands (**14a–f**, **18a,b**, **23a,b**, **26a,b**, **31**, and **34**) for the human β-AR. Using alkyl- or polyether-based linker extension from the nitrogen atom of the conserved aryloxypropanolamine portion of propranolol, alprenolol, and pindolol generated a range of fully deprotected congeners, functionally equipped to undergo a high yielding chemoselective acylation with the commercially available fluorochrome BODIPY 630/650-X, SE (Molecular Probes). No protecting group manipulations were required following fluorophore attachment. This new range of fluorescent β-blockers displayed high affinity for the $\beta2$-AR, with some (**18a** and **26a**) also possessing moderate affinity for the $\beta1$-AR. An evaluation of the pharmacology of the new fluorescent derivatives was undertaken. Compound **18a**, in particular, displayed a log K_D of -9.53 as an antagonist of functional responses mediated by the human $\beta2$-AR and a log K_D of -8.46 as an antagonist of the high-affinity catecholamine site of the $\beta1$-AR.

Critically, we were further able to utilize these molecules to visualize ligand–receptor interactions using confocal microscopy. Confirmation of selective fluorescent labeling of, for example, the human $\beta2$-AR in single living cells was achieved by incubating CHO-$\beta2$ cells expressing the human $\beta2$-AR with a 3 nM concentration of **18a**. This resulted in clear labeling of the cell membrane, with the receptor specific proportion of this binding blocked by preincubation with the nonfluorescent $\beta2$-selective antagonist ICI 118551. This fluorescent propranolol derivative should therefore be invaluable in future mechanistic studies aimed at unraveling the intricacies of cell signaling via this prototypical GPCR.
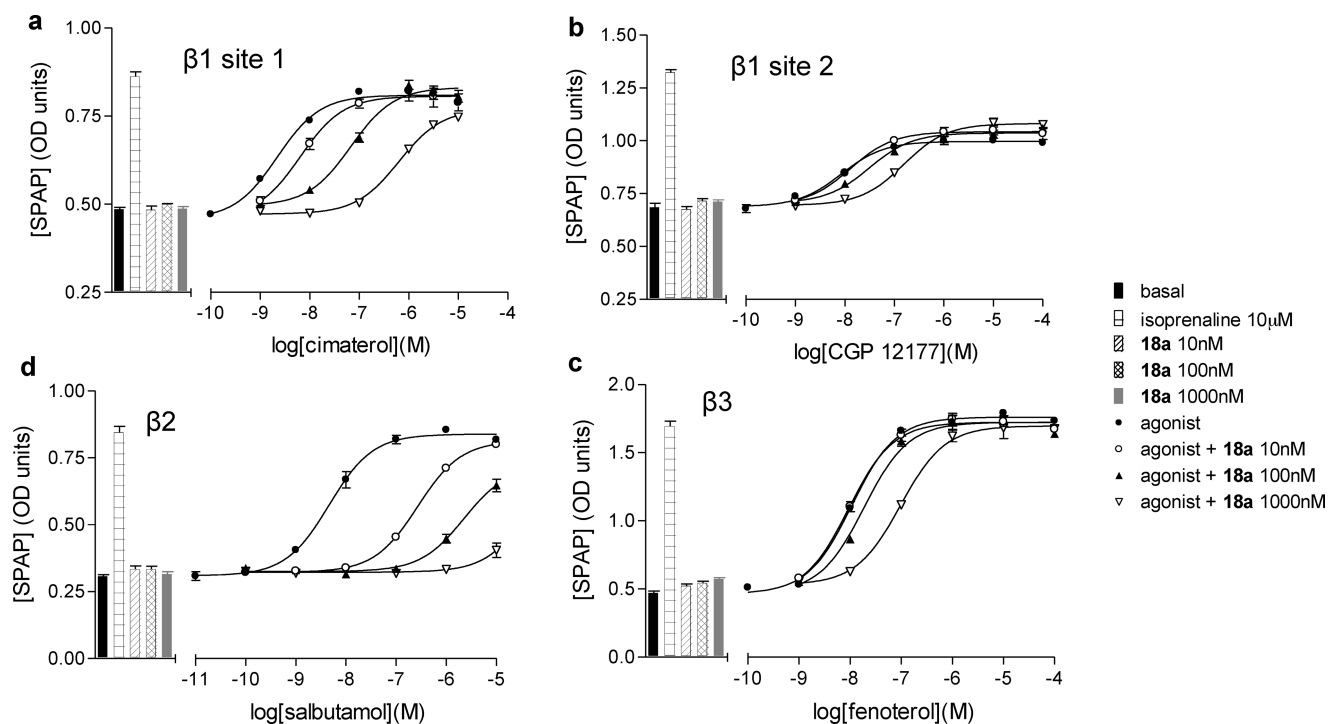
Figure 4. Fluorescent ligand **18a** is a competitive antagonist at all three human β-AR as measured via a whole cell reporter gene assay. CRE-SPAP production in the absence and presence of 10, 100, and 1000 nM compound **18a** in (a) $\beta1$ cells following stimulation by cimaterol, (b) $\beta1$ cells following stimulation by CGP 12177, (c) $\beta2$ cells following stimulation by salbutamol, and (d) $\beta3$ cells following stimulation by fenoterol. Bars represent basal CRE-SPAP production, that in response to 10 μM isoprenaline, and that in response to 10, 100, or 1000 nM compound **18a** alone. Data points are mean $\pm$ SE of triplicate determinations. These single experiments are representative of (a) five, (b) seven, (c) 10, and (d) four separate experiments. The Schild slope for part (a) is 1.08.
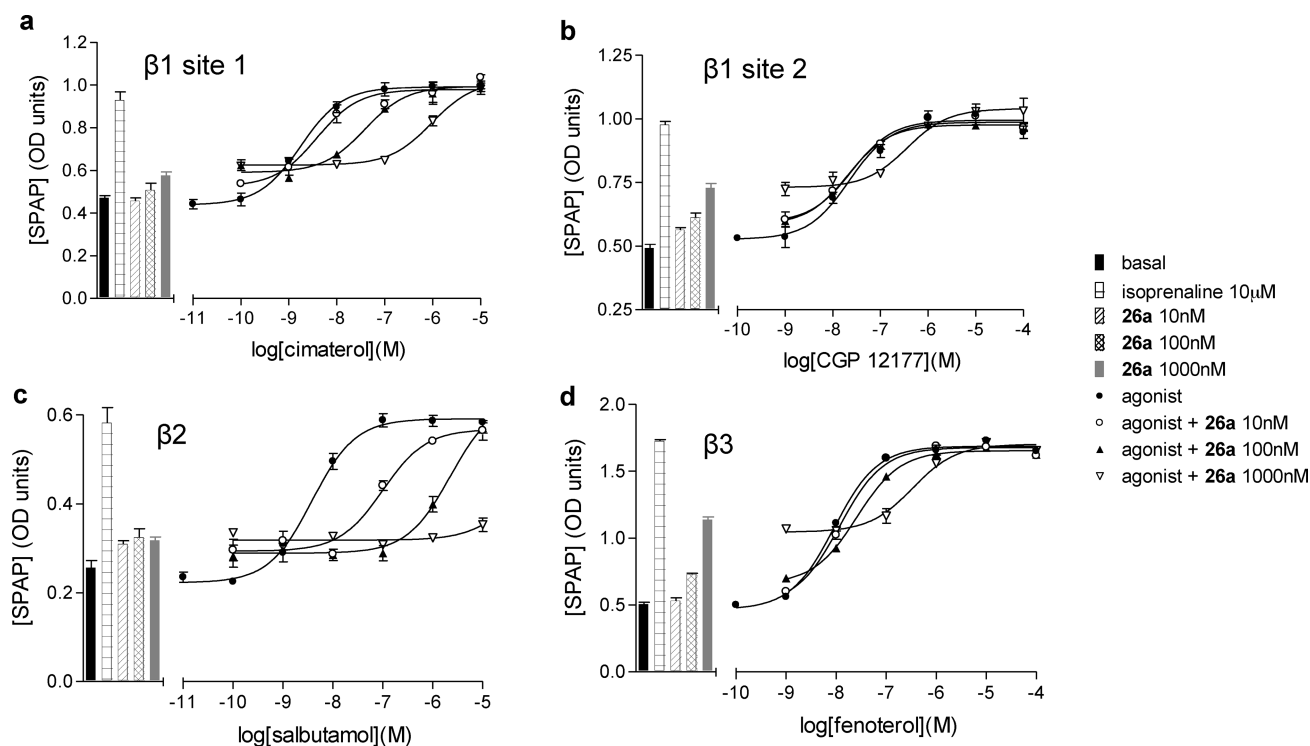

