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Adventures with the β_2-adrenoceptor Receptor: A Career Long Interest in Agonising One of the Most Widely Studied GPCRs

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Abstract: Drug discovery is a multi-disciplinary effort in which groups with expertise in a range of areas combine in a unified way to achieve a common goal: to deliver a clinical candidate to evaluate a hypothesis for improving human health. As a medicinal chemist this environment has provided multiple opportunities to be involved in cross-discipline interactions that have been both rewarding and led to outcomes that would not have been possible without an intimate interdisciplinary curiosity. Within this article I aim to share some of my experiences with the β_2-adrenoceptor that have fostered such synergistic relationships with several disciplines, but in particular with *in vitro* pharmacologists looking at different ways to stimulate this G protein-coupled receptor (GPCR). This interest now spans over a quarter of a century and has been intertwined with the delivery of three clinical candidates.

Keywords: β_2-Adrenoceptor agonist · Asthma · COPD · Intrinsic efficacy

Robin A. Fairhurst obtained his PhD in synthetic organic chemistry under the supervision of Prof. Harry Heaney at the University of Loughborough (UK) before postdoctoral studies with Prof. Philip Magnus at the University of Texas at Austin (USA). He began his industrial career in 1995 with Ciba Central Research Laboratories in Macclesfield (UK). Following the merger between Ciba and Sandoz he joined the Novartis Respiratory Disease Area in Horsham (UK), contributing to the discovery of inhaled treatments for reversible airway diseases. In 2007 he moved to the Novartis Oncology Disease area in Basel (CH) where he has worked on several kinase inhibitor projects and more recently on the transcription factor HIF2α. During this time, he has led and contributed to nine projects that have delivered clinical candidates including indacaterol, alpelisib and roblitinib and been a recipient of the Novartis Leading Science award and now the recipient of the SCS Senior Industrial Science Award.

1. Introduction

GPCRs represent a super family of structurally related cell surface receptors which historically have been highly successful targets for the pharmaceutical industry.[1] The β_2-adrenoceptor has been at the forefront of this success, having been the first GPCR to be cloned and expressed and to have a ligand-bound X-ray crystal structure solved.[2,3] As a therapeutic target, agonists of the β_2-adrenoceptor have been widely used as bronchodilators for the treatment of the reversible airway diseases asthma and chronic obstructive airways disease (COPD).[4] A treatment option that has evolved greatly over the last century since the nonselective endogenous adrenergic-agonist adrenaline was isolated from the adrenal glands of livestock and administered intravenously to patients to control acute asthma exacerbations.[5]

2. Indacaterol as a Once-daily Inhaled β_2-adrenoceptor Agonist

Our first foray into the β_2-adrenoceptor agonist field was the recognition that a once-daily inhaled agent with an optimised profile had the potential to provide more effective and convenient treatment options for the reversible airway diseases asthma and COPD. The hypothesis being that such a dosing regimen would lead to increased patient compliance and as a result improved disease control. Such agents have gone on to define a third generation of inhaled ultra-long-acting β_2-adrenoceptor agonists which are currently used either, as single agents, or in combination with other classes of inhaled therapies.[6] The first to be approved for clinical use was *indacaterol*, developed from the first Novartis inhaled β_2-adrenoceptor agonist project, and which has gone on to be joined by *olodaterol* and *vilanterol* as the other third generation agents in this class.[4,7] Fig. 1 highlights the evolution of inhaled β_2-adrenoceptor agonist treatments for the treatment of reversible airway diseases.

The first generation of inhaled β_2-adrenoceptor agonists, also termed short-acting β_2-adrenoceptor agonists, are characterised by *isoprenaline* and *salbutamol* which exhibit improved profiles compared to *adrenaline*. *Isoprenaline* has greater selectivity for β- versus α-adrenoceptors and is a high efficacy agonist that has been used to benchmark β-adrenoceptor intrinsic efficacy (the relative ability of a drug-receptor complex to produce a maximum functional response). *Salbutamol* shows further selectivity for β_2-adrenoceptors over the other family members, and as a result a greatly reduced level of β_1-adrenoceptor mediated cardiovascular side effects. Although a lower intrinsic efficacy agonist of the β_2-adrenoceptor, *salbutamol* has been shown to be an effective bronchodilator in the majority of patients. Both *isoprenaline* and *salbutamol* have rapid onsets of action and relatively short durations of action, providing 3 to 6 hours of bronchodilation when administered by inhalation to patients at their clinically approved

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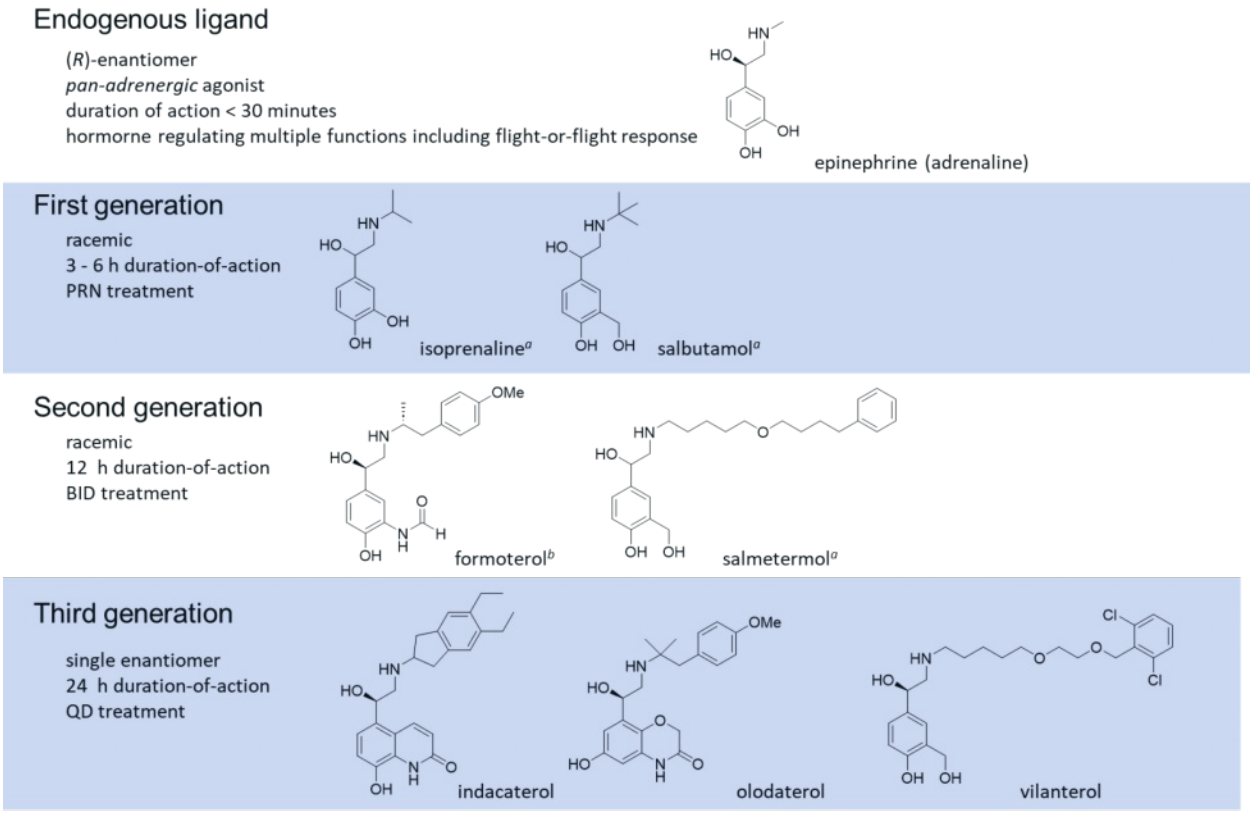


Fig. 1. Evolution of inhaled β_2-adrenoceptor agonists for the treatment of reversible airway diseases. [a]racemic mixture. [b]racemic mixture of the *l*-diastereoisomer.

doses. As a result, these agents are still in use today as 'rescue medications' on an as needed (*pro re nata*, PRN) basis to alleviate the intermittent symptoms of bronchoconstriction.

The second generation of β_2-adrenoceptor agonists, also termed long-acting β_2-adrenoceptor agonists, are characterised by *formoterol* and *salmeterol* which exhibit longer durations of action when compared to first-generation treatments. These agents were developed to overcome nocturnal symptoms and enabled patients to experience uninterrupted sleep and deliver durations of action in the region of 12 hours following a single dose. The main difference between *formoterol* and *salmeterol* being their onsets of action following inhaled delivery. *Salmeterol* exhibits a much longer onset of action of 10 to 20 minutes to achieve a perceived benefit with maximal bronchodilation taking > 1 hour. In contrast *formoterol* exhibits a rapid onset of action within 5 minutes of dosing. Both compounds are typically used in more severe patient populations with persistent airway obstruction as chronic maintenance-treatments administered twice daily (*bis in die*, BID). This maintenance setting also supported the inclusion of *formoterol* and *salmeterol* into fixed-combination products with inhaled corticosteroids that resulted in further improvements in patient compliance and outcomes.[8] Additionally, the faster onset of action has allowed *formoterol* to fill a dual role as both a maintenance therapy and as a rescue medication.[9]

To create the next (third) generation of inhaled β_2-adrenoceptor agonists a project was started in 1998 within the Novartis Respiratory Disease Therapeutic Area that ultimately led to *indacaterol*. The goal of the project being to identify an agent with what was considered to be the optimal Target Product Profile (TPP): a duration of action suitable for once-daily dosing (*quaque die*, QD), as the most convenient dosing regimen to maximise compliance; a rapid onset of action (< 5 min) to associate inhalation with relief as a way to further improve compliance and offer the option to function as both a rescue and as a maintenance treatment; medium potency, to provide a daily dose in the optimal range for inhaled formulation *via* a dry-powder delivery device (> 50 µg), to support the use as both a single-agent and in fixed-dose combination products; well tolerated, with minimal systemic β_2-adrenoceptor activation at the given dose; a single stereoisomer to meet the state-of-the-art requirements for a drug substance.[10] Molecules with such profiles now make up the third generation of ultra-long-acting inhaled β_2-adrenoceptor agonists treatments.

One unusual feature of the TPP compared to the vast majority of drug discovery projects was to include an upper limit to the potency. Multiple factors need to be optimized to arrive at a viable drug candidate and in almost every instance higher potency would be a desirable attribute to incorporate. However, in the case of inhaled delivery dose-to-dose variability needs to remain within a defined range, and when the delivered dose falls below 50 µg the technical challenge to achieve the required level of reproducibility increases.[11] Additionally, low doses lead to a high level of dilution in the fixed amount of excipient (typically lactose) delivered by a dry powder inhalation device. Consistent distribution of the small percentage of drug within the excipient and the long-term stability of the dilute formulations both further add to the technical challenge. Therefore, a lower dose limit of 50 µg was targeted to deliver a robust inhalation product that could also be readily co-formulated with other inhaled drugs.

