Long Receptor Residence Time of C26 Contributes to Super Agonist Activity at the Human β2 Adrenoceptor

Elizabeth M. Rosethorne, Michelle E. Bradley, Karolina Gherbi, David A. Sykes, Afrah Sattikar, John D. Wright, Emilie Renard, Alex Trifilieff, Robin A. Fairhurst, and Steven J. Charlton

Novartis Institutes for Biomedical Research, Horsham, West Sussex, United Kingdom (E.M.R., E.M.R., K.G., D.A.S., A.S., J.D.W., S.J.C.); Novartis Institutes for Biomedical Research, Basel, Switzerland (E.R., A.T., R.A.F.); and School of Life Sciences, Queen’s Medical Centre, University of Nottingham, Nottingham, United Kingdom (E.M.R., K.G., D.A.S., S.J.C.)

Received August 19, 2015; accepted January 13, 2016

ABSTRACT

Super agonists produce greater functional responses than endogenous agonists in the same assay, and their unique pharmacology is the subject of increasing interest and debate. We propose that receptor residence time and the duration of receptor signaling contribute to the pharmacology of super agonism. We have further characterized the novel β2 adrenoceptor agonist C26 (7-[(R)-2-((1R,2R)-2-benzyloxypropylamino)-1-hydroxyethyl]-4-hydroxybenzothiazolone), which displays higher intrinsic activity than the endogenous ligand adrenaline in cAMP accumulation, β-arrestin-2 recruitment, and receptor internalization assays. C26 recruited β-arrestin-2, and internalized the Green Fluorescent Protein (GFP)-tagged β2 adrenoceptor at a slow rate, with half-life (t1/2) values of 0.78 ± 0.04 and 0.78 ± 0.04 hours, respectively. This was compared with 0.31 ± 0.04 and 0.34 ± 0.01 hours for adrenaline-mediated β-arrestin-2 recruitment and GFP-β2 internalization, respectively. The slower rate for C26 resulted in levels of β-arrestin-2 recruitment increasing up to 4-hour agonist incubation, at which point the intrinsic activity was determined to be 124.3 ± 0.77% of the adrenaline response. In addition to slow functional kinetics, C26 displayed high affinity with extremely slow receptor dissociation kinetics, giving a receptor residence half-life of 32.7 minutes at 37°C, which represents the slowest dissociation rate we have observed for any β2 adrenoceptor agonist tested to date. In conclusion, we propose that the gradual accumulation of long-lived active receptor complexes contributes to the increased intrinsic activity of C26 over time. This highlights the need to consider the temporal aspects of agonist binding and signaling when characterizing ligands as super agonists.

INTRODUCTION

Inhaled long-actin β2 adrenoceptor agonists (LABAs), e.g., formoterol and salmeterol, which have a duration of action of approximately 12 hours, have been widely used for the treatment of chronic obstructive pulmonary disease, providing symptomatic relief by inducing bronchodilation via relaxation of airway smooth muscle. More recently, a further class of β2 adrenoceptor agonists has been developed, termed ultra LABA (e.g., indacaterol), which have a duration of action of 24 hours after a single inhaled dose. 7-[(R)-2-((1R,2R)-2-benzyloxypropylamino)-1-hydroxyethyl]-4-hydroxybenzothiazolone (C26) was originally identified as a high-affinity β2 adrenoceptor agonist with a predicted long duration of action. In addition to its high

ABBREVIATIONS: ANOVA, analysis of variance; BSA, bovine serum albumin; C26, 7-[(R)-2-((1R,2R)-2-benzyloxypropylamino)-1-hydroxyethyl]-4-hydroxybenzothiazolone, Chemical Abstracts Services number [663925-01-7]; CGP12177A, 4-[3-[[1,1-Dimethyllethyl]amino][2-hydroxypropoxy]-1,3-dihydro-2H-benimidazol-2-one hydrochloride; CHO-β2 adrenoceptor, CHO-arrestin-β2; PathHunter CHO-K1 β2 adrenoceptor/β-arrestin-2 cells; DHAl, dihydroalprenolol; DMSO, dimethylsulfoxide; DHA, dihydroalprenolol; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; FTY720, 2-amino-2-[2-(4-oclyphenyl)ethyl]propane-1,3-diol; GFP, Green Fluorescent Protein; GPCR, G protein–coupled receptor; GTPyS, guanosine S’-3’-O-(thiotriophosphate); HBSS, Hanks’ balanced salt solution; Kd, observed association rate; Kdiss, dissociation rate; Kassoc, association rate; LABA, long-actin β2 adrenoceptor agonist; MK-677, (R)-1-(2-methylalanyl-O-benzyl-D-seryl)-1-(methylsulfonyl)-1,2-dihydrospiro[indole-3,4’-piperidine]; pEC50, negative log of EC50; TRH, thyrotropin-releasing hormone; U2OS-GFP-β2, U2OS cells stably transfected with GFP-tagged human β2 adrenoceptor.
In general, endogenous agonists of G protein–coupled receptors (GPCR), such as adrenaline for the β2 adrenoceptor, have low affinity but high efficacy, meaning that few receptors need to be activated to achieve a maximum response. In addition, although a limited number of agonists have been studied to date, they also appear to rapidly dissociate from the receptor (Sykes and Charlton, 2012), thus reducing the risk of persistent signaling and over-stimulation of a pathway which may be detrimental to the cell or organism, and allowing rapid resetting of the system.