Figure 5. Partial agonism effects of compound **26a** are competitively antagonized at all three human β-AR. CRE-SPAP production in the absence and presence of 10, 100, and 1000 nM compound **26a** in (a) $\beta1$ cells following stimulation by cimaterol, (b) $\beta1$ cells following stimulation by CGP 12177, (c) $\beta2$ cells following stimulation by salbutamol, and (d) $\beta3$ cells following stimulation by fenoterol. Bars represent basal CRE-SPAP production, that in response to 10 μM isoprenaline, and that in response to 10, 100, or 1000 nM compound **26a** alone. Data points are mean $\pm$ SE of triplicate determinations. These single experiments are representative of (a) five, (b) eight, (c) four, and (d) five separate experiments.

Table 3. Partial Agonist Actions of Alprenolol and Pindolol[a] Derivatives at the Three Human β-AR[b]

	$\beta 1$ %[c]	n	$\beta 2$ %[c]	n	$\beta 3$ %[c]	n
alprenolol	46.4 ± 3.1	13	52.8 ± 1.8	7	62.4 ± 3.2	12
23a	40.8 ± 2.6	10	12.2 ± 1.4	4	56.6 ± 3.4	9
23b	33.1 ± 2.5	4	11.7 ± 2.3	3	60.6 ± 5.0	5
26a	28.9 ± 2.0	12	26.9 ± 2.1	5	63.5 ± 3.6	5
26b	30.9 ± 1.8	14	22.9 ± 2.3	6	49.6 ± 2.3	13
pindolol	64.3 ± 2.6	8	52.3 ± 1.9	3	85.2 ± 3.2	7
31	31.1 ± 2.0	4	10.8 ± 1.0	4	49.4 ± 4.9	4
34	20.8 ± 1.8	8	11.0 ± 1.5	4	53.2 ± 3.5	8

[a] The propranolol derivatives did not stimulate significant agonist responses. [b] Values are mean $\pm$ SE from n separate experiments. [c] Agonist action is expressed as a percentage of the CRE-SPAP responses to 10 μM isoprenaline in each experiment. As the top of the concentration response curve was not achieved by many fluorescent ligands at the maximum possible experimental concentration of 1 μM, the response to alprenolol, pindolol, and their derivatives at 1 μM is given.

■ EXPERIMENTAL SECTION

General Chemistry Methods. Chemicals and solvents (HPLC grade) were purchased from the standard suppliers and were used without further purification. BODIPY fluorophores were obtained from Molecular Probes, Cambridge Bioscience, Cambridge, UK. Merck Kieselgel 60 (230−400 mesh) for flash chromatography was supplied by Merck KgaA (Darmstadt, Germany), and deuterated solvents were purchased from Goss International Limited. Unless otherwise stated, reactions were carried out at ambient temperature. Reactions were monitored by thin layer chromatography on commercially available precoated aluminum backed plates (Merck Kieselgel 60 F254). Visualization was by examination under UV light (254 and 366 nm). General staining employed KMnO₄. A solution of ninhydrin (EtOH) was used for the visualization of primary amines. All organic extracts collected after aqueous workup procedures were dried over anhydrous magnesium sulfate, filtered under gravity, and evaporated to dryness. Organic solvents were evaporated in vacuo at a temperature of <35 °C (water bath). Purification using preparative layer chromatography (PLC) was carried out using Fluka silica gel GF254 on glass plates (200 mm × 200 mm × 1 mm).