At the outset we reviewed the literature and envisaged that controlling the lipophilicity of the compounds prepared within the project would be key to achieving the duration and onset of action targeted for the above TPP. An inhaled β_2-adrenoceptor agonist with the optimal level of lipophilicity was anticipated to partition into the phospholipid bilayer within the lung to provide a reservoir of compound. As clearance mechanisms slowly deplete this reservoir over time, sufficient drug would remain available locally within the lung to maintain an efficacious concentration for the desired 24 hour duration of effect. This rationale for lung retention has been termed the diffusion microkinetic theory.[12] The presence of the

secondary amine within the β_2-adrenoceptor agonist pharmacophore was anticipated to be a key component driving the lung retention through a charged interaction with the phospholipid phosphate head group. However, a ceiling to the level of lipophilicity was also envisioned above which potency and onset of action would be negatively impacted. Too high a level of phospholipid partitioning would limit the 'free-drug' available to drive efficacy and slow the rate of distribution throughout the lung slowing the onset of action. As a result, chemical series were sought that allowed homologation in a region of the molecule that would have minimal impact on the β_2-adrenoceptor activity and enable the systematic modulation of lipophilicity. From established SAR the ethanolamine *N*-substituent of the β_2-adrenoceptor pharmacophore was anticipated to be the ideal site for modulating these properties. Fig. 2 highlights the ethanolamine *N*-substituent as one of the three structural elements of the β_2-adrenoceptor agonist pharmacophore.

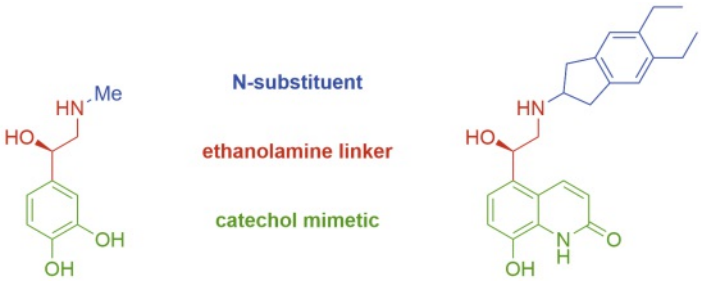


Fig. 2. Structural elements of the β_2-adrenoceptor agonist pharmacophore highlighted for adrenaline and indacaterol.

In addition, a rationale analysis of other factors controlling onset of action was made based upon receptor theory.[13] From this analysis higher intrinsic efficacy agonists were considered to be best suited to deliver faster onsets of action due to a lower level of receptor occupancy being required to induce an equivalent level of pathway stimulation. Essentially, greater efficiency in activating the β_2-adrenoceptor was anticipated to facilitate a faster response and also enable the response to be maintained for longer. In the case of bronchodilation, the downstream pathway stimulation that was typically measured for β_2-adrenoceptor activation was cyclic adenosine monophosphate (cAMP) production.

Two of the series that were explored based upon the above criteria are represented by the general structures **1** and **2** in which the R groups within the *N*-substituent provided the sites for modification to regulate the profile of the molecules, (Fig. 3). For series **1**, the 2-*N*-formylphenol catechol mimetic was selected based upon the high intrinsic-efficacy agonist *formoterol*.[14] Optimisation of R^1 resulted in the 4-phenylbutyl analogue QAC455 being identified as the analogue with the best balance of β_2-adrenoceptor potency, onset, and duration of action. The duration of action being 5-fold longer than measured for *formoterol* at an equally effective dose level (EC_{80}) using a super-fused guinea-pig tracheal strip *in vitro* model, a system widely used for the characterisation of inhaled β_2-adrenoceptor agonists.[15] The octanol partitioning of QAC455 was determined to be 93-fold higher compared to *formoterol* based upon the measured $logD_{7.4}$ values of 2.76 and 0.79 respectively. Overall, the data obtained with series **1** were consistent with the diffusion microkinetic theory in that higher lipophilicity was associated with longer durations of action.

One approach that was used routinely within the project to compliment calculated logP and logD values was the measurement of phospholipid partitioning using immobilised artificial membrane chromatography (IAM-HPLC).[16] This technique provided a ranking of compounds based upon their elution rates with a phosphatidylcholine monolayer as the stationary phase with the data expressed as chromatographic hydrophobicity index (CHI_{IAM}) values. Both the throughput of this method and the use of a phospholipid partitioning phase made CHI_{IAM} the preferred readout. $LogD_{7.4}$ values were only measured for selected examples and showed a good correlation with CHI_{IAM}. Similarly, we also used a human serum albumin (HSA) stationary phase to routinely rank compounds with respect to plasma protein binding (PPB), to understand how this parameter impacted upon the profile of the compounds as discussed below.[17] Table 1 shows some representative CHI_{IAM} and HSA-binding data.

Table 1. CHI_{IAM} and HSA-binding data compared to clogP values for indacaterol and reference inhaled β_2-adrenoceptor agonists.

Compound	CHI_{IAM} pH 7.4	clogP	%HSA
salbutamol	21.0	0.06	29.9
formoterol	40.4	1.26	31.7
salmeterol	56.7	3.06	91.1
carmoterol	37.7	1.31	57.1
indacaterol	59.7	2.97	95.7
QAC455	>70	4.05	–
2a	549	1.91	89.8
2b	>70	4.02	98.6

For series **2**, the 8-hydroxyquinolinone catechol mimetic was selected based upon the high intrinsic-efficacy agonist *carmoterol*, Fig. 3.[18] Substitution with the two R^2 groups in the 5- and 6-positions of the indane moiety was found to be optimal for β_2-adrenoceptor agonist activity and the symmetrical substitution also avoided an increased level of stereochemical complexity. Optimisation of R^2 resulted in the diethyl analogue, *indacaterol*, being identified at an early stage of the project as having the targeted profile based upon β_2-adrenoceptor potency, onset, and duration of action. The duration of action of *indacaterol* was determined to be > 10-fold longer than measured for *formoterol* at an equally effective dose level (IC_{80}) using the super-fused guinea-pig tracheal strip *in vitro* model. The profile for the more lipophilic dibutyl homologue of **2a** (R^2 = *n*Bu, clogP 4.0) was once more in line with our analysis based upon the diffusion microkinetic theory. Compound **2a** exhibited a slightly reduced level of potency compared

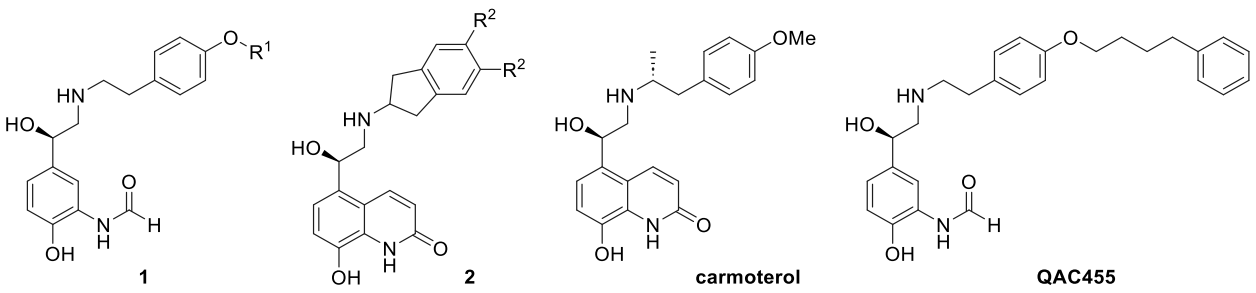


Fig. 3. Structures of the 2-*N*-formylphenol **1** and 8-hydroxyquinoline **2** series, carmoterol and QAC455.

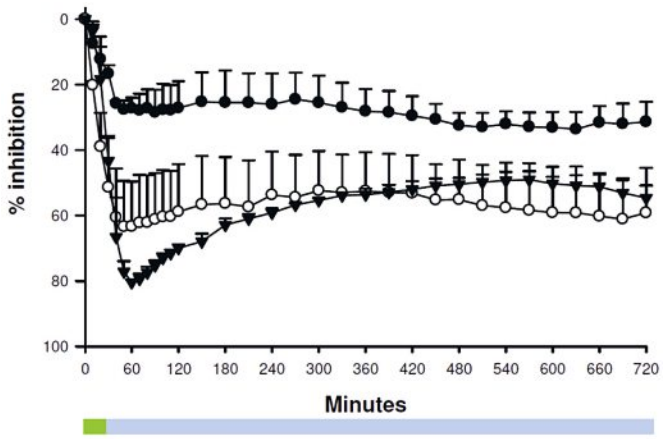


Fig. 4. Effect of **2a** on the inhibition of the electrically stimulated contraction of guinea pig tracheal strip. The preparations were superfused for the first 30 min (green bar) with **2a** at concentrations of ● 10 nM, ○ 30 nM, ▼ 100 nM followed by buffer for the following 690 min washout phase (blue bar). Onset of action is the time at which the maximal response was achieved, and half-lives were determined from the time required for the percentage inhibition to decay to 50% of the maximal response during the washout phase for the lowest concentration with > 80% maximal inhibition. Data are expressed as mean ± standard error of the mean. Reprinted with permission from *J. Med. Chem*. **2010**, *53*, 3675. Copyright 2010 American Chemical Society.

to *indacaterol* and an extended onset of action based upon the maximum inhibition being achieved 25 minutes after the end of the 30-minute drug infusion period, (Fig. 4). The lower lipophilicity of the dimethyl analogue **2b** (R^2 = Me, clogP 1.9) resulted in a duration of action of 1.4 hours in the same *in vitro* system with the anticipated rapid onset of action.

Although intrinsic efficacy and lipophilicity provided useful criteria for identifying several interesting molecules from the initial *in vitro* testing, the *in vivo* models provided readouts of how these molecules fulfilled all aspects of the TPP. Throughout all of these assays and models *indacaterol* was consistently equivalent or superior to the other β_2-adrenoceptor agonists tested. In a methacholine-induced bronchoconstriction model in the rhesus monkey with test compounds dosed at an equally effective dose level (80% of maximal bronchoprotection) *indacaterol* exhibited a longer duration of effect and improved therapeutic index when compared to the reference β_2-adrenoceptor agonists *salbutamol*, *salmeterol* and *formoterol*.[19] This rhesus monkey model proved to be a key discriminator for late-stage optimisation and in particular to identify compounds with the potential for improved therapeutic indices. The therapeutic index being assessed against increased heart rate, a readout that was anticipated to be driven by systemic β_2-adrenoceptor activation and one of the effects considered to be dose limiting in the clinical setting.[20] Selected data for *indacaterol* and the reference β_2-adrenoceptor agonists are included in Table 2.

Although targeting a defined range of β_2-adrenoceptor agonist potency and intrinsic efficacy in combination with a defined level of lipophilicity had been found to consistently deliver compounds with interesting *in vitro* profiles, some compounds were clearly superior in terms of their intrinsic durations of action and systemic β_2-adrenoceptor safety profiles when tested in the rhesus monkey model. Analyses of these data suggested that more than just the three above parameters of lipophilicity, potency and intrinsic efficacy needed to be considered to rationalise these observations and this resulted in further hypotheses being explored.