It is generally assumed that the interaction between an endogenous agonist and its receptor, as a result of strong evolutionary pressure, is likely to be as efficient as it can possibly be. If such a case were true, the maximal asymptote of the concentration–response curves of endogenous agonists, fitted to experimental data (Strange, 2008) (intrinsic activity, \( E_{\text{max}} \)), would always be equal to the system maximum. However, there is a class of synthetic ligands, such as MK-677 ((R)-1′-(2-methylallyl-O-benzyl-D-seryl)-1-(nordansulfonyl)-1,2-dihydropiperidino[indole-3,4′-piperidin)] at the gonadotropin-releasing hormone receptor or R-Des-TRH (thyrotropin-releasing hormone) at the TRH receptor (Engel et al., 2006; Bennett et al., 2009), that have been demonstrated to produce higher maximal responses than their respective endogenous ligands, leading to the term super agonist being coined. Such terminology has been met with mixed views in the pharmacology community, and the unique pharmacology of super agonists is now the subject of increasing interest and debate.

Evidence from structural studies on the β2 adrenoceptor (Nygaard et al., 2013) demonstrated that the change in conformation upon activation of a receptor depends as much upon the presence of G protein as it does on agonist, such that agonist binding alone is not able to stabilize a fully active conformation of the receptor. The “mobile” or “fluid” receptor hypothesis describes the active receptor complex as having three entities, receptor, effector, and ligand, that are able to interact in a cooperative manner (de Haën, 1976; Jacobs and Cuatrecasas, 1976). In simplest the terms, a high-efficacy ligand will therefore act as a positive allosteric modulator, increasing the association of the effector protein with the receptor. As allosteric interactions are reciprocal, it also follows that association of the effector molecule with the receptor can be considered to exert positive allosteric modulation of ligand binding. Conversely, dissociation of effector (or association of inactive effector complex) could be considered to negatively regulate ligand binding, thus reducing its affinity for the receptor. One could therefore speculate that even the fully efficacious endogenous agonists may not be able to maximally activate receptors in cellular systems where high levels of cytosolic guanine nucleotides uncouple the receptor from G proteins and drive it away from the active state (Ehler, 2008), thus leaving scope for “super agonism” to occur.

In this study, we used a number of functional and binding assay systems to fully characterize the pharmacology of C26. We demonstrate that C26 displays interesting pharmacological properties, as it shares binding characteristics with low-efficacy partial agonists, but appears in functional assays to be a kinetically driven super agonist in terms of its intrinsic activity.

Materials and Methods

Materials. PathHunter cell lines, lysis, and Flash detection reagents were purchased from DiscoveRx (Birmingham, UK). CO2-independent medium, Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/l D-glucose, 0.58 g/l L-glutamine and 0.11 g/l pyruvate, Ham’s F12 medium supplemented with GlutaMAX, McCoy’s 5A medium, heat-inactivated fetal bovine serum (FBS), geneticin, hygromycin, blasticidin, penicillin, streptomycin, trypsin-EDTA, Dulbecco’s phosphate-buffered saline solution, Hanks’ balanced salt solution (HBSS) without phenol red, and HEPES were all purchased from Life Technologies (Paisley, UK). ALPHAScreen cAMP detection kit, [3H]-DHA (dihydroxyprodrenol), and 384- and 96-well ViewPlates were purchased from PerkinElmer Life Sciences (Waltham, MA). GloSensor cAMP reagent and GloSensor cAMP plasmid were purchased from Promega (Hampshire, UK). Bovine serum albumin (BSA), Tween-20, adrenaline (epinephrine), isoprenaline, dimethylsulfoxide (DMSO), GTP, and ascorbic acid were purchased from Sigma-Aldrich (Poole, UK). C26 was synthesized in house (Beattie et al., 2010).

Cell Culture. A431 cells (human carcinoma cell line; Life Technologies) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/l D-glucose, 0.11 g/l pyruvate, and FBS (10% v/v). PathHunter CHO-K1 human adrenoceptor: β-arrestin-2 cells (CHO-arrestin-β2) were maintained in Ham’s F12 nutrient mix supplemented with FBS (10% v/v), hygromycin B (0.2 mg/ml), and geneticin (0.5 mg/ml). Chinese hamster ovary cells stably transfected with the human β2 adrenoceptor (CHO-β2) prepared in house were maintained in Ham’s F12 Nutrient Mix GlutaMAX-1, supplemented with FBS (10% v/v) and geneticin (0.5 mg/ml). U2OS cells stably transfected with Green Fluorescent Protein (GFP)-tagged human β2 adrenoceptor (U2OS-GFP-β2) prepared in house (Rosethorne et al., 2015) were maintained in McCoy’s 5A medium supplemented with FBS (10% v/v) and geneticin (0.5 mg/ml). All cells were grown adherently and maintained at 37°C in 5% CO2-humidified air. For routine culture and for experiments, all of the cells were harvested using trypsin-EDTA. For radioligand binding studies, membranes were prepared from CHO-β2 cells as described in Sykes et al. (2009).