Melting points were recorded on a Gallenkamp 3A 3790 apparatus and are uncorrected. FT-infrared spectra were recorded as thin films or KBr discs in the range of 4000−600 cm⁻¹ using an Avatar 360 Nicolet FT-IR spectrophotometer. Optical rotation was measured on a Bellingham-Stanley ADP220 polarimeter. Mass spectra and HRMS TOF-ES mass spectra were recorded on a Waters 2795 Separation Module/ Micromass LCT platform. Proton nuclear magnetic resonance spectra were recorded on a Bruker-AV 400 (400 MHz) spectrometer. Carbon nuclear magnetic resonance spectra were recorded at 100.6 MHz. Unless otherwise stated, spectra were recorded in CDCl₃. Chemical shifts (δ) are recorded in ppm with reference to the chemical shift of the deuterated solvent/an internal tetramethylsilane standard. Coupling constants (J) are recorded in hertz and the signal multiplicities described by s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; dd, doublet of doublets. Spectra were assigned using appropriate COSY, DEPT, and HMQC sequences. Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters Millennium 995 LC system using columns, gradients, and flow rates as described. The eluent was monitored using photodiode array detection. Mobile phases were solvent A, water; solvent B, acetonitrile (both containing 0.06% v/v trifluoroacetic acid) degassed by helium bubble and sonication, respectively. The purities of all compounds tested in

biological systems were therefore assessed as being ∼95%. Monoprotected diamines [benzyl 2-aminoethylcarbamate, *tert*-butyl 2-aminoethylcarbamate, *tert*-butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate] were purchased (Sigma-Aldrich) or were synthesized [benzyl 4-aminobutylcarbamate, benzyl 8-aminooctylcarbamate, benzyl 2-(2-(2-aminoethoxy)-ethoxy)ethylcarbamate] as previously described.[36,51]

(S)-2-((Naphthalen-1-yloxy)methyl)oxirane (10a). A portion of 60% NaH in mineral oil (610 mg, equivalent to 366 mg of NaH, 15.26 mmol, 1.1 equiv) was dispersed in anhydrous DMF (2 mL) under an atmosphere of nitrogen at room temperature. To this vigorously stirred suspension was added a solution of 1-naphthol (2.00 g, 13.87 mmol) in anhydrous DMF (8 mL). The resulting pale green suspension was stirred at room temperature for 30 min. A solution of (S)-glycidyl nosylate (3.632 g, 14.01 mmol, 1.01 equiv) in anhydrous DMF (5 mL) was added dropwise to the reaction mixture. Once addition was complete, the mixture was heated at 60 °C overnight before cooling and quenching with aqueous saturated NH₄Cl. The mixture was concentrated to dryness under reduced pressure and the resulting residue dispersed in water (100 mL). The aqueous slurry was extracted with Et₂O (3 × 50 mL), and the combined organic extracts were washed with aqueous 1 M NaOH (50 mL) and brine (50 mL). After drying over anhydrous MgSO₄, the organic extracts were concentrated under reduced pressure. The crude product was further purified by flash column chromatography (eluent EtOAc/ petroleum ether 40−60 0:100 to 30:70 over 10 column volumes) to afford a colorless oil (2.44 g, 88%). $[\alpha]_D^{26}$ = +16.7° (c 1.65, CHCl₃); lit.[52] $[\alpha]_D^{20}$ = +17.3° (c 2.0, CHCl₃). ¹H NMR: δ 2.86 (1H, dd, J = 4.9/2.7 Hz), 2.98 (1H, dd, J = 4.8/4.2 Hz), 3.48−3.54 (1H, m), 4.16 (1H, dd, J = 11.0/5.6 Hz), 4.41 (1H, dd, J = 11.0/3.2 Hz), 6.82 (1H, d, J = 7.0 Hz), 7.37 (1H, dd, J = 7.9 Hz), 7.43−7.54 (3H, m), 7.76−7.85 (1H, m), 8.27−8.35 (m, 1H). ¹³C NMR: δ 44.91, 50.39, 69.10, 105.12, 121.00, 122.16, 125.47, 125.73, 125.84, 126.65, 127.59, 134.66, 154.38. HRMS (TOF ES⁺): calcd for C₁₃H₁₃O₂ 201.0910, found 201.0904 (MH⁺).

(S)-2-((2-Allylphenoxy)methyl)oxirane (20a). A portion of 60% NaH in mineral oil (656 mg, equivalent to 394 mg NaH, 16.40 mmol, 1.1 equiv) was dispersed in anhydrous DMF (2 mL) under an atmosphere of nitrogen at room temperature. To this vigorously stirred suspension was added a solution of 2-allylphenol (2.00 g, 14.91 mmol) in anhydrous DMF (8 mL). The resulting pale green suspension was stirred at room temperature for 30 min. A solution of (S)-glycidyl nosylate (3.903 g, 15.05 mmol, 1.01 equiv) in anhydrous DMF (5 mL) was added dropwise to the reaction mixture. Once addition was complete, the mixture was heated at 60 °C overnight before cooling and quenching with aqueous saturated NH₄Cl. The mixture was concentrated to dryness under reduced pressure and the resulting residue dispersed in water (100 mL). The aqueous slurry was extracted with Et₂O (3 × 50 mL), and the combined organic extracts were washed with aqueous 1 M NaOH (50 mL) and brine (50 mL). After drying over anhydrous MgSO₄, the organic extracts were concentrated under reduced pressure. The crude product was further purified by flash column chromatography (eluent EtOAc/petroleum-ether 40−60 0:100 to 30:70 over 10 column volumes) to afford a clear yellow oil (2.97 g, quantitative). $[\alpha]_D^{21}$ = +8.97° (c 4.18, DCM). ¹H NMR: δ 2.78 (1H, dd, J = 5.0/2.7 Hz), 2.90 (1H, dd, J = 4.9/4.2 Hz), 3.33−3.39 (1H, m), 3.41 (2H, br d, J = 6.6 Hz), 3.99 (1H, m, J = 11.0/5.4 Hz), 4.23 (1H, dd, J = 11.0/3.1 Hz), 5.00−5.11 (2H, m), 5.93−6.06 (1H, m), 6.84 (1H, d, J = 8.2 Hz), 6.92 (1H, ddd, J = 7.4/7.4/1.1 Hz), 7.10−7.23 (2H, m). ¹³C NMR: δ 34.50, 44.80, 50.44, 68.88, 111.73, 115.62, 121.31, 127.46, 129.19, 130.11, 137.03, 156.28. HRMS (TOF ES⁺): calcd for C₁₂H₁₅O₂ 191.1067, found 191.1060 (MH⁺).