The pharmacokinetic profile of a compound clearly had a role to play in the level of systemic exposure and hence the duration and extent to which systemic β_2-adrenoceptors could be stimulated to define the therapeutic index. Two routes to the systemic circulation were envisioned following inhaled delivery. Firstly, the slow redistribution of the inhaled portion of the drug from the phospholipid reservoir within the lung directly into the systemic circulation is an unavoidable contribution. Secondly, even with state-of-the-art inhalation devices and good inhalation technique a large proportion of an inhaled dose is swallowed (up to 90%).[21] The swallowed portion of the dose then has the possibility to be absorbed through the gastrointestinal tract and enter the systemic circulation, effectively following the oral route of administration. The property space explored above to fulfil the TPP was anticipated to be associated with good membrane permeability and a high likelihood for a high level of absorption of the swallowed component. Two elements were seen to be important to managing the impact of the drug entering the systemic circulation. Firstly, higher protein binding would reduce the free-drug levels and flatten-out the response to any initial spike in systemic β_2-adrenoceptor activation directly after dosing. Secondly, metabolism to β_2-adrenoceptor-inactive metabolites was envisioned to be a way that systemic β_2-adrenoceptor activation could be minimised. In particular, the phenolic groups present in the β_2-adrenoceptor agonist pharmacophore had been shown to be a site for phase II metabolism (glucuronidation / sulfation), and the resulting conjugates were anticipated to be effectively inactive on the β_2-adrenoceptor.[22] In the case of *indacaterol* the glucuronide was the pri-

Table 2. *In vitro* β_2-adrenoceptor activity and physical chemistry data for indacaterol and reference inhaled β_2-adrenoceptor agonists. [a]Affinity for the human β_2-adrenoceptor was assessed in a radioligand binding assay.[18] [b]Elevation of cAMP measured in A431 cells endogenously expressing the β_2-adrenoceptor, intrinsic efficacy is expressed relative to the maximum response obtained with formoterol.[18] [c]Super-fused guinea-pig tracheal strip assay.[19] [d]pK_a values were determined by UV titration.[18] [e]Apparent permeability coefficients (Papp) were calculated from the apical to basolateral rate of flux across Caco-2 membranes.[18] [f]Intrinsic clearance (CL_{int}) was determined with rat liver microsomes incubated with the test compounds and UDP-glucuronic cofactors to assess their susceptibility towards glucuronidation.[18]

Compound	β_2 Ki (nM)[a]	β_2 EC_{50} (nM) / intrinsic efficacy (%)[b]	Tracheal strip IC_{50} (nM) / OoA (min) / DoA (h)[c]	pKa amine / phenol[d]	Papp A-B ($x10^{-6}$ cm/s)[e]	UGT CL_{int} (µL/min/mg)[f]
salbutamol	1,828	68 / 45	17 / 28 / 0.9	10.1 / 9.4	1.9	-
formoterol	23	1.3 / 100	0.40 / 28 / 1.2	9.1 / 8.1	1.3	214
salmeterol	0.39	0.32 / 30	3.5 / 120 / > 12	9.9 / 9.0	1.8	91
carmoterol	3.2	0.76 / 95	0.30 / 28 / 1.6	7.3 / 8.6	-	-
indacaterol	76	11 / 75	7.9 / 35 / > 12	6.7 / 8.3	9.5	378
QAC455	9.2	-	71 / 28 / 6.6	10.2 / 8.4	7.0	228
2a	119	14 / 74	20 / 55 / > 12	-	2.9	342
2b	522	39 / 74	48 / 42 / 1.4	-	1.8	389

mary metabolite formed which exhibited > 100-fold lower affinity for the β_2-adrenoceptor.[18] Consistent with this notion for limiting the systemic side effects of *indacaterol* exhibited a higher rate of glucuronidation and higher PPB when compared to the other long-acting β_2-adrenoceptor agonists of interest, (Table 2).

Based upon the above preclinical profile *indacaterol* entered early development in 2000 as the hemi-maleate salt and clinical studies revealed that the molecule fulfilled all aspects of the TPP. Clinical development progressed to a first health authority approval in 2009 as a once-daily maintenance treatment for COPD.[7] *Indacaterol* is now approved for use in > 100 countries worldwide for the treatment of reversible airway diseases and marketed as: a single agent as Onbrez® Breezhaler® and Arcapta® Neohaler®; as part of a fixed dose dual combination product with glycopyrronium bromide as Ultibro® Breezhaler® and Xoterna® Breezhaler®; as part of a fixed dose dual combination product (acetate salt) with mometasone furoate as Atectura® Breezhaler® and Utibron® Neohaler®; as part of a fixed dose triple combination product (acetate salt) with glycopyrronium bromide and mometasone furoate as Enerzair® Breezhaler® and Zimbus® Breezhaler®. The daily dose of *indacaterol* in these products ranges from 55 to 300 µg. From a drug discovery perspective, the experience from the discovery of *indacaterol* and the molecules' successful development provided us with both a scientific grounding in the area and the impetus to build upon this expertise moving forwards.

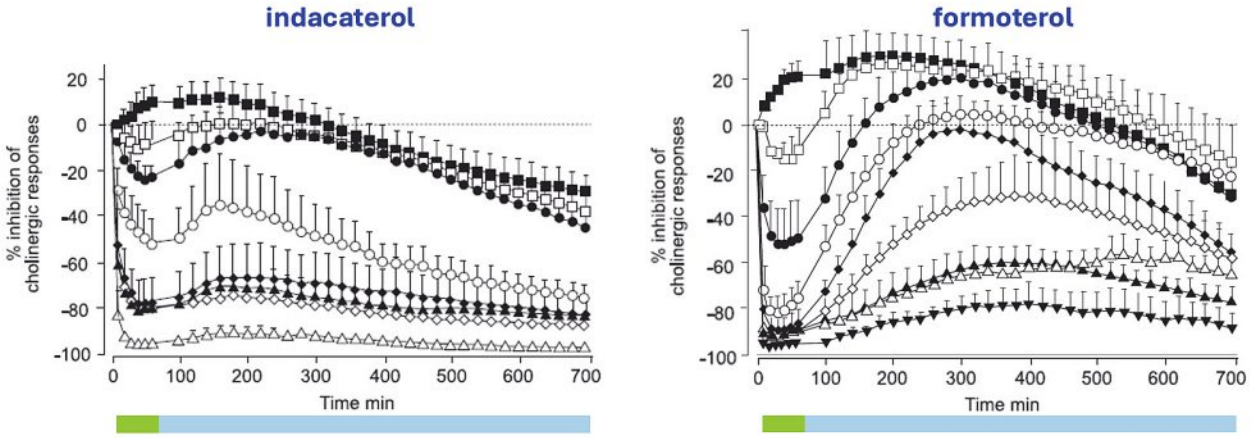
3. Securing a Lead Position Following the Identification of *Indacaterol*

Having identified and advanced the molecule that was to go on to become *indacaterol* into development, two new questions arose within the project: firstly, could we identify a backup molecule, to increase our overall chance of success towards delivering an approved β_2-adrenoceptor agonist for the treatment of reversible airways diseases; secondly, could we understand what made *indacaterol* successful as a once-daily inhaled candidate molecule at the molecular level, to better understand how it differentiated from other compounds in the same class, and as a result be in a stronger position to design future inhaled therapies. How we tackled these questions is covered in the following sections and little did we know at this point as to how long and interesting this journey would become.

3.1 *Exploring How Inhaled β_2-adrenoceptor Agonists Work at the Molecular Level*

The extended period required to undertake a drug discovery effort in combination with the interdisciplinary nature of the teams conducting the work inevitably cultivates the evolution of interesting hypotheses to explain the observed findings. Some of these hypotheses stand the test of time and have the potential to enlighten biomedical research, but all too often the pragmatic nature of drug discovery means only the immediate and obviously enabling ones are investigated. One benefit of a molecule advancing into development was the extended opportunity to support and learn more about the molecule going forwards. In the case of *indacaterol* the targeted benefits were considered to be twofold, as mentioned above: to highlight the treatments benefits and to enable future drug discovery efforts. A collection of the most interesting areas we explored are discussed below in this section.

On entering early preclinical development we looked for ways to build confidence that *indacaterol* would have the targeted profile in man before the start of the clinical trials. To support this, two studies were conducted using resected human tissue with the groups of Molimard (isolated human bronchi) and Sturton (precision-cut lung slices). The aim of these studies was to characterise the onset and duration of action profile of *indacaterol versus* the twice-daily β_2-adrenoceptor agonists *fomoterol* and *salmeterol*. The isolated human bronchi organ-bath model provides a way to assess the relaxant effect of β_2-adrenoceptor agonists with intact human tissue. In this model *indacaterol* behaved as a fast onset of action and high efficacy agonist with a long duration of effect that was longer than determined for *formoterol* and comparable to *salmeterol*, (Fig. 5).[23] The human precision-cut lung slice model provided a way to assess the bronchodilator effect of β_2-adrenoceptor agonists on small airways. In this model *indacaterol* showed an intrinsic efficacy comparable to *formoterol*, an onset of action that was comparable to *formoterol* and faster than *salmeterol*, and a longer duration of action than both *formoterol* and *salmeterol*.[24] The data from these human *ex vivo* studies helped



Compound	IC_{50} (nM)	DoA conc (nM)	DoA (h)
indacaterol	110	100	> 12
formoterol	1.1	1	0.59

Fig. 5. Effect of indacaterol and formoterol on the electrical field–induced contraction of human bronchi: compounds were added to the bath for the first 60 min (green bar) followed by buffer over the 11 h washout period (blue bar); traces are for ■ control; indacaterol at □ 10 nM, ● 30 nM, ○ 100 nM, ♦ 300 nM, ◊ 1 µM, ▲ 3 µM, Δ 10 µM; formoterol at □ 0.3 nM, ● 1 nM, ○ 3 nM, ♦ 10 nM, ◊ 30 nM, ▲ 100 nM, Δ 300 nM, ▼ 1 µM; duration of action was calculated from the concentration closest to the IC_{50} as the time taken to reach 50% of the maximal response. Data are expressed as mean ± standard error of the mean. Reproduced with permission of the © ERS 2024: *Eur. Resp. J*. **2007**, *29* 575; https://doi.org/10.1183/09031936.00032806 Published 28 February 2007.

to bridge our understanding and provided increased confidence that *indacaterol* would deliver on the TPP in the clinical setting.

At the outset of the project, we had analysed the available in-house and external data and hypothesised that higher intrinsic efficacy would be beneficial to deliver a candidate fulfilling the TPP, in particular the rapid onset-of-action component. To support this notion, we went on to perform a study with reported β_2-adrenoceptor agonists of varying intrinsic efficacies including *indacaterol*.[25] The rate of cAMP accumulation was measured in primary human bronchial smooth muscle cells and in a second HEK 293 cell line with endogenous β_2-adrenoceptor expression. In both cell lines we were able to show that the rate of cAMP accumulation correlated well with the intrinsic efficacies of the β_2-adrenoceptor agonists when compared at either their K_d or EC_{80} values, Fig. 6A. Interestingly, the rate of cAMP accumulation showed no correlation with lipophilicity, a factor that clearly did impact upon this parameter as discussed further below, (Fig. 6B). Therefore, these data suggested that the two factors acted independently of each other to regulate the onset of action.