Measurement of cAMP Using A431 Cells Endogenously Expressing Human β2 Adrenoceptor. A431 cells were seeded overnight in white, 96-well ViewPlates at 20,000 cells/well in culture medium. Complete medium with 10% FBS was removed and replaced with assay buffer [HBSS without phenol red, 5 mM HEPES, and 0.1% (v/v) BSA]. Cells were then stimulated with a range of concentrations of agonist for 1 hour at room temperature. The incubation was terminated by the addition of lysis buffer [dH2O, 0.3% (v/v) Tween-20] containing 20 U/ml streptavidin-coated donor beads, biotinylated cAMP, and anti-cAMP acceptor beads. A cAMP standard curve (10,000–0.001 nM) was constructed in each experiment, and lysis buffer containing the bead mix was added to the standard curve at the same time it was added to the wells of the assay. The assay plate and standard curve plate were incubated overnight in the dark at room temperature. The plate was then read on the EnVision plate reader (PerkinElmer Life Sciences). The levels of cAMP produced were always within the linear part of the standard curve. For time-course experiments, A431 cells were transiently transfected with the GloSensor cAMP plasmid using LipofectamineR (R) 2000 (Life Technologies, Paisley, UK) according to the manufacturer’s instructions. Twenty-four hours later, cells were stimulated with a range of concentrations of each agonist, and luminescence was monitored over time (every minute for 25 minutes). Responses were obtained for an EC50 concentration of isoprenaline (32 nM) and equieffective concentrations of C26 (0.3 nM) and adrenaline (63 nM) concentrations of human β2 adrenoceptor which produce the same amount of cAMP as the EC50 concentration of isoprenaline after 8-minute treatment in this assay. To account for the interassay variation in levels of cAMP which were produced in each experiment, data were normalized to the amount of cAMP produced compared with the maximum isoprenaline response.
Measuring \( \beta \)-Arrestin-2 Recruitment to the Human \( \beta_2 \) Adrenoceptor in CHO-\( \beta \)-Arrestin-\( \beta_2 \) Cells. \( \beta \)-Arrestin-2 recruitment was monitored using an enzyme-fragment complementation assay (DiscoveRx). CHO-\( \beta \)-arrestin-\( \beta_2 \) cells were seeded overnight in white, 384-well ViewPlates at 10,000 cells/well in culture medium. Spent medium was removed and replaced with assay buffer (HBSS supplemented with 10 mM HEPES, 0.1% (w/v) BSA). Cells were then stimulated with a range of concentrations of agonist for 2 hours at 37°C, after which time Flash detection reagent was added and luminescence read on the EnVision plate reader. In addition, time courses of \( \beta \)-arrestin-2 recruitment were performed, whereby a range of concentrations of each agonist was added to the cells, and Flash detection reagent was added at different times from 0 to 4 hours. To construct association curves, responses were obtained for an EC50 concentration of isoprenaline (13.5 nM) and equeeffective concentrations of C26 (0.29 nM) and adrenaline (110 nM). To account for the interassay variation in levels of luminescence produced in each experiment, data were normalized to the maximum isoprenaline response.

Measuring Human \( \beta_2 \) Adrenoceptor Internalization Using U2OS-GFP-\( \beta_2 \) Cells. U2OS-GFP-\( \beta_2 \) cells were seeded overnight in black, 384-well ViewPlates at 3000 cells/well in culture medium. Spent medium was removed and replaced with cell culture medium containing 100 mg/ml cycloheximide to inhibit protein biosynthesis and generation of new receptors inside the cell. After 4 hours, the cycloheximide-containing medium was replaced with assay buffer (CO2-independent medium supplemented with 5 mM HEPES, 0.05% (w/v) ascorbic acid, 0.05% (w/v) human serum albumin, and 100 mg/ml cycloheximide) containing 1 \( \mu \)M Hoechst to label nuclei. Cells were then stimulated with a range of concentrations of agonist for 2 hours at 37°C, after which time receptor internalization was quantified on the InCell Analyzer 2000 (GE Healthcare, Buckinghamshire, UK) using 409/447 nm (excitation/emission) settings to visualize nuclei (5-ms exposure) and 506/536 nm (excitation/emission) isothiocyanate settings for GFP (200-ms exposure). All images were collected using a Nikon 20 x 0.45 NA objective and a large-chip charge-coupled device camera (2048 x 2048 pixels; Nikon Instruments Inc., Melville, NY). In addition, time courses of receptor internalization were performed, whereby an EC50 concentration of isoprenaline, as determined in this assay at 2 hours (80 nM), and an equeeffective concentration of C26 (0.2 nM) and adrenaline (300 nM) were added to the cells, and receptor internalization was monitored on the InCell Analyzer 2000 every 5 minutes for 2 hours, using the aforementioned settings. For agonist washout studies, cells were first imaged to enable monitoring of baseline levels of internalized receptors, incubated with EC50 concentrations of agonist (described earlier) for 2 hours at 37°C, and imaged again prior to washing five times in fresh assay buffer using an automated Biomek FX (Beckman Coulter, High Wycombe, UK) and fresh tips for each washout step. Following agonist washout, the assay plate was imaged on the InCell Analyzer 2000 every 5 minutes for 4 hours, using the aforementioned settings. Cellular image analysis was performed using the InCell Analyzer Workstation 3.7.1 (GE Healthcare) to measure the presence of vesicles (1–3 \( \mu \)m) containing the GFP-tagged \( \beta_2 \) adrenoceptor. To account for the interassay variation in levels of fluorescence produced in each experiment, data were normalized to the maximum isoprenaline response.

Common Procedures Applicable to All Radioligand Binding Experiments. All radioligand experiments were conducted in 96 deep-well plates. In all cases, nonspecific binding was determined in the presence of 1 \( \mu \)M propranolol. After the indicated incubation period, bound and free radiolabels were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life Sciences) onto 96-well GBP filter plates and rapidly washed three times with ice-cold HEPES (75 mM, pH 7.4). After drying (\( \sim \)4 hours), 40 \( \mu \)l of Microscint 20 (PerkinElmer Life Sciences) was added to each well, and radioactivity was quantified using single photon counting on a TopCount microplate scintillation counter (PerkinElmer Life Sciences). Aliquots of radiolabel were also quantified to accurately determine how much radioactivity was added to each well, using liquid scintillation spectrometry on an LS 6500 scintillation counter (Beckman Coulter). In all experiments, total binding never exceeded 10% of that added, limiting complications associated with depletion of the free radioligand concentration (Carter et al., 2007).