(±)-4-(Oxiran-2-ylmethoxy)-1H-indole (28). 4-Hydroxy-1H-indole (100 mg, 0.75 mmol) and Cs₂CO₃ (257 mg, 0.79 mmol, 1.05 equiv) were placed in a 10 mL MW vial, prior to the addition of (±)-epichlorohydrin (3.0 mL). The mixture was heated in the MW reactor at
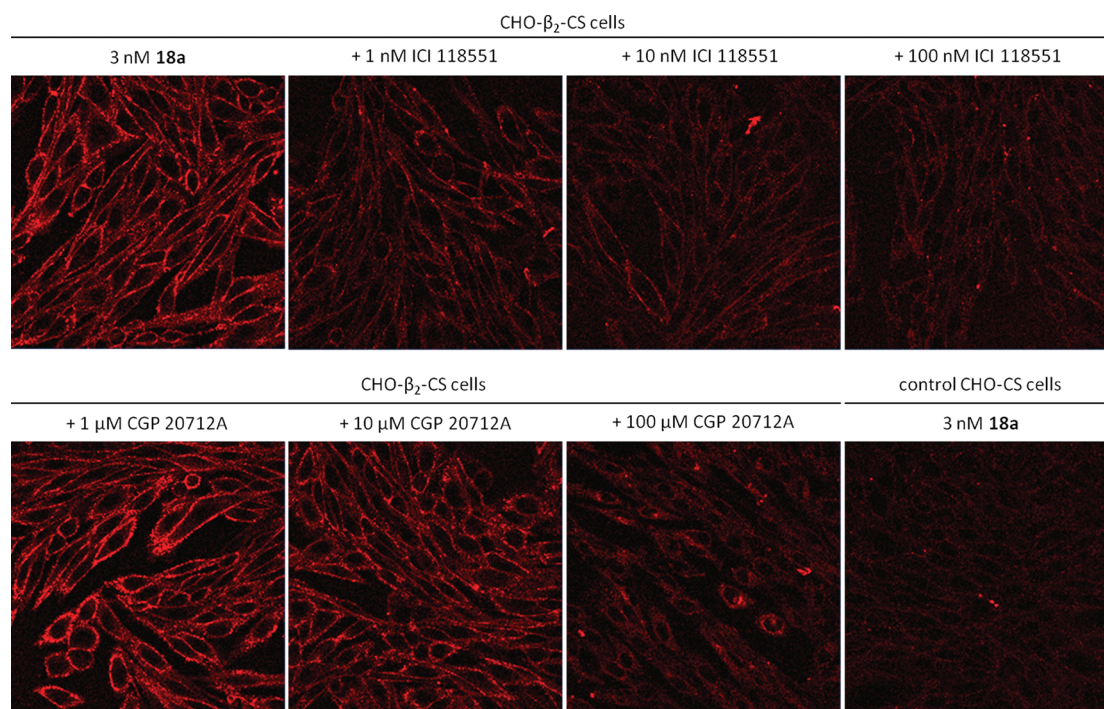
Figure 6. Confocal visualization of the effect of ICI 118551 or CGP 20712A on the binding of the BODIPY-630/650 derivative of propranolol **18a** to CHO K1 cells expressing the β2-AR. Antagonists were incubated with cells for 30 min prior to addition of 3nM **18a** for 10 min. Imaging of cells was undertaken in the continued presence of fluorescent ligand. The lower right-hand panel shows the binding of **18a** to control CHO-K1 cells.

120 °C (dynamic program, max. pressure 250 psi, max. power 300 W) for 30 min. The reaction mixture was diluted with water (20 mL) before extracting with DCM (3 × 10 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 before concentration under reduced pressure. The crude product was further purified by flash column chromatography (eluent EtOAc/petroleum ether 40−60 0:100 to 30:70 over 16 column volumes), to afford a pale blue solid (0.138 g, 97%). Mp: 56−58 °C. ^{1}H NMR: δ 2.83 (1H, dd, J = 4.9/2.7 Hz), 2.94 (1H, dd, J = 4.9/4.2 Hz), 3.44−3.52 (1H, m), 4.14 (1H, dd, J = 11.2/5.6 Hz), 4.38 (1H, dd, J = 11.2/3.2 Hz), 6.54 (1H, d, J = 7.5 Hz), 6.68−6.75 (1H, m), 7.03 (1H, d, J = 8.2 Hz), 7.08 (1H, dd, J = 2.8 Hz), 7.12 (1H, dd, J = 7.9 Hz), 8.30 (1H, br s). ^{13}C NMR: δ 44.97, 50.48, 68.90, 99.90, 100.85, 105.11, 118.87, 122.63, 122.95, 137.50, 152.28. HRMS (TOF ES$^+$): calcd for $C_{11}H_{12}NO_2$ 190.0863, found 190.0856 (MH$^+$).

($\pm$)-Benzyl 4-(2-Hydroxy-3-(naphthalen-1-yloxy)propyl-amino)butylcarbamate (12e). A solution of 2-(napthalen-1-ylox-ymethyl)oxirane (**10b**) (0.20 g, 1 mmol) and benzyl 4-aminobutylcar-bamate (**11b**) (0.56 g, 2.5 mmol) in DMF/H_2O 9:1 (5 mL) was heated at 85 °C for 16 h. The solvent was evaporated under reduced pressure and the crude material was purified by flash column chromatography on silica employing a gradient from 0 to 10% MeOH in CH_2Cl_2 as eluent to afford the title compound as a yellow solid (0.245 g, 58%). Mp 90−91 °C. ^{1}H NMR: 1.49−1.61 (4H, m), 2.45−2.74 (4H, m), 2.86 (1H, dd, J = 12.1, 7.5), 2.95 (1H, dd, J = 12.1, 3.7), 3.12−3.25 (2H, m), 4.09−4.25 (3H, m), 5.09 (2H, s), 5.19 (1H, br s), 6.81 (1H, d, J = 7.5), 7.27−7.40 (6H, m), 7.42−7.52 (3H, m), 7.76−7.84 (1H, m). ^{13}C NMR: 27.5, 27.9, 41.0, 49.5, 52.2, 66.7, 68.5, 70.8, 105.1, 120.8, 121.9, 125.4, 125.7, 126.0, 126.6, 127.7, 128.2, 128.2, 128.6 (3 × CH), 134.6, 136.8, 154.4, 156.6. IR (NaCl, film): 2933, 1701, 1269, 1242, 1102, 772 cm^{-1}. MS m/z (TOF ES$^+$): 423 (MH$^+$, 100%). HRMS (TOF ES$^+$): calcd for $C_{25}H_{30}N_2O_4$ 423.2284, found 423.2247.