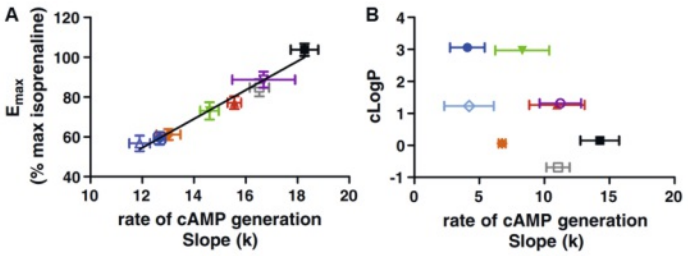


Fig. 6. Impact of intrinsic efficacy on β_2-adrenoceptor agonist onset of action: (A) correlation of rate of cAMP generation with intrinsic efficacy (percentage maximal response versus isoprenaline) with compounds dosed at their EC_{80} concentration; (B) correlation of rate of cAMP generation with lipophilicity (clogP). Reprinted with permission from, 'Efficacy is a contributing factor to the clinical onset of bronchodilation of inhaled β_2-adrenoceptor agonists', Elizabeth M. Rosethorne, Robert J. Turner, Robin A. Fairhurst, Steven J. Charlton, Naunyn-Schmiedeberg's *Archives of Pharmacology,* 382. Copyright **2010** Springer-Verlag.

One key aspect in the identification of *indacaterol*, and discussed above, was the pharmacokinetic profile. Lung distribution through the modulation of lipophilicity was used to rationalise the onset and duration of action, and metabolism and protein binding was used to rationalise the systemic side effect profile. However, these properties were clearly interrelated to varying degrees. For example, duration of action can be extended by increasing the inhaled dose of a β_2-adrenoceptor agonist. A consequence being that this will lead to a higher level of systemic exposure and increased potential for unwanted cardiovascular and metabolic side-effects. Thus, highlighting the therapeutic index benefit of a molecule with a high intrinsic duration of action that does not require 'over-dosing' to achieve the targeted duration of effect. Collectively the parameters discussed above could be considered as the macroscopic PK properties of *indacaterol* (lipophilicity, clearance, PPB). One area which generated several hypotheses and follow up studies focused on understanding the microscopic PK properties of *indacaterol*, at the cellular distribution and receptor interaction level.[26] Cell membranes are organised into discrete regions with differing lipid compositions and charge distributions and the proteins that interact with them do so in a spatially defined manner. This offered up the potential for localised effects within the membrane at the cellular and sub-cellular levels that could bring further refinement to the understanding of the profile of a compound beyond the macroscopic PK properties. The hypothesis being: asymmetric distribution within the phospholipid bilayer would be advantageous if a molecule colocalised with the target protein (β_2-adrenoceptor).

The interaction of a molecule with phospholipid membranes is a function of both the molecules lipophilicity and charged state. For β_2-adrenoceptor agonists the *N*-substituent can be modelled to reside within the hydrocarbon chains of the bilayer core whilst the protonated amine of the ethanolamine linker interacts with the phosphate group. An *in silico* model was generated with dodecyl sulfate derived micelles as a surrogate for the phospholipid bilayer to study this interaction, (Fig. 7A). Additionally, the phenol present in the catechol mimetic is oriented into the solvent and the pK_a of this group can lead to different amounts of the phenoxide or phenol form being present in the equilibria, adding further complexity to the species ensemble. For *indacaterol* a greater acidity was determined for the 8-hydroxyquinolinone group when compared to the catechol mimetics present in *formoterol* and *salmeterol*, (Table 2). Consequently, for *indacaterol* the zwitterion is the predominant species under physiological conditions as opposed to the protonated amine in the case of *formoterol* and *salmeterol*. To assess the extent of any impact on the interaction with phospholipid membranes we used IAM-HPLC to determine the CHI_{IAM} values.[16] At the level of the bulk interaction with phospholipid the CHI_{IAM} value determined for *indacaterol* showed no large deviation from the other β_2-adrenoceptor agonists based upon what would be predicted from their overall lipophilicity values (clogP), (Fig. 7B). However, an external collaboration with the Krämer group highlighted subtle differences at the microscopic PK level.[27] When compared to *salmeterol*, *indacaterol* showed similar liposome partitioning except when a lipid-raft partition phase was used. In this instance *indacaterol* exhibited a 2-fold greater affinity to the lipid-raft phase, the region within the cell membrane where the β_2-adrenoceptors are localised. Additionally, surface plasmon resonance studies with immobilised liposomes showed that the association rate was two-times faster with *indacaterol* compared to *salmeterol*. Rationalising these data, a greater preference to accumulate in the lipid-raft regions proximal to the β_2-adrenoceptor and a faster membrane association rate could help to explain the longer duration of action and faster onset of action of *indacaterol*.

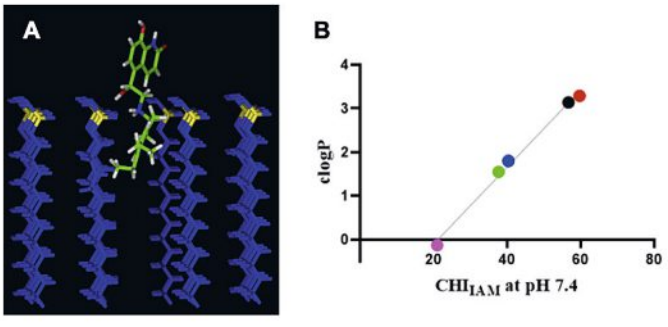


Fig. 7. Exploring the phospholipid β_2-adrenoceptor interaction: (A) *in silico* model of indacaterol bound within a dodecyl sulfate micelle; (B) correlation between clogP and CHI_{IAM} for indacaterol and the reference β_2-adrenoceptor agonists.

The greater affinity for lipid raft regions within the cell membrane also integrated nicely into the rebinding theory that membrane partitioning generated a higher concentration proximal to the membrane spanning β_2-adrenoceptors, and this higher local concentration contributed to increased ligand-receptor affinity.[28] Through the measurement of the β_2-adrenoceptor binding kinetics and phospholipid affinity we demonstrated that the degree of phospholipid binding directly affects the kinetic association rate (k_{on}), but not the dissociation rate (k_{off}).[29] The outcome being that

ligands with higher phospholipid binding (CHI_{IAM}) were found to produce faster association rates and as a result lower dissociation rate (K_d) values (higher affinities). When corrected for phospholipid binding the k_{on} was found to be comparable across the ligands studied and CHI_{IAM}-corrected K_d values correlated well with the measured K_d values, (Fig. 8A). Additionally, molecular dynamics simulations with ligand bound β_2-adrenoceptor complexes generated SAR for the key ligand-binding interactions which correlated well when the same phospholipid binding correction was applied.[30] These studies highlighted that for ligands with strong partitioning into phospholipid bilayers there was a concentrating effect proximal to the membrane bound protein (β_2-adrenoceptor) that would enhance affinity relative to the ligand concentration in bulk solvent. The ligand concentrating effect can be viewed as a concentration gradient at the water-lipid interface, but also raises the possibility that ligands can enter the target protein directly from the phospholipid bilayer.[31] Fig. 8B shows a cartoon in which the above observations are condensed into a set of equilibria to summarise the mechanisms through which *indacaterol*, and other lipophilic (basic) drugs can be retained in the lung following inhaled delivery as an extended version of the plasmalemma diffusion microkinetic theory.

Throughout all these studies *indacaterol* was consistently found to be equivalent or superior to the reference long-acting β_2-adrenoceptor agonists based upon our TPP, a profile that was supported by the ongoing clinical studies. However, the differences were often small and reflect how the sum of multiple marginal gains can lead to superior product. For example, the difference in duration of action between the second and third generations of inhaled β_2-adrenoceptor agonists is in principle only 2-fold, but this has a marked impact on their clinical outcomes.

3.2 *NVP-QAN746 as a Backup Once-daily Inhaled β_2-adrenoceptor Agonist*

A key component of defining what to aim for with a backup project is understanding the potential weaknesses of the front runner compound. In the case of *indacaterol* defining a backup TPP was challenging as no weaknesses had been observed as the compound progressed into early development (or arose during the development of *indacaterol*). Two unknowns, and hence concerns, at that point in the project were discovering an unacceptable toxicological finding and identifying a physical form suitable for use in a dry-powder inhalation device. To address these concerns a structurally different molecule to *indacaterol*, but with a comparable preclinical pharmacology profile was regarded to be the best approach, and this vision became the TPP for the backup project.

In the middle of 1999 as we were starting the backup project the disclosure of the 4-hydroxybenzothiazolone adrenaline analogue S1319 by the Kirin Brewery as a β_2-adrenoceptor agonist attracted our interest. S1319 was isolated from the marine sponge *Dysidea sp.* and highlighted the 4-hydroxybenzothiazolone as a catechol mimetic that could be used as a structurally different replacement for the 8-hydroxyquinolinone moiety in *indacaterol*.[32] From both an ionization and potency comparison the 4-hydroxybenzothiazolone catechol mimetic compared favourably with the 8-hydroxyquinolinone of *indacaterol*, (Fig. 9). The pK_a of the 4-hydroxybenzothiazolone was predicted to be within one log unit of *indacaterol* and the β_2-adrenoceptor agonist potency of S1319 was reported to be comparable to *formoterol*, suggesting a high level of activity for this catechol mimetic. Additionally, an initial SAR analysis had indicated that the phenolic residue of bicyclic catechol mimetics exhibited a higher susceptibility towards glucuronidation when compared to the monocyclic versions found in, for example: *formoterol*, QAC455 and *salmeterol*. This metabolic difference potentially offered a way to help to minimise systemic exposure through increased systemic clearance, ultimately contributing to an enhanced therapeutic index towards systemic β_2-adrenoceptor activation. Thus, one avenue that became attractive for the backup project was to explore S1319 analogues in which the *N*-methyl group was modified to regulate the properties/profile of the series using the learnings from the discovery of *indacaterol*.