Determination of Agonist Affinity. Affinity estimates of unlabeled agonists were determined using two slightly different assay formats depending on whether the agonist affinity (\( K_i \)) determined was to be used in competition kinetic experiments or whether it was to be used to investigate GFP sensitivity. In each case, \(^{3}H\)-DHA competition experiments were performed at equilibrium, and \(^{3}H\)-DHA was used at a concentration of approximately 600 pM (25,000 cpm final assay volume of 1.5 ml). Radioligand was incubated in the presence of the indicated concentration of unlabeled agonist and CHO-\( \beta_2 \) membranes (30 \( \mu \)g/ml) with gentle agitation for 180 minutes. \( K_i \) values determined for kinetic experiments were performed in binding buffer (HBSS supplemented with 20 mM HEPES, 0.1% (w/v) BSA, 5% (v/v) DMSO, 0.01% (w/v) ascorbic acid, and 100 \( \mu \)M GTP), and experiments were run at 37°C. \( K_i \) values determined to investigate GFP sensitivity were performed in assay buffer (20 mM HEPES, 1 mM MgCl2, 0.1% (w/v) BSA, 5% (v/v) DMSO, and 0.01% (w/v) ascorbic acid) ±30 \( \mu \)M guanosine 5’-O-(thio)triphosphate (GTPγS), and the experiments were run at room temperature.

Competition Binding Kinetics. To accurately determine association rate (\( k_{on} \)) and dissociation rate (\( k_{off} \)) values, observed association rate (\( K_{obs} \)) was calculated for at least three different concentrations of \(^{3}H\)-DHA in binding buffer. The appropriate concentration of radioligand was incubated with CHO-\( \beta_2 \) membranes (30 \( \mu \)g/ml) in binding buffer with gentle agitation (final assay volume 1000 \( \mu \)l). Exact concentrations were calculated in each experiment by liquid scintillation counting. Free radioligand was separated by rapid filtration at multiple time points to construct association kinetic curves, and the resulting data were globally fitted to the association kinetic model to derive a single best fit estimate for \( k_{on} \) and \( k_{off} \) as described previously by Sykes et al. (2009).

The kinetic parameters of C26 were assessed using a competition kinetic binding assay, as described by Sykes et al. (2009), in binding buffer. This approach involves the simultaneous addition of both radioligand and competitor to the receptor preparation, so that at t = 0, all receptors are unoccupied. Approximately 600 pM \(^{3}H\)-DHA (a concentration which avoids ligand depletion in this assay volume) was added simultaneously with the unlabeled compound (at t = 0) to CHO-\( \beta_2 \) membranes (30 \( \mu \)g/ml) in 500 \( \mu \)l of binding buffer. The degree of \(^{3}H\)-DHA bound to the receptor was assessed at multiple time points by filtration harvesting and liquid scintillation counting, as described previously. Nonspecific binding was subtracted from each time point, meaning that t = 0 was always equal to 0. Each time point was conducted on the same 96 deep-well plate incubated at 37°C with constant agitation. Reactions were considered stopped once the membranes reached the filter, and the first wash was applied within 1 second. Three different concentrations of unlabeled competitor were tested to ensure the rate parameters calculated were independent of ligand concentration. All compounds were tested at 30-, 10-, and 3-fold their respective \( K_i \) and data were globally fitted to simultaneously calculate \( k_{on} \) and \( k_{off} \).

Measuring Inhibition of Contraction of Isolated Guinea Pig Tracheal Strips. Male Dunkin-Hartley guinea pigs (350–700 g; Charles River, Margate, UK) were killed by exposure to an increasing concentration of CO2; the trachea was removed and placed in oxygenated (95% O2/5% CO2)/Krebs-Henseleit solution (118 mM NaCl, 25 mM NaHCO3, 11.1 mM glucose, 4.8 mM KCl, 205 mM CaCl2, 1.2 mM MgSO4, 1.2 mM Na2PO4, 1.2 mM KH2PO4). The method used for the electrically stimulated tracheal strip is a modification of a previously published technique (Coleman and Nials, 1989). The trachea was cut into rings of 4–5 cartilage bands in width, which were subsequently opened into strips by cutting the cartilage opposite the smooth muscle mass to create a strip with equal lengths of trachea on each side of the muscle band. Each strip was set up in a superfusion system and attached to an isometric force transducer (Fort 10; World Precision Instruments, Stevenage, Hertfordshire, UK) under a resting
tension of 1 g and superfused (1 ml/min) with oxygenated Krebs-Henseleit solution at 37°C. Following a 1-hour equilibration period, phasic contractile responses were induced by electrical stimulation with 5-second trains of square wave pulses of 10 V, 10-Hz frequency, and 0.2-ms duration every 2 minutes. After a 1-hour equilibration period using the aforementioned stimulation parameters, a baseline contractile response was determined over a 20-minute period before the superfusion fluid was changed to Krebs-Henseleit solution containing C26 for 30 minutes. After this time, the superfusion fluid was changed back to compound-free Krebs-Henseleit solution for the remaining 11.5 hours of the experiment.

The onset of action of guinea pig tracheal contraction in response to electrical stimulation for each concentration of C26 was taken as the time from the start of compound superfusion until maximum inhibition of contraction to electrical stimulation was observed. The duration of action was defined as the time taken from the end of compound superfusion to 50% recovery from maximum inhibition. The percentage of maximum inhibition at each concentration of C26 was used to construct a concentration-response curve to calculate potency.

**Data Analysis.** All experiments were analyzed by either linear or nonlinear regression using Prism 6.0 (GraphPad Software, San Diego, CA). Competition displacement binding data and agonist concentration effect curves were fitted to sigmoidal (variable slope) curves using a four-parameter logistic equation. IC50 values obtained from the inhibition curves were converted to Kᵢ values using the method of Cheng and Prusoff (1973).