(S)-$tert$-Butyl 2-(3-(2-Allylphenoxy)-2-hydroxypropylamino)-ethylcarbamate (21a). A solution of (S)-2-((2-allylphenoxy)-methyl)oxirane (**20a**) (0.105 g, 0.553 mmol) and $tert$-butyl

2-aminoethylcarbamate (0.221 g, 1.38 mmol) in DMF/H_2O 9:1 (3 mL) was heated at 85 °C for 16 h. The solvent was evaporated under reduced pressure and the target compound purified by flash column chromatography on silica (10% MeOH in CH_2Cl_2) to afford the title compound as a colorless oil (0.102 g, 53%). $[\alpha]_D^{26}$ = +3.1° (c 0.8, 15:85 MeOH/CH_2Cl_2). ^{1}H NMR: 1.44 (9H, s), 2.69−2.85 (2H, br s), 2.79 (2H, t, J = 5.8), 2.82 (1H, dd, J = 12.3, 7.7), 2.89 (1H, dd, J = 12.3, 4.1), 3.20−3.31 (2H, m), 3.38 (2H, d, J = 6.1), 3.98 (2H, d, J = 5.4), 4.06−4.14 (1H, m), 4.96−5.08 (3H, m), 5.97 (1H, ddt, J = 16.7, 10.4, 6.4), 6.83 (1H, d, J = 7.7), 6.91 (1H, t, J = 7.7), 7.13 (1H, dd, J = 7.7, 1.5), 7.17 (1H, td, J = 7.7, 1.5). ^{13}C NMR: 28.5 (3 × CH$_3$), 34.8, 40.3, 49.4, 51.8, 68.6, 70.5, 79.5, 111.5, 115.4, 121.1, 127.6, 128.6, 130.2, 137.4 156.4. HRMS (TOF ES$^+$): calcd for $C_{19}H_{31}N_2O_4$ 351.2284, found 351.2319 (MH$^+$).

($\pm$)-Benzyl 2-(2-(2-(3-(1H-Indol-4-yloxy)-2-hydroxypropy-lamino)ethoxy)ethoxy)ethylcarbamate (32). ($\pm$)-4-(Oxiran-2-ylmethoxy)-1H-indole (**28**) (100 mg, 0.53 mmol) and benzyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (**15**) (224 mg, 0.79 mmol, 1.5 equiv) were dissolved in propan-2-ol/acetonitrile/water (7:2:1, 5 mL) and heated in a MW reactor at 90 °C (dynamic program, max. pressure 250 psi, max. power 300 W) for 60 min. The reaction mixture was concentrated under reduced pressure and the residue purified by flash column chromatography (eluent 1 M methanolic NH$_3$/DCM 0:100 to 10:90 over 12 column volumes), to give 100 mg of a pale yellow viscous oil (40%). ^{1}H NMR: δ 8.72 (1H, br s), 7.23−7.41 (5H, m), 7.03−7.10 (2H, m), 7.00 (1H, d, J = 8.1 Hz), 6.61 (1H, br s), 6.46 (1H, d, J = 7.4 Hz), 5.67−5.88 (1H, br m), 5.07 (2H, s), 3.98−4.27 (3H, m), 3.53−3.65 (6H, m), 3.50 (2H, t, J = 4.9 Hz), 3.35 (2H, dt, J = 5.1/5.1 Hz), 2.72−2.95 (5H, m). ^{13}C NMR: δ 156.73, 152.32, 137.46, 136.66, 128.55, 128.16, 128.12, 122.94, 122.60, 118.77, 104.97, 100.70, 99.62, 70.51, 70.20, 70.11, 68.46, 66.69, 51.97, 48.98, 40.91. IR (NaCl, film): 3319, 1707, 1253, 1090, 727 cm^{-1}. HRMS (TOF ES$^-$): calcd for $C_{25}H_{31}N_3O_6$ 470.2297, found 470.2283 (MH$^-$).

Method A. Cbz-Group Hydrogenolysis. To a solution of the Cbz-protected amine (100−130 μM) in MeOH/H_2O (9:1) was added

10% Pd/C (1:1 w/w with protected amine). The solvent was briefly degassed and hydrogen was added via balloon; the mixture was then stirred at room temperature for 1 h. The reaction mixture was filtered through Celite and the pad was washed with MeOH (×2). The solvent was evaporated under reduced pressure to afford the deprotected amine, which was used without further purification.

(±)-1-(4-Aminobutylamino)-3-(napthalenyl-1-yloxy)propan-2-ol (13e). The compound was prepared via method A to yield a colorless oil (quant.). ^{1}H NMR (CD$_3$OD): 1.63−1.79 (4H, m), 2.71−2.84 (2H, m), 2.89 (2H, t), 2.92 (1H, dd, J = 12.3, 8.7), 3.03 (1H, dd, J = 12.3, 3.6), 4.14 (1H, dd, J = 9.8, 5.3), 4.18 (1H, dd, J = 9.8, 5.3), 4.28 (1H, dtd, J = 8.7, 5.3, 3.6), 6.91 (1H, dd, J = 7.6, 0.9), 7.34−7.50 (4H, m), 7.76−7.82 (1H, m), 8.26−8.34 (1H, m). ^{13}C NMR (CD$_3$OD): 27.5, 27.7, 40.7, 49.7, 52.9, 69.2, 71.8, 106.0, 121.6, 122.9, 126.1, 126.9, 127.0, 127.4, 128.5, 136.0, 155.7. IR (NaCl, film): 3354, 2939, 1579, 1398, 1270, 1241, 1102, 795, 772 cm^{-1}. HRMS (TOF ES$^+$): calcd for C$_{17}$H$_{25}$N$_2$O$_2$ 289.1916, found 289.1895 (MH$^+$).

(S)-1-(2-Allylphenoxy)-3-(2-aminoethylamino)propan-2-ol (22a). (S)-tert-Butyl 2-(3-(2-allylphenoxy)-2-hydroxypropylamino)-ethylcarbamate (21a) (0.04 g, 0.114 mmol) was dissolved in a 2 M solution of HCl in dioxane (2 mL) and the reaction mixture was stirred at room temperature for 18 h. The solvent was evaporated under reduced pressure and the resultant residue was azeotroped sequentially with toluene and EtOAc to afford the dihydrochloride salt of the title compound as a white solid (0.036 g, 98%), which was used without further purification. Mp: 142−145 °C. $[\alpha]_D^{26}$ = −2.2° (c 0.46, 15:85 MeOH/CH$_2$Cl$_2$). ^{1}H NMR (CD$_3$OD): 3.28 (1H, dd, J = 12.5, 9.8), 3.36−3.49 (7H, m), 4.01 (1H, dd, J = 10.0, 5.7), 4.09 (1H, dd, J = 10.0, 4.8), 4.32−4.40 (1H, m), 4.98−5.06 (2H, m), 5.94−6.05 (1H, m), 6.91 (1H, td, J = 7.7, 1.0), 6.95 (1H, d, J = 7.7), 7.13 (1H, dd, J = 7.7, 1.7), 7.18 (1H, td, J = 7.7, 1.7). ^{13}C NMR (CD$_3$OD): 35.3, 36.8, 45.9, 52.0, 66.8, 71.0, 112.7, 115.7, 122.3, 128.5, 129.8, 131.0, 138.5, 157.4. IR (KBr): 3299, 2956, 1600, 1491, 1455, 1258, 1214, 764 cm^{-1}. HRMS (TOF ES$^+$): calcd for C$_{14}$H$_{23}$N$_2$O$_2$ 251.1760, found 251.1773 (MH$^+$).