One area which should not be underestimated in medicinal chemistry is the role of synthesis, and being able to prepare the desired molecules efficiently is fundamental to the progress/success of a project. Although routes to 4-hydroxybenzothiazolone analogues had been described at the start of the backup project we chose to look for a more efficient alternative. In particular, the group of Stanetty had described a benzyne-mediated cyclisation to give 2-alkoxybenzothiazoles which we believed could provide

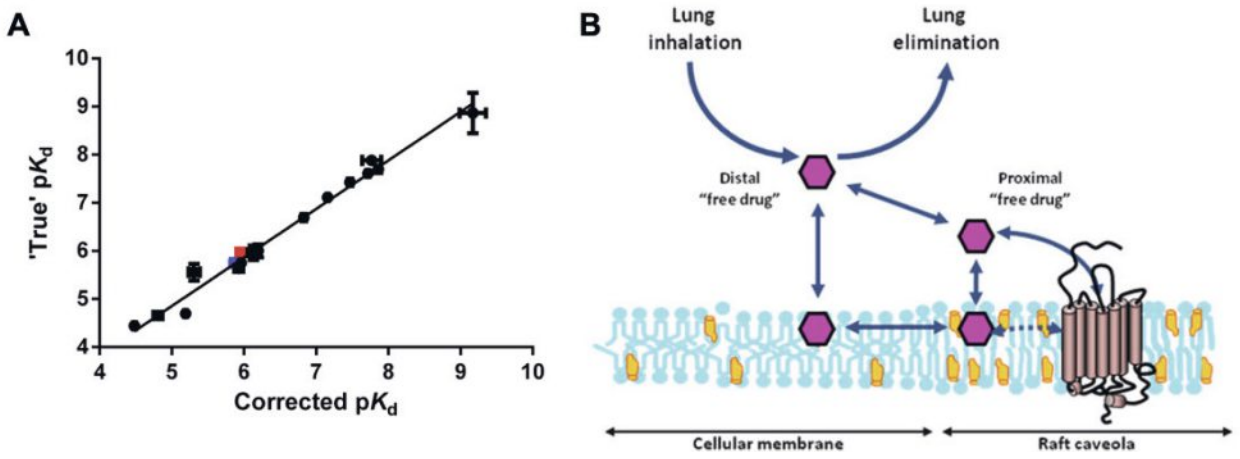
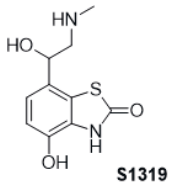


Fig. 8. Impact of the phospholipid partitioning on β_2-adrenoceptor agonist receptor-affinity supporting an expanded lung interaction model: (A) correlation between the phospholipid binding corrected pK_d and measured 'True' pK_d (r^2=0.98); (B) cartoon showing the expanded plasmalemma diffusion microkinetic theory. Figure 8A used with permission of *American Society for Pharmacology & Experimental Therapeutics* from, 'Observed Drug-Receptor Association Rates Are Governed by Membrane Affinity: The Importance of Establishing "Micro-Pharmacokinetic/Pharmacodynamic Relationships" at the β_2-Adrenoceptor', David A. Sykes, Cheryl Parry, John Reilly, Penny Wright, Robin A. Fairhurst, and Steven J. Charlton, 85, **2014**; permission conveyed through Copyright Clearance Center, Inc. Figure 8B reprinted with permission from, *The Design of the Indacaterol Molecule*, Robin A. Fairhurst, Steven J. Charlton, and Alexandre Trifilieff in, 'Indacaterol the first once-daily long-acting beta2 agonist for COPD', editor A. Trifilieff. Copyright Springer Basel **2014**.



S1319

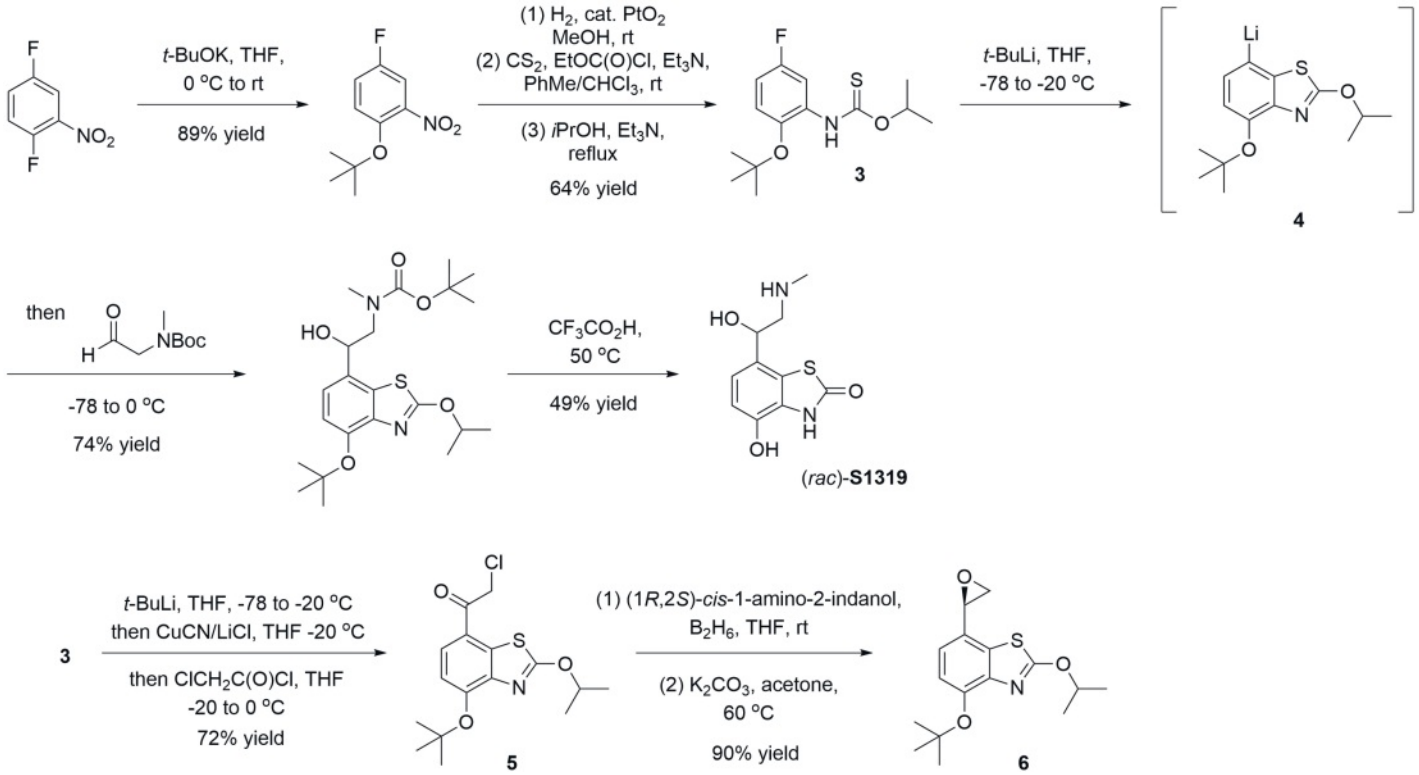
β_2-adrenoceptor activity	
affinity K_d (nM)[a]	51
functional pIC_{50}[b]	10.6

Fig. 9. Structure of S1319 with selected data reported by the Kirin Brewery. [a]Affinity was assessed using a radioligand binding assay with the β_2-adrenoceptor antagonist reporter ^{3}H-CGP12177. [b]Functional activity was assessed in an organ-bath experiment with guinea-pig trachea *via* the inhibition of histamine-induced contraction.[32]

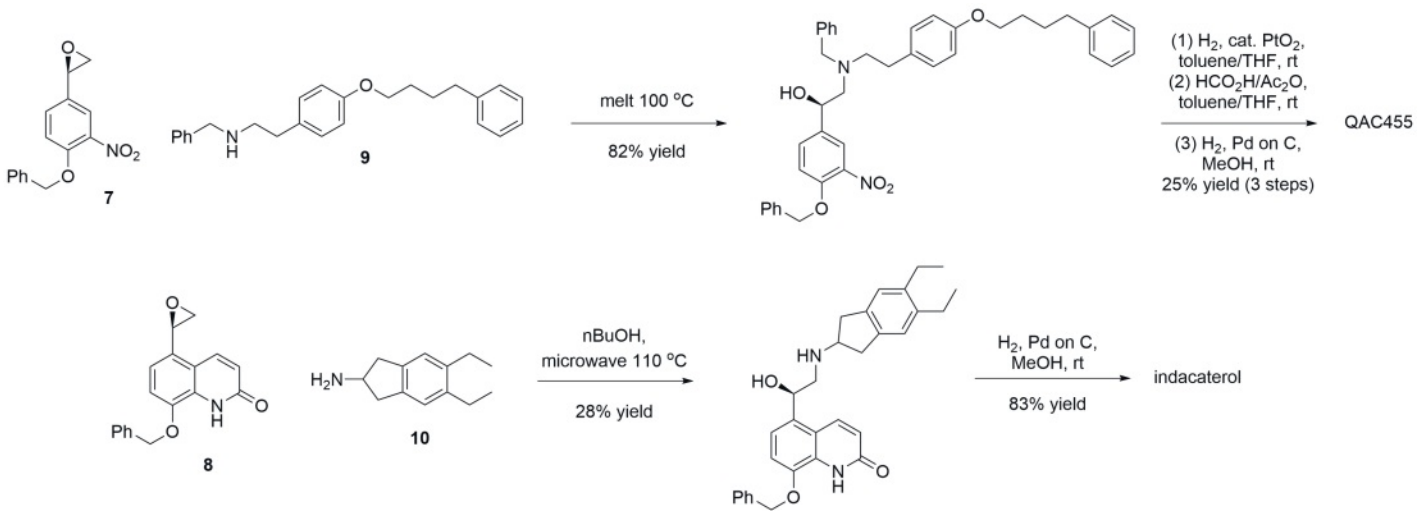
an efficient route to a key intermediate for preparing the targeted analogues.[33] This possibility was initially explored through the development of a racemic synthesis of S1319 that used a *tert*-butyl ether in the cyclisation precursor **3** to direct deprotonation to the desired position between the fluoro and thiocarbamate moieties. This lithiated species then underwent benzyne formation and cyclisation to give the intermediate **4**, (Scheme 1).[34] The corresponding methyl ether analogue of **3** led to significant deprotonation *ortho* to the ether moiety as an unproductive competing reaction. Expanding the scope of the approach, transmetalation of the putative 7-lithiated thiazole intermediate **4** to the lower-order cyanocuprate enabled a clean acylation to occur with chloroacetyl chloride which gave the chloroketone **5**.[35] This process served the discovery phase of the project to produce **5** on the multigram scale, but then went on to be further optimized in early development by direct acylation of the lithiated intermediate **4** with the Weinreb amide of chloroacetic acid at - 40 °C to deliver **5** efficiently in kilogram quantities.[36] Asymmetric reduction of **5** gave the (*R*)-chlorohydrin which could be cyclized to the key-intermediate epoxide **6** for exploring the *N*-substituent SAR.

From the initial phase of the project epoxides had proven to be the most versatile intermediates for preparing analogues with variation of the ethanolamine *N*-substituent following the S_N2 addition of primary amines, or their equivalents (typically *N*-benzylated secondary amines). Such an approach would be considered a late-stage diversification opportunity and applied in this way synthetic organic chemistry is very much a tool to answer the questions in hand in the most efficient way possible. In this setting overall yields are often a secondary consideration to speed and flexibility. The adage 'there are only two yields in medicinal chemistry: either you have sufficient material to perform the next set of studies, or you do not, nicely capture the essence of what synthetic chemistry needed to deliver in this phase of a project where the majority of compounds do not progress beyond the next set of studies. What immediately became apparent for this epoxide opening reactions was the diversity in reactivity between what appeared to be electronically similar unsubstituted aryl epoxides. Conditions that worked adequately for one epoxide across a range of primary amines yielded minimal, or no, product with a different epoxide and the same primary amine set. Shown in Scheme 2 are the conditions that were used in the discovery phase with the protected *ortho*-nitrobenzyloxyphenyl epoxide **7** and the 8-hydroxyquinolinone epoxide **8** for the preparation of QAC455 and *indacaterol* as representative examples. In the case of QAC455, the secondary *N*-benzylated amine **9** opened the epoxide **7** efficiently when the two reactants were melted together in equal quantities. In the case of *indacaterol*, the quinolinone epoxide **8** was opened with a slight excess of the aminoindane **10** (15 mol%) following microwave heating for 1 hour at 110 °C in *n*-butanol. In the former the *N*-benzylated secondary amines **9** were required to avoid double *N*-alkylation, a by-product that was not formed in significant amounts with the epoxide **8**. What supported this approach was that once identified these reaction conditions yielded workable quantities of the ethanolamine products across the range of amine nucleophiles of interest.