To monitor the rate of cAMP accumulation, the area under the curve was fitted for the data for the initial 10 minutes of accumulation. To calculate the rate of β-arrestin-2 recruitment and GFP-β₂ internalization, data were analyzed using nonlinear regression, one-phase exponential association to determine the rate constant k. The rate half-time was then calculated as 0.69/k. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post-test (Fig. 2A; Table 1). In addition, C26, adrenaline, and isoprenaline were able to increase relative intrinsic activity compared with formoterol in a cAMP assay using A431 cell lysates (Beattie et al., 2010). We revisited this assay specifically to investigate the relative intrinsic activity of C26 (Fig. 1) with respect to the endogenous agonist adrenaline, in addition to the more stable full agonist isoprenaline, in whole cells.

Isoprenaline and adrenaline showed comparable potency and efficacy values in these cells, whereas C26 was almost 500-fold more potent in the cAMP assay. We were able to confirm that C26 does indeed have higher intrinsic activity with respect to adrenaline and isoprenaline, giving 117.8 ± 7.6% of the isoprenaline response (P = 0.034, one-way ANOVA followed by Bonferroni post-test) (Fig. 2A; Table 1). In addition, C26, adrenaline, and isoprenaline were able to recruit β-arrestin-2 and cause GFP-β₂ adrenoceptor internalization in a concentration-dependent manner (Fig. 2, B and C; Table 1). In both assays, C26 also demonstrated greater intrinsic activity compared with adrenaline and isoprenaline, with E_max values of 119 ± 6.7% (P = 0.027) and 121 ± 6.9% (P = 0.0021) of the maximal isoprenaline response (one-way ANOVA followed by Bonferroni post-test), in the β-arrestin-2 recruitment and GFP-β₂ adrenoceptor internalization assays, respectively.

To determine the kinetics of the onset of cAMP accumulation, β-arrestin-2 recruitment, and GFP-β₂ adrenoceptor internalization, cells were stimulated with concentrations of ligands that gave an equieffective response to an EC80 concentration of isoprenaline. For cAMP accumulation, there was a slight lag between compound addition and complementation of the GloSensor-luciferase enzyme; therefore, the area under the curve was calculated for the first 10 minutes of stimulation. Using this method, we determined that C26 was

\[
\text{Efficacy} = \frac{K_A}{E_{C50}}
\]

\[
\text{Efficacy} = \frac{E_{\text{max}} \times K_A}{E_{C50}}
\]

In addition, we used the following equations to calculate alternative values for agonist efficacy using experimental data from cAMP accumulation, β-arrestin-2 recruitment, and β₂ adrenoceptor internalization (Strange, 2008):

**Results**

**Functional Characterization of C26.** We previously reported that a novel 4-hydroxybenzothiazolone series of β₂ adrenoceptor agonists have both high potency and increased relative intrinsic activity compared with formoterol in a cAMP assay using A431 cell lysates (Beattie et al., 2010). We revisited this assay specifically to investigate the relative intrinsic activity of C26 (Fig. 1) with respect to the endogenous agonist adrenaline, in addition to the more stable full agonist isoprenaline, in whole cells.

Isoprenaline and adrenaline showed comparable potency and efficacy values in these cells, whereas C26 was almost 500-fold more potent in the cAMP assay. We were able to confirm that C26 does indeed have higher intrinsic activity with respect to adrenaline and isoprenaline, giving 117.8 ± 7.6% of the isoprenaline response (P = 0.034, one-way ANOVA followed by Bonferroni post-test) (Fig. 2A; Table 1). In addition, C26, adrenaline, and isoprenaline were able to recruit β-arrestin-2 and cause GFP-β₂ adrenoceptor internalization in a concentration-dependent manner (Fig. 2, B and C; Table 1). In both assays, C26 also demonstrated greater intrinsic activity compared with adrenaline and isoprenaline, with E_max values of 119 ± 6.7% (P = 0.027) and 121 ± 6.9% (P = 0.0021) of the maximal isoprenaline response (one-way ANOVA followed by Bonferroni post-test), in the β-arrestin-2 recruitment and GFP-β₂ adrenoceptor internalization assays, respectively.

To determine the kinetics of the onset of cAMP accumulation, β-arrestin-2 recruitment, and GFP-β₂ adrenoceptor internalization, cells were stimulated with concentrations of ligands that gave an equieffective response to an EC80 concentration of isoprenaline. For cAMP accumulation, there was a slight lag between compound addition and complementation of the GloSensor-luciferase enzyme; therefore, the area under the curve was calculated for the first 10 minutes of stimulation. Using this method, we determined that C26 was

\[
\text{Efficacy} = \frac{K_A}{E_{C50}}
\]