(±)-13-Amino-1-(1H-indol-4-yl)-1,8,11-trioxa-5-azatridecan-3-ol (33). The compound was prepared via method A to yield a colorless oil (88%). ^{1}H NMR (CD$_3$OD): 2.86−3.02 (6H, m), 3.58−3.69 (8H, m), 4.12 (1H, dd, J = 9.9, 6.0), 4.15 (1H, dd, J = 9.9, 4.7), 4.16−4.24 (1H, m), 6.41 (1H, d, J = 3.0), 6.64 (1H, d, J = 7.6), 6.91 (1H, t, J = 7.8), 7.16 (1H, dd, J = 7.8, 0.7), 7.20 (1H, d, J = 3.3). ^{13}C NMR (CD$_3$OD): 41.1, 49.8, 52.9, 69.6, 69.6, 70.4, 71.3, 71.3, 71.7, 102.7, 103.6, 114.6, 120.4, 125.2, 128.2, 131.1, 146.7. IR (NaCl, film): 2919, 1576, 1253, 1091, 728 cm^{-1}. HRMS (TOF ES$^+$): calcd for C$_{17}$H$_{27}$N$_3$O$_4$ 338.2080, found 338.2062 (MH$^+$).

Method B. Coupling of Amines with BODIPY 630/650-X, SE. BODIPY 630/650-X, SE (1−2.5 mg, 1 equiv) and amine (2−4 equiv) were dissolved in anhydrous DMF (1 mL) [in the case of the alprenolol congener HCl salts, 2 equiv (with respect to the congener) of N,N-diisoproylethylamine (DIPEA) was also added (method B1)] and stirred under a nitrogen atmosphere, with the exclusion of light, for 2 h. The solvent was evaporated under reduced pressure and this crude mixture was purified by PTLC on silica using (10:90 MeOH/CH$_2$Cl$_2$) as eluent to give compounds **14a−f, 18a,b, 23a,b, 26a,b, 31**, and **34** as blue amorphous solids. When submitted to analytical HPLC [YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B or 5−100% B, 30 min, monitored using photodiode array detection between 190 and 800 nm; mobile phases, solvent A, H$_2$O; solvent B, MeCN (both A and B containing 0.06% TFA as additive)] all fluorescent conjugates were observed to elute as single and symmetrical peaks at the retention time t_R given below for each compound. For the initial conjugates synthesized within the racemic propranolol series, we further analyzed compound purity by performing a second analytical HPLC [Jones C^4, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min, monitored using photodiode array detection between 190 and 800 nm; solvent A, H$_2$O; solvent B, MeCN (both A and B containing 0.06% TFA as additive)]. Again, all

target compounds eluted as homogeneous single peaks (with the expected reduction in retention time). The identities of all the compounds were further analyzed by HRMS (TOF ES$^+$). The purities of all compounds tested in biological systems were determined as being ∼95%.

(S)-N-[2-(2-Hydroxy-3-(naphthalen-1-yloxy)propylamino)-ethyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (14a). The compound was prepared via method B (78%). t_R 9.61 min (YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{44}$H$_{47}$BF$_2$N$_5$O$_5$S 806.3359, found 806.3425 (MH$^+$).

(S)-N-[2-(2-Hydroxy-3-(naphthalen-1-yloxy)propylamino)-butyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (14b). The compound was prepared via method B (80%). t_R 15.68 min (YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 5−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{46}$H$_{51}$BF$_2$N$_5$O$_5$S 834.3672, found 834.3690 (MH$^+$).

(S)-N-[2-(2-Hydroxy-3-(naphthalen-1-yloxy)propylamino)-octyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (14c). The compound was prepared via method B (71%). t_R 10.22 min (YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{50}$H$_{59}$BF$_2$N$_5$O$_5$S 890.4298, found 890.4278 (MH$^+$).

(±)-N-[2-(2-Hydroxy-3-(naphthalen-1-yloxy)propylamino)-ethyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (14d). The compound was prepared via method B (98%). t_{R1} 10.37 min (YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min). t_{R2} 7.45 min (Jones C^4, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{44}$H$_{47}$BF$_2$N$_5$O$_5$S 806.3359, found 806.3279 (MH$^+$).

(±)-N-[4-(2-Hydroxy-3-(naphthalen-1-yloxy)propylamino)-butyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (14e). The compound was prepared via method B (77%). t_{R1} 9.48 min (YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min). t_{R2} 7.54 min (Jones C^4, 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min). ^{1}H NMR (CD$_3$OD): 1.22−1.33 (2H, m), 1.47−1.64 (6H, m), 1.66−1.76 (2H, m), 2.16 (2H, t, J = 7.3), 3.06 (2H, t, J = 8.0), 3.16−3.33 (6H, m), 4.13 (1H, dd, J = 9.9, 5.7), 4.20 (1H, dd, J = 9.9, 5.0), 4.34−4.41 (1H, m), 4.55 (2H, s), 6.83 (1H, d, J = 4.2), 6.90 (1H, d, J = 7.1), 7.02 (2H, app d, J = 8.8), 7.11 (1H, d, J = 4.2), 7.11 (1H, d, J = 4.2), 7.18 (1H, d, J = 4.4), 7.19 (1H, dd, J = 5.0, 3.8), 7.33 (1H, s), 7.37 (1H, t, J = 7.9), 7.43−7.56 (4H, m), 7.57−7.63 (3H, m), 7.81 (1H, dd, J = 7.1, 1.5), 8.12 (1H, dd, J = 3.9, 1.0), 8.22 (1H, br t, J = 6.0), 8.24−8.28 (1H, m). HRMS (TOF ES$^+$): calcd for C$_{46}$H$_{51}$BF$_2$N$_5$O$_5$S 834.3672, found 834.3622 (MH$^+$).