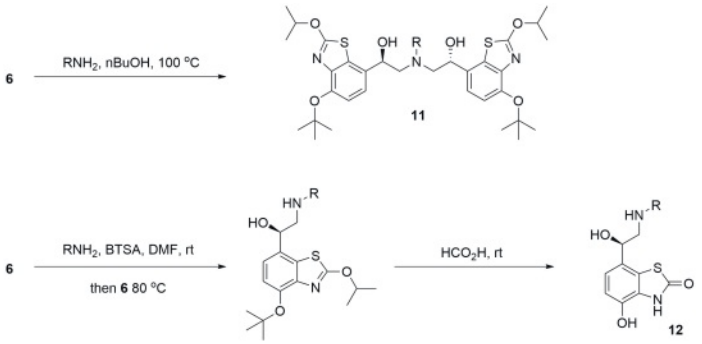
Interestingly, the benzothiazole epoxide **6** was found to be bench stable but appeared to be more reactive than either **7** or **8**, and underwent solvolysis under relatively mild conditions (TLC, HPLC). As a result, epoxide **6** was best analysed with ^{1}H nmr in non-nucleophilic solvents (*e.g.* $CDCl_3$). Attempted ring opening of **6** with all but the most hindered primary amines led to the tertiary amines **11** as the major product *via* a double addition



Scheme 1. Synthesis of the natural product S1319 and the key chiral epoxide intermediate **6**.



Scheme 2. Epoxide opening reactions used in the discovery syntheses of QAC455 and indacaterol.



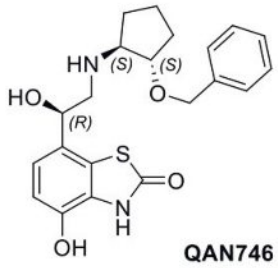
Scheme 3. Epoxide opening and final deprotection steps to prepare the 4-hydroxybenzothiazolone β_2-adrenoceptor agonist **12**.

with insufficient amounts of the targeted secondary amines being formed to be useful for further synthesis. This overreaction could be readily controlled by the *in situ* mono-silylation of the primary amines prior to epoxide opening, (Scheme 3).[35] The benefit of these conditions were two-fold, to avoid the use of *N*-benzylated secondary amines with the associated additional deprotection step, and the larger number of readily available primary amines to select from. Importantly, these epoxide opening conditions proved to be reliable with a structurally diverse set of primary amines which greatly expanded the options for the series/project. Finally, acid catalysed dealkylation cleanly deprotected the 4-hydroxybenzothazolone moiety to give the targeted compounds **12** with high chemical and optical purity following purification.

The benefit of having a robust synthetic platform in place to prepare the 4-hydroxybenzothiazolone β_2-adrenoceptor analogues **12** was that it allowed a parallel synthesis approach to be considered to accelerate/broaden the scope during the initial stage of the optimisation. Additionally, experience gained from the identification of *indacaterol* enabled a lipophilicity window to be defined to provide the highest probability of delivering interesting compounds with respect to their onset and duration of action profiles. Capitalising on these observations led to the application of a properties based parallel synthesis approach. Following the above design hypothesis, a set of 105 commercial and proprietary primary amines were assembled that when modelled as the *N*-substituent of the 4-hydroxybenzothiazolone scaffold **12** were calculated to satisfy the targeted lipophilicity range ($logD_{7.4}$ 2.4 to 3.5). These amines underwent a successful parallel synthesis campaign with the epoxide **6** using the *in situ* silylation ring-opening approach described above with a > 90% success rate.[35] Screening the set *in vitro* for β_2-adrenoceptor agonist activity, functional β_1-adrenoceptor selectivity and susceptibility towards glucuronidation enabled the analogues to be identified with the desired potency and selectivity whilst satisfying the hypothesized property space for a wide therapeutic window in combination with a rapid onset and long duration of action.

Following up on the interesting examples from the parallel synthesis effort identified a number of 1,2-disubstituted cyclopentylamine substituted analogues of **12** as being particularly interesting.[36] This type of *N*-substituent had not been described previously in the β_2-adrenoceptor space, and hence fulfilling one of the TPP objectives with structural novelty in both the 4-hydroxybenzothiazolone catechol mimetic and the ethanolamine *N*-substituent. Further profiling along this 1,2-disubstituted cyclopentylamino SAR path ultimately resulted in the identification of QAN746 as the backup candidate molecule with a preclinical profile comparable to *indacaterol* and satisfying the second TPP objective. Interestingly, even though a number of novel 1,2-disubstituted cyclopentylamine analogues were prepared following on from the parallel synthesis output, QAN746 was part of the original parallel synthesis run and ultimately considered to have the best overall profile to satisfy the TPP.[36] As for *indacaterol* another example of the 'optimal' compound being prepared at an early point in a medicinal chemistry optimisation and subsequent activities only serving to confirm the assessment. Key aspects of the preclinical profiles of *indacaterol* and QAN746 are compared in Fig.10.

Preclinical assessment of QAN746 supported that the compound could be taken forward into clinical evaluation. A key part of this evaluation was the identification of the *hemi*-maleate salt form and demonstrating that it was suitable for use in a dry powder inhalation device. A phase I study was initiated in 2004 with QAN746 administered by inhalation to assess the molecules suitability as a once-daily bronchodilator. In stark contrast to *indacaterol* the trial with QAN746 showed the molecule to be a competent bronchodilator, but not to provide 24 h bronchoprotection at a dose that had an acceptable level of systemic side effects. The most prevalent side effects being tremor, tachycardia and hypokalemia which were considered to be β_2-adrenoceptor mediated. Consequently, the development of QAN746 was terminated following the dose-escalation study. This experience highlighted the challenge in translating preclinical findings into patients, and that even with good science and intelligent decision making some level of good fortune is required to achieve the desired clinical outcome. This particularly applies to parenteral approaches where design principals are less well developed compared to the more widely studied oral route of administration.



Compound	QAN746	indacaterol
β_2 K_i (nM)	2.5	21
β_2 EC_{50} (nM) / intrinsic efficacy (%)	0.25 / 103	1.5 / 91
pK_a amine, phenol	8.5, 7.5	8.3, 6.7
$logD_{7.4}$ / $logK_{IAM}$	1.25 / 1.21	1.77 / 1.90
plasma protein binding	96.1%	95.1-96.2%

Fig. 10. Structure of QAN746 and table comparing key data with indacaterol. Data were measured as described for Table 2.

3.3 *Exploring the* β_2*-adrenoceptor Agonist Pharmacophore*

From the start of the project, we had been keen to explore the existing SAR surrounding the β_2-adrenoceptor in addition to expanding into previously unexplored regions. For example, the initial design rationale for QAC455 had included the exosite binding region located outside the catechol binding site.[4] Although the SAR correlated to some extent with the exosite model, this approach had helped to clarify the importance of lipophilicity and intrinsic efficacy in defining the onset and duration of action profile at an early point in the project.

With *indacaterol* in hand small structural changes were investigated in all three regions of the β_2-adrenoceptor to understand how they impacted upon the SAR.[37] Interestingly, the analogues **13** and **14**, which differed by only a single bond from the parent compound highlighted how quickly the profile of *indacaterol* could be eroded, (Fig. 11). When dosed at an equally effective dose level in the rhesus monkey model (ED_{80}) the cyclised analogue **13** was found to be 1.7-fold less potent and exhibited a significantly shorter duration of bronchoprotection compared to *indacaterol*. In contrast, the 3,4-dihydroquinolinone analogue **14** was found to be 4.2-fold less potent and produced a comparable duration of bronchoprotection to *indacaterol*, but this was accompanied by a higher and more sustained systemic acting β_2-adrenoceptor activation as measured by heart-rate increase.[10] These marked differences in *in vivo* profiles highlighted the sensitivity of the SAR controlling the key aspects of the TPP upon small structural changes, differences that were not readily predicted from the compounds *in vitro* profiles.

In most cases the modifications to *indacaterol* led to equivalent or lower levels of β_2-adrenoceptor agonist activity. One exception was found to be the addition of a methyl group onto the α-carbon of the aminoindane moiety which produced **15**. When compared to *indacaterol*, **15** was found to be a 7-fold more potent agonist of the β_2-adrenoceptor and to exhibit a surprisingly high level of intrinsic efficacy (116% *versus formoterol*). The higher affinity of **15** was found to be driven by a 145-fold slower dissociation rate from the receptor at 37 °C ($t_{½}$ 29 minutes), and such slow-dissociation profiles are atypical for agonists of the β_2-adrenoceptor, Table 3. The efficiency of the GPCR endogenous-ligand interaction is thought to have been optimised through evolutionary pressure, and ligands with greater efficacy than the endogenous/reference ligands are unusual and have been termed super-agonists.[38] Another example of a slowly dissociating β_2-adrenoceptor agonist is the *N*-phentermine analogue BI-167107, and the slow dissociation of this ligand was reported to be a key property that enabled the first agonist cocrystal structure to be solved with the β_2-adrenoceptor.[39] As discussed above, although high potency is a desirable feature for almost every drug discovery project inhaled delivery remains an exception. The anticipated low human dose for **15** made it unsuitable for development as an inhaled dry-powder formulation due to the technical challenge to achieve consistent dose-to-dose reproducibility.[11] However, the high affinity, slow dissociation rate and high intrinsic-efficacy super-agonist profile made this a very interesting β_2-adrenoceptor agonist from a receptor pharmacology perspective.

Having observed the super-agonist profile with **15**, we were prepared when one of the 4-hydroxybenzothiazolone library members C26 also showed a similar profile with a very high β_2-adrenoceptor affinity (K_i 0.5 nM) and long residence time measured at 37 °C ($t_{½}$ 33 minutes), Table 3.[35] This was interesting from a structural perspective as both **15** and BI-167107 contained a quaternary carbon at the α-position of the *N*-substituent, a structural feature we had hypothesised to be important to achieve the super-agonist profile. In contrast, C26 bore a secondary carbon at this position, and interestingly was also a diastereoisomer of the backup candidate QAN746. Again, the high potency and resulting low anticipated human dose prevented C26 from being considered as a viable development candidate. Further studies with C26 showed it to be a potent β_2-adrenoceptor agonist (EC_{50} 0.032 nM) with a high intrinsic efficacy (140% *versus formoterol*) in a cellular assay reading out cAMP, and which confirmed the super-agonist profile, (Fig. 12).[41]

During the backup project we had become increasingly interested in a number of established and emerging concepts in the field of GPCR signalling and in particular the role of intrinsic efficacy and the concept of biased agonism.[42] What the benzothiazole series and C26 in particular allowed us to do was start to explore these concepts with interesting ligands in hand.