\[
\text{Efficacy} = \frac{E_{\text{max}} \times K_A}{E_{C50}}
\]
accumulation of cAMP (significantly slower than adrenaline and isoprenaline for the means Data are normalized to the 2-hour isoprenaline response and expressed as internalization in U2OS cells (C) using adrenaline, C26, and isoprenaline. Fig. 2. duplicate. Adrenaline and isoprenaline also recruited was sustained for longer than either isoprenaline or adrenaline, but resulted in greater intrinsic activity over time, we investigated the binding kinetics of these compounds to determine if dissociation rates contributed to this effect. Isoprenaline and C26 were able to produce concentration-dependent inhibition of [3H]-DHA binding in CHO-β2 cell membranes (Fig. 4, A and B). In the case of C26, a mean standard deviation of 9.81 ± 0.09 was calculated, which is consistent with the mean pKi (9.78 ± 0.14) determined from studies by Beattie et al. (2010) using [3H]-CGP12177A (4-[[1,1-Dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride) and membranes prepared from sf9 cells expressing human β2 adrenoceptor. As G proteins stabilize the high-affinity conformation of the receptor, we also investigated the effect of including GTPγS in these assays to uncouple prebound receptor–G protein complexes and reveal the low-affinity site. Using this approach, we showed that, for isoprenaline (Fig. 4A), there was a shift from a two-site curve fit in the absence of GTPγS (mean pKi values of 8.84 ± 0.45 and 6.55 ± 0.15 for high- and low-affinity sites, respectively) to a single-site fit in the presence of GTPγS (mean pKi 6.59 ± 0.11, P = 0.003). In contrast, there was no shift in the inhibition of [3H]-DHA binding by C26 when performed in the presence of GTPγS (Fig. 4B). This is similar to the data produced for the antagonist propranolol (Fig. 4C), where the pKi in the absence of GTPγS was determined to be 9.14 ± 0.30. and there was no shift in the curve in the presence of GTPγS.

**TABLE 1**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>pEC50 (n)</th>
<th>Intrinsic Activity</th>
<th>pEC50 (n)</th>
<th>Intrinsic Activity</th>
<th>pEC50 (n)</th>
<th>Intrinsic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>7.5 ± 0.13 (5)</td>
<td>91.4 ± 5.7</td>
<td>7.5 ± 0.06 (3)</td>
<td>100.9 ± 1.0</td>
<td>7.24 ± 0.14 (4)</td>
<td>95.3 ± 4.9</td>
</tr>
<tr>
<td>C26</td>
<td>10.2 ± 0.07 (5)</td>
<td>117.8 ± 7.6</td>
<td>9.76 ± 0.05 (6)</td>
<td>118.5 ± 6.7</td>
<td>9.87 ± 0.08 (4)</td>
<td>121.0 ± 6.9**</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>7.8 ± 0.06 (3)</td>
<td>102.4 ± 1.3</td>
<td>8.42 ± 0.05 (6)</td>
<td>100.5 ± 1.2</td>
<td>7.65 ± 0.06 (8)</td>
<td>99.0 ± 1.85</td>
</tr>
</tbody>
</table>

pEC50, standard abbreviation.

*p < 0.05; **p < 0.01; one-way ANOVA followed by Bonferroni’s multiple comparisons relative to adrenaline.
The kinetic parameters for C26 were then determined indirectly by monitoring how the association rate of [3H]-DHA was altered in the presence of increasing concentrations of C26 (as described in Sykes et al., 2010). Using this method (Fig. 4D), the $k_{on}$ and $k_{off}$ values were determined to be $3.70 \pm 0.47 \times 10^3$ (M$^{-1}$ min$^{-1}$) and $0.0212 \pm 0.003$ (min$^{-1}$), respectively. In addition, the time taken for half of the bound C26 molecules to dissociate from the receptor ($t_{1/2}$) was calculated to be 32.7 minutes.

**Duration of Action of C26.** As C26 displays very slow receptor kinetics, we postulated this would also lead to persistent signaling and a long duration of action. To test this hypothesis, we monitored the retention of receptors in intracellular vesicles and the relaxation effect of C26 on electrical field–induced contraction of isolated guinea pig tracheal strips following agonist washout.

Using the GFP-$\beta_2$ internalization assay, which is readily reversible, equieffective concentrations of isoprenaline, C26, and adrenaline were incubated with U2OS GFP-$\beta_2$ cells for 2 hours. After this time, the cells were washed and bathed in assay buffer while the level of $\beta_2$ internalization was monitored over time. The amount of internalized receptors produced by isoprenaline and adrenaline began to decrease around 15 minutes after agonist washout, and continued to decrease steadily until baseline levels were achieved within 2 hours post agonist washout. In contrast, even at 4 hours post agonist washout, C26 held $\sim 80\%$ of internalized receptors inside the cell, and the rate of decline of internalized vesicles was very slow (Fig. 5A). Estimated mean $t_{1/2}$ values representing the loss of GFP-$\beta_2$ vesicles from within U2OS cells were determined to be 44.2 $\pm$ 3.4, 1229 $\pm$ 289, and 40.0 $\pm$ 8.1 minutes for isoprenaline, C26, and adrenaline, respectively (span fixed to 100\%).

Following this, we assessed the duration of the relaxant effects of C26 in isolated guinea pig tracheal strips after 30-minute incubation with ligand. C26 was able to maximally inhibit the contraction of electrical field–induced contraction of isolated guinea pig tracheal strips (Fig. 5B), with a potency (pEC$50$) of 10.37 $\pm$ 0.04. The onset and duration of action of C26 were derived using the concentration closest to the pEC$50$ value—in this case, 30 pM. At this concentration, the onset of action of C26 was determined to be $5.2 \pm 0.4$ hours , and the duration of action of more than 12 hours (Fig. 5C).
In this study, we investigated the kinetics of binding and signaling of C26, a novel super agonist, at the β2 adrenoceptor. The signaling properties of C26 were explored in three different functional assays: cAMP accumulation, β-arrestin-2 recruitment, and receptor internalization. At all three levels of receptor signaling, C26 produced responses greater than the endogenous agonist adrenaline and the previously described full agonist isoprenaline, leading to its classification as a super agonist based on its intrinsic activity. In this way, C26 shares properties with other synthetic ligands that have been shown to have higher intrinsic activity than their respective endogenous ligands, such as the nonpeptide growth hormone secretagogue MK-677 (Holst et al., 2005; Bennett et al., 2009) and the thyrotropin-releasing hormone analog R-Des-TRH (Engel et al., 2006).