(±)-N2-(2-Hydroxy-3-(naphthalen-1-yloxy)propylamino)-octyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (14f). The compound was prepared via method B (69%). t_R 14.21 min (YMC C^8, 150 × 4.6 mm, 1 mL min^{-1}, 5−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{50}$H$_{59}$BF$_2$N$_5$O$_5$S 890.4298, found 890.4321 (MH$^+$).

(S)-N-[3-Hydroxy-1-(naphthalen-1-yl)-1,8,11-trioxa-5-aza-tridecan-13-yl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacen-3-yl)vinyl)phenoxy)-acetamido)hexanamide (18a). The compound was prepared

via method B (87%). t_R 10.75 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{48}$H$_{55}$BF$_2$N$_5$O$_7$S 894.3883, found 894.3849 (MH$^+$).

(±)-*N*-[3-Hydroxy-1-(naphthalen-1-yl)-1,8,11-trioxa-5-aza-tridecan-13-yl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)phenoxy)-acetamido)hexanamide (18b). The compound was prepared via method B (81%). t_R 9.52 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{48}$H$_{55}$BF$_2$N$_5$O$_7$S 894.3883, found 894.3926 (MH$^+$).

(*S*)-*N*-[2-(3-(2-Allylphenoxy)-2-hydroxypropylamino)ethyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)phenoxy)acetamido)hexan-amide (23a). The compound was prepared via method B1 (79%). t_R 9.33 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min). ^{1}H NMR (CD$_3$OD): 1.24−1.35 (2H, m), 1.54 (2H, tt, *J* = 7.6, 7.6), 1.60 (2H, tt, *J* = 7.6, 7.6), 2.17 (2H, t, *J* = 7.5), 2.82−2.94 (3H, m), 3.00 (1H, dd, *J* = 12.5, 3.5), 3.23−3.31 (2H, m), 3.33−3.40 (4H, m), 3.92 (1H, dd, *J* = 9.6, 5.4), 3.97 (1H, dd, *J* = 9.6, 5.1), 4.07−4.16 (1H, m), 4.57 (2H, s), 4.96−5.04 (2H, m), 5.97 (1H, ddt, *J* = 16.9, 10.3, 6.5), 6.82−6.92 (3H, m), 7.05 (2H, app d, *J* = 9.0), 7.08−7.19 (4H, m), 7.20 (1H, d, *J* = 3.8), 7.21 (1H, d, *J* = 3.8), 7.35 (1H, s), 7.55 (2H, br d, *J* = 7.7), 7.59−7.65 (3H, m), 8.12 (1H, dd, *J* = 3.8, 1.0). HRMS (TOF ES$^+$): calcd for C$_{43}$H$_{49}$BF$_2$N$_5$O$_5$S 796.3516, found 796.3474 (MH$^+$).

(±)-*N*-[2-(3-(2-Allylphenoxy)-2-hydroxypropylamino)-ethyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (23b). The compound was prepared via method B1 (29%). t_R 13.33 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 5−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{43}$H$_{49}$BF$_2$N$_5$O$_5$S 796.3516, found 795.3345 (MH$^+$).

(*S*)-*N*-[3-Hydroxy-1-(2-(prop-2-en-1-yl)phenyl)-1,8,11-trioxa-5-azatridecan-13-yl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)-phenoxy)acetamido)hexanamide (26a). The compound was prepared via method B1 (53%). t_R 13.35 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 5−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{47}$H$_{57}$BF$_2$N$_5$O$_7$S 884.4040, found 884.4086 (MH$^+$).

(±)-*N*-[3-Hydroxy-1-(2-(prop-2-en-1-yl)phenyl)-1,8,11-trioxa-5-azatridecan-13-yl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)-phenoxy)acetamido)hexanamide (26b). The compound was prepared via method B1 (68%). t_R 9.57 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{47}$H$_{57}$BF$_2$N$_5$O$_7$S 884.4040, found 884.4045 (MH$^+$).

(±)-*N*-[2-(2-Hydroxy-3-(1*H*-indol-4-yloxy)propylamino)-ethyl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)phenoxy)acetamido)-hexanamide (31). The compound was prepared via method B (39%). t_R 9.19 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min; solvent A, H$_2$O; solvent B, MeCN, both A and B contain 0.06% TFA as additive). HRMS (TOF ES$^+$): calcd for C$_{42}$H$_{46}$BF$_2$N$_6$O$_5$S 795.3312, found 795.3345 (MH$^+$).

(±)-*N*-[3-Hydroxy-1-(1*H*-indol-4-yl)-1,8,11-trioxa-5-azatridecan-13-yl]-6-(2-(2-(4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)vinyl)phenoxy)-acetamido)hexanamide (34). The compound was prepared via method B (68%). t_R 9.40 min (YMC C[8], 150 × 4.6 mm, 1 mL min^{-1}, 35−100% B, 30 min). ^{1}H NMR (CD$_3$OD): 1.21−1.32 (2H, m), 1.51

(2H, tt, *J* = 7.6, 7.6), 1.57 (2H, tt, *J* = 7.6, 7.6), 2.14 (2H, t, *J* = 7.4), 3.11−3.22 (3H, m), 3.25 (2H, t, *J* = 6.9), 3.28−3.37 (2H, m), 3.44−3.50 (1H, m), 3.47 (2H, t, *J* = 5.6), 3.53−3.58 (2H, m), 3.58−3.63 (2H, m), 3.72 (2H, t, *J* = 4.8), 4.13 (1H, dd, *J* = 9.8, 5.4), 4.18 (1H, dd, *J* = 9.8, 5.0), 4.26−4.35 (1H, m), 4.55 (2H, s), 6.43 (1H, d, *J* = 3.2), 6.63 (1H, d, *J* = 7.7), 6.85 (1H, d, *J* = 4.5), 6.92 (1H, t, *J* = 7.8), 7.03 (2H, app d, *J* = 9.0), 7.11−7.15 (2H, m), 7.17−7.22 (4H, m), 7.35 (1H, s), 7.55 (2H, br d, *J* = 8.3), 7.58−7.63 (3H, m), 8.12 (1H, dd, *J* = 3.8, 1.0). HRMS (TOF ES$^+$): calcd for C$_{46}$H$_{54}$-BF$_2$N$_6$O$_7$S 883.3836, found 883.3886 (MH$^+$).