Biased agonism is an interesting concept where a GPCR can signal through more than one pathway and the extent to which each of these pathways are activated can vary depending upon the applied agonist. Of particular interest to the medicinal chemist is the notion that each pathway is activated by a distinct population of receptor conformations, thus offering the possibility that pathway selectivity can be controlled through an understanding of the SAR.[43] In the case of the β_2-adrenoceptor the most studied signalling pathways are through the heterotrimeric G protein Gs activation of adenylate cyclase leading to cAMP accumulation and through β-arrestin. β-arrestins were for a long time considered to be negative regulators of GPCRs by turning off signalling through the internalisation of activated receptors. This regulation was seen as a way to auto regulate the system and avoid overstimulation. More recently β-arrestins have been shown to be involved in controlling GPCR signalling beyond receptor internalization and the canonical cAMP pathway.[44]

Further profiling of C26 confirmed the high affinity, slow-binding profile, and high intrinsic efficacy for the cAMP pathway *versus* isoprenaline (118%).[41] C26 was also shown to be a super agonist of the β-arrestin pathway in both recruitment and internalization assays (126% *versus* isoprenaline). Together these data suggested no functional bias between the two pathways, consistent with data subsequently reported for other commonly studied β_2-adrenoceptor agonists.[45] However, the kinetic profile of C26 highlighted the compounds' unusual nature when com-

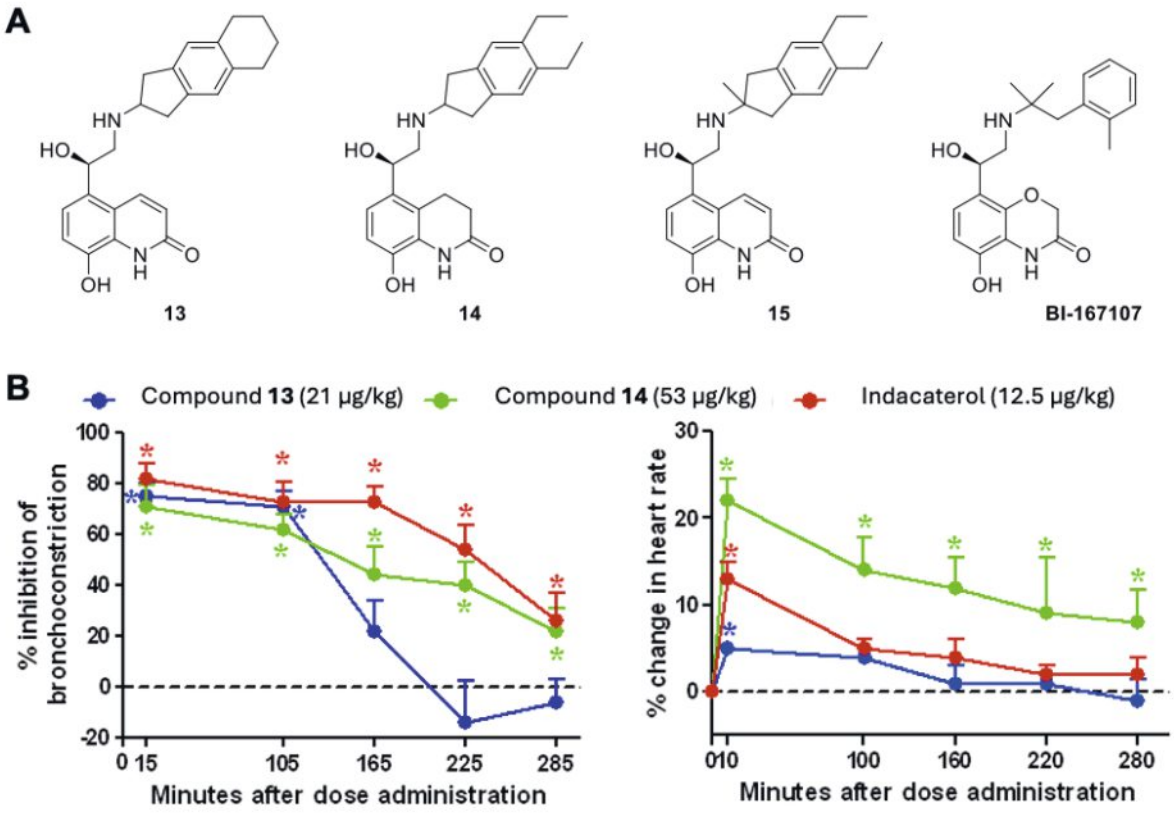


Fig. 11. Structures of the close analogues of indacaterol and BI-167107 with data from the *rhesus* monkey model for analogues **13** and **14** compared to indacaterol: (A) chemical structures of the compounds; (B) time course for the inhibition of the methacholine-induced bronchonstriction and heart-rate changes in the *rhesus* monkey for analogues **13** and **14** compared to indacaterol at the ED_{80} dose level. Data are expressed as mean ± standard error of the mean. Significance, $p < 0.05$, indicated by * is against the respective control values.[18] Figure 11B reprinted with permission from *The Design of the Indacaterol Molecule*, Robin A. Fairhurst, Steven J. Charlton, and Alexandre Trifilieff in, 'Indacaterol the first once-daily long-acting beta2 agonist for COPD', editor A. Trifilieff. Copyright Springer Basel **2014**.

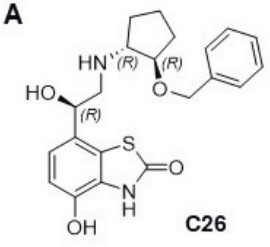
pared to other β_2-adrenoceptor agonists. At early timepoints C26 functioned as a partial agonist relative to adrenaline in terms of cAMP production and then as a super agonist at later time points, (Fig. 12B). With C26 the recovery from receptor internalisation was dramatically increased following washout when compared at *equi*-effective concentrations (EC_{80}) to adrenaline and isoprenaline ($t_{½}$ 20.5 *versus* 0.74 and 0.67 hours respectively), (Fig. 12C). These data supported the notion that the internalised population of β_2-adrenoceptors can contribute efficiently to the signalling output of a cell.[46] In the guinea-pig tracheal strip model C26 showed an extended onset of action and long duration of action, (Fig.12D). These data challenged our earlier observations made with the commonly studied β_2-adrenoceptor agonists regarding the relationships between intrinsic efficacy and onset of action, and binding kinetics and duration of action.[25,29] The profiling of C26 only served to raise more questions than were answered relating to how GPCRs function, and ligands of this type we still believe have a role to play in increasing our understanding of how this family of receptors function and in particular the temporal aspects of signalling.

GPCR intrinsic efficacy was also an area that interested us, and how the continuum from inverse agonist, through antagonist, and onto partial, full, and super-agonist could be exploited. We became interested in the possibility of preparing lower intrinsic-efficacy agonists with sufficient potency to explore as bronchodilators. The question at the time was to see if there was an exploitable difference between the receptor reserve in airway smooth muscle and the tissues driving the β_2-adrenoceptor-mediated side effects. Contradicting this hypothesis, the data for the lower intrinsic efficacy agonists *salbutamol* and *salmeterol* suggested that the therapeutic index of an inhaled β_2-adrenoceptor agonist would not be improved through exploring such a profile. However, we felt there were levels of intrinsic efficacy and physical property space that had not been fully explored by the ligands reported up to that point in time.

Table 3. β_2-adrenoceptor binding kinetics of the super-agonists **15** and C26 compared to the reference compounds.[40]

Compound	k_{on} ($M^{-1}min^{-1}$)	k_{off} (min^{-1})	$t_{½}$ (min)	pK_d (k_{off}/k_{on})
salbutamol	2.05×10^7	4.06	0.17	6.65
formoterol	2.15×10^8	3.29	0.21	7.83
indacaterol	8.74×10^7	3.48	0.20	7.37
15	8.99×10^7	0.021	29	9.63
C26	4.7×10^7	0.024	33	9.29

A



β_2-adrenoceptor activity	
K_i (nM)	0.5
EC_{50} (nM) / intrinsic efficacy (%)	0.032 / 140
Tracheal-strip IC_{50} (nM) / OoA (min) / DoA (h)	0.23 / 312 / > 12

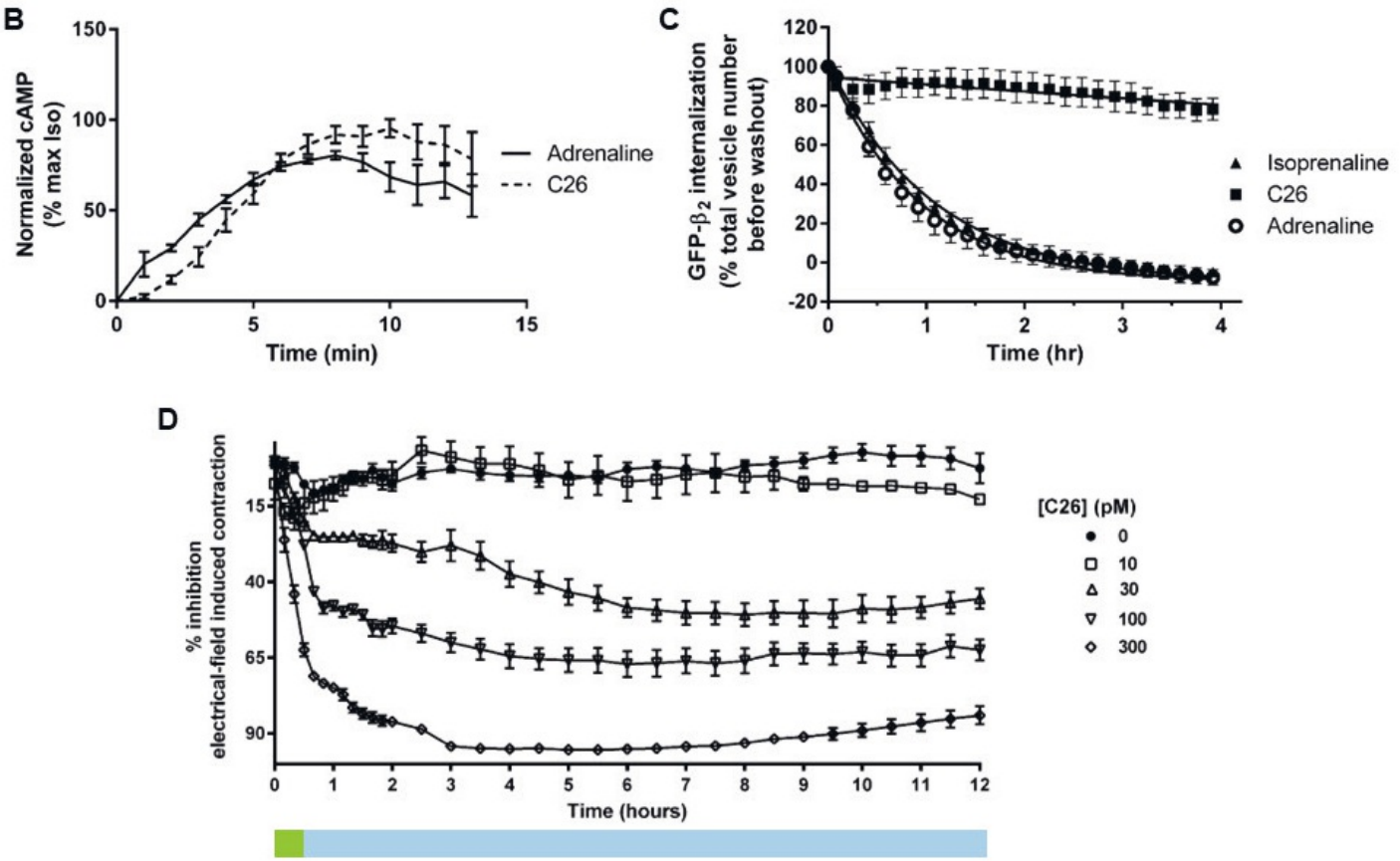
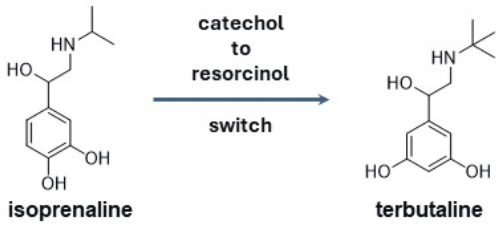