In addition to monitoring the intrinsic activity of C26, we also investigated its kinetics in the signaling assays. We have previously demonstrated a correlation between high-efficacy agonists and an increased rate of cAMP accumulation (Rosethorne et al., 2010), which would predict a faster onset of action for C26 than for either adrenaline or isoprenaline based on their respective endogenous ligands, such as the nonpeptide growth hormone secretagogue MK-677 (Holst et al., 2005; Bennett et al., 2009) and the thyrotropin-releasing hormone analog R-Des-TRH (Engel et al., 2006).

In this study, we investigated the kinetics of binding and signaling of C26, a novel super agonist, at the β2 adrenoceptor. The signaling properties of C26 were explored in three different functional assays: cAMP accumulation, β-arrestin-2 recruitment, and receptor internalization. At all three levels of receptor signaling, C26 produced responses greater than the endogenous agonist adrenaline and the previously described full agonist isoprenaline, leading to its classification as a super agonist based on its intrinsic activity. In this way, C26 shares properties with other synthetic ligands that have been shown to have higher intrinsic activity than their respective endogenous ligands, such as the nonpeptide growth hormone secretagogue MK-677 (Holst et al., 2005; Bennett et al., 2009) and the thyrotropin-releasing hormone analog R-Des-TRH (Engel et al., 2006).

In addition to monitoring the intrinsic activity of C26, we also investigated its kinetics in the signaling assays. We have previously demonstrated a correlation between high-efficacy agonists and an increased rate of cAMP accumulation (Rosethorne et al., 2010), which would predict a faster onset of action for C26 than for either adrenaline or isoprenaline based on their relative intrinsic activities. However, we found the opposite was the case, such that C26 produced the slowest rate of cAMP accumulation, β-arrestin-2 recruitment, and receptor internalization. Maximal levels of cAMP accumulation were achieved 2–3 minutes after adrenaline and isoprenaline reached peak cAMP accumulation, and maximal levels of C26-mediated β-arrestin-2 recruitment and receptor internalization were only achieved after at least 4-hour treatment as opposed to 2 hours with adrenaline and isoprenaline. Using kinetic radioligand binding assays, we demonstrated that C26 has a half-life of 32.7 minutes at 37°C. Compared with isoprenaline and adrenaline (0.23 and 0.14 minute, respectively), this represents the slowest dissociation rate we have observed for any β2 adrenoceptor agonist tested to date (Sykes and Charlton, 2012). As C26 demonstrates slow dissociation from the β2 adrenoceptor, it is likely that this compound will also be slow to reach equilibrium, thus delaying its onset of action. This is supported by the steepness of the Hill slope for C26, which often occurs under nonequilibrium conditions. We have repeated these experiments in 10-fold the assay volume (data not shown) to eliminate the possibility that the steep slope is due to ligand depletion at the lower concentrations of C26 (Carter et al., 2007). The slow binding kinetics of C26 translated into a slow onset and long duration of action, with C26 inhibition of electrically stimulated contraction of guinea pig tracheal strips being sustained for at least 12 hours after agonist washout. We also observed that, in the absence of a phosphodiesterase inhibitor, the cAMP generated by C26 was sustained for longer than that generated by either adrenaline or isoprenaline, which may suggest that C26 continues to promote cAMP generation over longer periods. The slow kinetic binding and signaling appear to be properties unique to C26, as other high-affinity, long-acting β2 agonists have been shown to rapidly dissociate from the receptor (Sykes and Charlton, 2012). It is believed that, for many of the LABAs, such as salmeterol, the high lipophilicity
of these compounds contributes to their long duration of action (Anderson et al., 1994). In the case of C26, it is likely that its slow dissociation from the receptor provides a significant contribution, as it has a longer duration of action than would be predicted from its lipophilicity (calculated log P of C26 52.42 ± 6.54; salmeterol 5.39 ± 0.49; from ALOGPS v2.1, http://www.vcclab.org) (Tetko et al., 2005), although we cannot rule out a contribution of tissue retention. The persistent internalization of GPR-β2 receptors caused by C26 even after agonist washout provides another potential factor that could contribute to the long duration of action of C26. It has previously been thought that signal transduction mediated by GPCR is confined to the plasma membrane, and once receptors are phosphorylated and internalized, these processes are switched off. However, recent evidence suggests that internalized receptors are capable of forming an active ternary complex by accessing effectors from subcellular compartments, which contributes to the total cellular signaling. This has been demonstrated for a range of receptors, including the parathyroid- and thyroid-stimulating hormone receptors, as well as the S1P1 and β2 adrenoceptor (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009; Irannajad et al., 2013). Indeed, slow dissociation kinetics have been linked to the ability of FTY720 (2-amino-2-[2-(4-octylphenyl) ethyl]propane-1,3-diol) to cause persistent internalization of the S1P1 receptor (Sykes et al., 2014). As such, the prolonged receptor internalization caused by C26 may provide an additional mechanism of long duration of action at the β2 adrenoceptor, which may be further exploited by targeting drugs to intracellular compartments, e.g., by incorporating physiochemical properties that facilitate ion trapping (Goldman et al., 2009). The continued signaling of certain agonists from internalized compartments may contribute to the overall magnitude of intracellular signaling as well as the duration of responses, which may underlie signaling bias by directing receptors to alternative pathways/compartments or degradation. C26 may therefore be a useful tool to study this phenomenon in more detail.