General Pharmacology Methods: Cell Lines and Cell Culture. CHO-K1 stably expressing a six cyclic AMP response (CRE) secreted placental alkaline phosphatase (SPAP) reporter gene and either human β1 (1146 fmol/mg of protein), human β2 (466 fmol/mg of protein), or human β3-AR (790 fmol/mg of protein) were used throughout this study.[44] Cells were grown in Dulbecco's modified Eagle's medium nutrient mix F12 (DMEM/F12) containing 10% fetal calf serum and 2 mM L-glutamine in a 37 °C humidified 5% CO$_2$:95% air atmosphere.

[3]H-CGP 12177 Whole Cell Binding. Cells were grown to confluence in white-sided tissue culture treated 96-well view plates. ^{3}H-CGP 12177 whole cell competition binding was performed as previously described,[45] using ^{3}H-CGP 12177 in the ranges of 0.71−3.14 nM (for β1 and β2) and 4.64−63.0 nM (β3) (total volume 200 μL per well).

CRE-SPAP Production. Cells were grown to confluence in clear plastic tissue culture treated 96-well plates, and CRE-SPAP secretion into the media was measured between 5 and 6 h after the addition of agonist as previously described.[36]

Confocal Imaging. Cells were grown in Labtek eight-well plates (Nunc Nalgene, Rochester, NY) for at least 18 h and grown to 80−100% confluence before imaging. Following washing of the cells in HEPES-buffered saline solution (HBSS; 25 mM HEPES, 10 mM glucose, 146 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 2 mM sodium pyruvate, 1.3 mM CaCl$_2$), live cell imaging was performed at room temperature using a Zeiss LSM 710 laser scanning confocal microscope and a Zeiss Plan-Neofluar 40 × 1.3 NA oil-immersion objective. A 633 nm HeNe laser was used for the excitation of the BODIPY630/650−18a conjugate with emission being detected using a 650 nm long-pass filter. The pinhole diameter (1 Airy Unit; 1.1 μm optical slice), laser power, and gain remained constant between experiments.

Data Analysis: Whole Cell Binding. All data points on each competition binding curve were performed in triplicate and each 96-well plate also contained six determinations of total and nonspecific binding. In all cases where a K_D value is stated, the competing ligand completely inhibited the specific binding of ^{3}H-CGP 12177. For compounds 14c and 14f, where inhibition of binding was only 66% and 85%, respectively, an apparent K_D value is stated. A one-site sigmoidal response curve was then fitted to the data using Graphpad Prism 2.01 and the IC$_{50}$ was then determined as the concentration required to inhibit 50% of the specific binding.

$$\%\text{uninhibited binding} = 100 - \frac{(100 \times A)}{(A + \text{IC}_{50})} + \text{NS} \qquad (1)$$

where *A* in the concentration of the competing ligand, IC$_{50}$ is the concentration at which half of the specific binding of ^{3}H-CGP 12177 has been inhibited, and NS is the nonspecific binding.

From the IC$_{50}$ value and the known concentration of ^{3}H-CGP 12177, a K_D value was calculated using the equation

$$K_D = \frac{\text{IC}_{50}}{1 + \{[^3\text{H-CGP 12177}]/K_D(^3\text{H-CGP 12177})\}} \qquad (2)$$

Inhibition of Agonist-Stimulated CRE-SPAP Production. Agonist responses were best described by a one-site sigmoidal

concentration response curve (eq 3)

$$\text{response} = \frac{E_{max} \times [A]}{IC_{50} + [A]} \qquad (3)$$

where E_{max} is the maximum response, $[A]$ is the agonist concentration, and EC_{50} is the concentration of agonist that produces 50% of the maximal response

The affinities of antagonist drugs were calculated (K_D values) from the shift of the agonist concentration responses in the presence of a fixed concentration of antagonist using eq 4:

$$DR = 1 + \frac{[B]}{K_D} \qquad (4)$$

where DR (dose ratio) is the ratio of the agonist concentration required to stimulate an identical response in the presence and absence of a fixed concentration of antagonist, $[B]$.

Where clear partial agonism was observed (e.g., Figure 5), the affinity was calculated according to the method[45] of Stephenson using eq 5:

$$K_D(\text{partial agonist}) = \frac{Y \times [P]}{1 - Y} \quad \text{where} \quad Y = \frac{[A_2] - [A_1]}{[A_3]} \qquad (5)$$

where $[P]$ in the concentration of the partial agonist, $[A_1]$ in the concentration of the agonist at the point where the partial agonist alone agonist causes the same response, $[A_2]$ is the concentration of agonist causing a given response above that achieved by the partial agonist, and $[A_3]$ the concentration of the agonist, in the presence partial agonist, causing the same stimulation as $[A_2]$.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. Full experimental procedures for compounds **12a−d, 12f, 13a−d, 13f, 14a−d, 14f, 16a,b, 17a, b, 18a,b, 21b, 22b, 23b, 24a,b, 25a,b, 26a,b, 29−31**. This material is available free of charge via the Internet at http://pubs.acs.org

■ AUTHOR INFORMATION

Corresponding Author
*B.K.: tel, +44-115-9513026; fax, +44-115-9513412; e-mail, barrie. kellam@nottingham.ac.uk. S.J.H.: tel, +44-115-8230082; fax, +44-115-8230081; e-mail, stephen.hill@nottingham.ac.uk.

Author Contributions
‖These authors contributed equally to this work

Notes
S.J.H. and B.K. are founding directors of the University of Nottingham spin-off company CellAura Technologies Ltd. R.M. is currently employed by CellAura Technologies Ltd.

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■ ABBREVIATIONS USED

β-AR, β-adrenoceptor; BODIPY 630/650 or BY630, 6-(((4, 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)-styryloxy)acetyl)aminohexanoic acid; BODIPY 630/650-X, SE, 6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)- styryloxy)acetyl)aminohexanoic acid succinimidyl ester; Cbz, benzyloxycarbonyl; CHO, chinese hamster ovary cell ; CGP 20712A, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol; CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one; FCS, fluorescence correlation spectroscopy; GPCR, G-protein coupled receptor; ICI 118551, 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol; LSCM, laser scanning confocal microscopy; MW, microwave; NBD, 7-nitrobenzo-2-oxa-1,3-diazole.

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