A number of different approaches have been used to quantify agonist efficacy, ranging from simple calculations, such as $K_A/EC_{50}$ or $E_{max} \times K_A/EC_{50}$ (Strange, 2008), to more thermodynamically complete systems, such as the cubic ternary complex model (Weiss et al., 1996). However, it is more common to fit data with empirical mathematical models such as the operational model of agonism to quantify agonist efficacy (Black and Leff, 1983). This model has been used to describe super agonists that are more efficient at transducing receptor binding events into a cellular response, such as iperoxo, which demonstrates supraphysiologic efficacy compared with acetylcholine at the muscarinic M2 acetylcholine receptor (Schrage et al., 2013). These models are of little use for C26, as both the $K_A/EC_{50}$ and $E_{max} \times K_A/EC_{50}$ calculations predict a lower efficacy for this compound than either adrenaline or isoprenaline, despite its higher intrinsic activity (Table 2). The operational model also struggles to correctly fit the data we have obtained with C26, either overestimating the maximal response of adrenaline and isoprenaline relative to C26, or overestimating the potency based on the affinity entered into the equation. This may be because the affinity measured in this study is not an accurate representation of the “functional affinity” at a specific pathway, due to effector-dependent changes in receptor conformation (Yan et al., 2008; Kenakin and Christopoulos, 2013). Although efforts have been made to measure this using receptor-effector fusions (Rasmussen et al., 2011), it remains very difficult to accurately measure the functional affinity of high full (or super) agonists.

Our data indicate that C26 was insensitive to guanine nucleotides, suggesting it either does not stabilize many receptors in the classic high-affinity state, or it has equal affinity for the high- and low-affinity states of the receptor, as has been reported previously for a number of different high-affinity GPCR agonists (Childers et al., 1993; Vanhauwe et al., 1999; Roberts et al., 2004; Schneider et al., 2009). One explanation for this is that ligands that do not affect the overall affinity of the receptor for the G protein, but rather accelerate the binding and dissociation of guanylnucleotides, will be functionally active but unable to discriminate two receptor states in binding studies (Waebroeck et al., 1997). This can be likened to the allosteric ternary complex model, where the G protein is defined as an allosteric modulator of ligand-receptor interactions (Leach et al., 2007). Using this model, C26 yields an α value of 1.5 ($r = K_Low/K_{High}$) (KLow being low affinity and KHigh being high affinity data).

![Fig. 6. Relative intrinsic activities of C26 and adrenaline for cAMP accumulation after incubation with EC_{50} concentrations of each agonist. Data are normalized to the maximal adrenaline response (8 minutes) and expressed as means ± S.E.M. for four independent experiments, run in duplicate. Statistical significance was determined using an unpaired t test ($P < 0.05$) at 3 versus 10 minutes.](image-url)

**Table 2**

<table>
<thead>
<tr>
<th>Adrenaline</th>
<th>C26</th>
<th>Isoprenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_A/EC_{50}$</td>
<td>44.7</td>
<td>1.05</td>
</tr>
<tr>
<td>$E_{max} K_A/EC_{50}$</td>
<td>4083</td>
<td>123</td>
</tr>
</tbody>
</table>

Efficacy calculations for adrenaline, C26, and isoprenaline.
respectively); data taken from Fig. 4B), which indicates that the presence of the G protein on the receptor had no effect on the affinity of C26 ($K_{\text{d}}^\text{low} \approx K_{\text{d}}^\text{high}$) (Christopoulos and Kenakin, 2002). In contrast, the presence of the G protein had a positive allosteric effect on the binding of isoprenaline to the $\beta_2$ adrenoceptor, resulting in $\alpha = 194$ ($K_{\text{d}}^\text{low} > K_{\text{d}}^\text{high}$; data taken from Fig. 4A). Despite having an $\alpha$ value close to 1, C26 has higher intrinsic activity than isoprenaline, suggesting its effect is to enhance the ability of the receptor to activate the effecter ($\beta > 1$)—for example, the ability to accelerate GDP-GTP exchange (Waelbroeck et al., 1997; Sykes et al., 2009).

In conclusion, we have fully characterized C26, a kinetically driven super agonist at the human $\beta_2$ adrenoceptor that displays higher intrinsic activity than either the endogenous agonist adrenaline or the full agonist isoprenaline. We have demonstrated that this compound displays extremely slow receptor dissociation kinetics for an agonist, and propose that this contributes to the increased intrinsic activity of C26 over time. This can most easily be visualized by observing the relative intrinsic activity of an EC80 concentration (determined after 8 minutes) of each agonist over time. In this model, C26 has a lower intrinsic activity at the early time points where it appears partial relative to adrenaline, versus the later time point where it has greater intrinsic activity than adrenaline (Fig. 6). This highlights the need to consider the temporal aspects of agonist binding and signaling when characterizing ligands as super agonists.

Authorship Contributions

**Participated in research design:** Rosethorne, Bradley, Sykes, Renard, Trifillief, Fairfax, Carlarton.

**Conducted experiments:** Rosethorne, Bradley, Gherbi, Sykes, Sattikar, Wright, Renard.

**Performed data analysis:** Rosethorne, Bradley, Gherbi, Sykes, Sattikar, Trifillief.

**Wrote or contributed to the writing of the manuscript:** Rosethorne, Bradley, Gherbi, Sykes, Fairfax, Carlarton.

References


Calebiro D, Nikolaevo VG, Gagliani MC, de Filippis T, Seelos A, Tichecht C, Persani L, Renard, Trifillief, Fairhurst, Charlton.

Conducted experiments: Rosethorne, Bradley, Gherbi, Sykes, Sattikar, Wright, Renard.

Performed data analysis: Rosethorne, Bradley, Gherbi, Sykes, Sattikar, Trifillief.

Wrote or contributed to the writing of the manuscript: Rosethorne, Bradley, Gherbi, Sykes, Fairfax, Carlarton.

Address correspondence to: Steven J. Charlton, School of Life Sciences, Queen’s Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK. E-mail: Steven.Charlton@nottingham.ac.uk