Fig. 12. Structure of C26 and selected *in vitro* data highlighting the super-agonist profile: (A) table showing the binding affinity, cellular activity, and guinea-pig tracheal strip activity of C26, data were measured as described for Table 2; (B) relative intrinsic activities of C26 and adrenaline for cAMP accumulation after incubation with EC_{80} concentrations of each agonist, data are normalised to the maximal adrenaline response (8 minutes). C26 appears as a lower intrinsic efficacy agonist up to the 6-minute timepoint and then goes on to show the super agonist profile beyond 6 minutes; (C) rate of loss of GFP-β_2-adrenoceptor internalized receptors in U2OS cells following washout of agonist. Equi-effective concentrations of adrenaline, C26, and isoprenaline were used equivalent to an EC_{80} concentration of isoprenaline; (D) Time course of inhibition of electrical field–induced contraction in isolated guinea pig tracheal strips using a range of different concentrations of C26. Data are expressed as means ± standard error of the mean.[41] Used with permission of *American Society for Pharmacology & Experimental Therapeutics* from, 'Long Receptor Residence Time of C26 Contributes to Super Agonist Activity at the Human β_2 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, 89, **2016**; permission conveyed through Copyright Clearance Center, Inc.

Receptor reserve relates to the proportion of receptors in a tissue that an agonist is required to interact with to produce a maximal response. The additional proportion of receptors beyond that point defines the receptor reserve for that agonist in that tissue. Therefore, lower intrinsic efficacy agonists can appear as partial agonists in tissues with lower receptor reserves. The question in hand being to establish if the tissues driving the systemic side effects (*e.g.* the cardiovascular system) possessed a lower receptor reserve compared to the tissue driving efficacy (airway smooth muscle). If the difference in receptor reserve proved to be in the desired direction and was of sufficient size, then a greater therapeutic index should be possible whilst maintaining the maximum bronchodilator effect on airway smooth muscle. To investigate this possibility, we explored approaches to generate β_2-adrenoceptor agonists with lower intrinsic efficacy compared to the agonists we had studied up to this point. Additionally, a second potential goal for this approach was to discover a way to systematically reduce potency to be able to exploit the *N*-substituents that had been discovered to be too potent for inhaled dry-powder delivery from the parallel synthesis campaign that identified QAN746, as discussed above.

These receptor pharmacology questions once more prompted medicinal chemistry ideas. In particular, the possibility that we could build on our findings from the benzothiazolone series to generate high-affinity and lower intrinsic-efficacy β_2-adrenoceptor agonists. Modification of the adrenaline catechol moiety to the corresponding *resorcinol* analogues, such as found in *terbutaline*, had been shown to be a way to reduce intrinsic efficacy and po-



Compound	isoprenaline	terbutaline
β_2 K_i (nM)	281	3,900
β_2 EC_{50} (nM) / intrinsic efficacy (%)	16 / 102	794 / 55

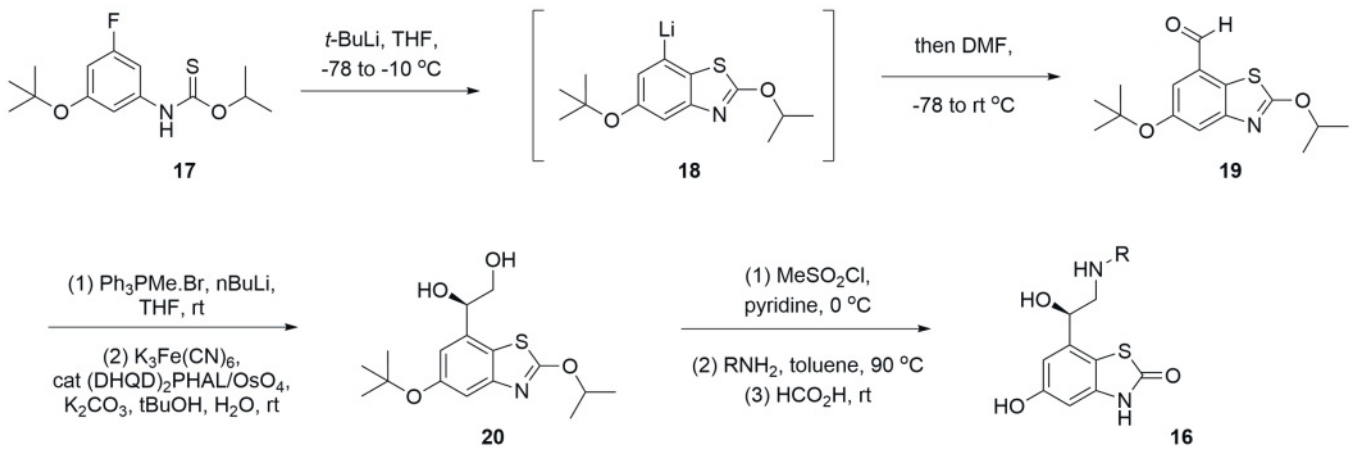
Fig. 13. Structures of the first generation β_2-adrenoceptor agonists isoprenaline and terbutaline highlighting the impact of the resorcinol phenol relationship.

tency from the early work in the area as part of the first generation of β_2-adrenoceptor agonists, (Fig. 1).[5] Applying this modification to our own work suggested the 5-hydroxybenzothiazolone catechol mimetic as a bioisostere to achieve the same profile. To access these targets, we reasoned that an extension of the synthetic approach used to prepare the 4-hydroxy isomers **16** could be applied, (Scheme 4).[47] The desired precursor **17** was readily prepared and underwent the benzyne-mediated cyclisation. In this instance the putative 7-lithiated intermediate **18** was discovered to react most efficiently with dimethyl formamide. The *tert*-butyl ether was again key to a high yielding outcome by directing the lithiation to the desired position. In contrast to the 4-*tert*-butoxy system the isomeric 5-*tert*-butoxy epoxide proved to be too reactive to be a bench-stable intermediate and again alternative conditions had to be identified to form the ethanolamine functionality. The 7-formyl analogue **19** was instead converted to the diol **20** *via* methylenation followed by asymmetric dihydroxylation. The primary alcohol **20** was then selectively activated by mesylation and the mesylate displaced with a range of primary amines in a reaction which likely involves the epoxide as the reactive species. This chemistry once more enabled a parallel synthesis campaign to be employed using the most interesting amines from the set used to identify QAN746. The syntheses worked well across the set of amines and the targeted compounds **16** were obtained after double dealkylation with formic acid.

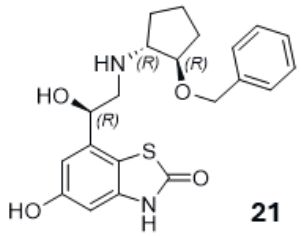
Evaluation of these analogues revealed that the targeted profile had been achieved in the vast majority of instances. Fig. 14 shows a comparison between the 4- and 5-hydroxy analogues C26 and **21** bearing an (*R,R*)-trans-benyloxycyclopentyl *N*-substituent. In this case taking the *N*-substituent that had been found to have the highest affinity and to produce super-agonist levels of activation from the 4-hydroxybenzothiazolone series was considered to be the most challenging comparison. The 5-hydroxybenzothiazolone **21** was satisfyingly found to be a partial agonist in the projects standard functional assay (maximum cAMP response 60% *versus formoterol*) with both the β_2-adrenoceptor affinity and functional potency lowered by 13-fold. This trend was evident across the 5-hydroxy series and allowed many of the amino substituents that had been deemed too potent in the 4-hydroxy series to be shifted into an acceptable potency range for inhaled delivery. However, although the targeted modulation of β_2-adrenoceptor potency and intrinsic efficacy had been achieved no other clear benefit was discovered upon further profiling these analogues as bronchodilators. Thus, the original question had been answered with partial success by expanding the breadth of *N*-substituents that could be utilised between the 4- and 5-hydroxy series based upon potency. However, the possibility to gain tissue selectivity and improve the therapeutic index through the differences in intrinsic efficacy did not play out in this setting, but the notion remained to use this approach to increase tolerability.

3.4 *Discovering an Application for the High Affinity Low Intrinsic Efficacy β_2-adrenoceptor Agonists*

Occasionally it is possible to have a solution looking for a problem to solve, and when we were approached by a Novartis team in 2008 looking to identify β_2-adrenoceptor agonists for the treatment of skeletal muscle atrophy we were immediately interested and thought of the 5-hydroxybenzothiazolone series. β_2-adrenoceptor agonists have been associated with this indication for some time, both as a legitimate therapeutic approach and as performance enhancing agents in the sports arena.[48] However, poor tolerability has limited their application due to the same systemic cardiovascular and metabolic side-effects described above for inhaled β_2-adrenoceptor agonists.[49] The team tackling this challenge went on to explore several β_2-adrenoceptor agonist series and hypotheses before settling on the 5-hydroxybenzothiazolone analogue 5-HOB as the candidate molecule to be advanced from the project, (Fig.15A).[50] Key to the selection of 5-HOB was the compounds ability to function as a high-efficacy agonist in readouts associated with anabolic effects in skeletal smooth muscle whilst being a partial agonist in readouts of metabolic and cardiovascular activation. Fig. 15B shows cAMP production in membranes generated from rat skeletal muscle and heart tissue: compared with *formoterol*, 5-HOB demonstrated comparable maximal efficacy in



Scheme 4. Synthetic route used to prepare the 5-hydroxybenzothiazolone series of β_2-adrenoceptor agonists **16**.



Compound	21	C26	QAN746
β_2 K_i (nM)	2.7	0.50	2.5
β_2 EC_{50} (nM) / intrinsic efficacy (%)	0.40 / 60	0.032 / 140	0.25 / 103

Fig. 14. Structure of the 5-hydroxybenzothiazolone analogue **21** with β_2-adrenoceptor affinity and functional activity compared to the isomers C26 and QAN746. Data were measured as described for Table 2.

skeletal muscle membranes and a significantly reduced efficacy in heart membranes. Fig. 15C shows data from a rat model following 4 weeks of daily treatment followed by a 5-week washout period. In this model *formoterol* and 5-HOB produced comparable increases in skeletal muscle mass throughout the experiment and *formoterol* produced a significant reduction in ejection fraction from the heart during the treatment period in contrast to 5-HOB which did not, indicating superior tolerability for 5-HOB. Overall, 5-HOB showed a favourable tissue selectivity for the treatment of skeletal muscle atrophy in preclinical models when compared to *formoterol* as the reference β_2-adrenoceptor agonist. These data supported the lower intrinsic efficacy of 5-HOB to be a key element contributing to the molecule's safety and efficacy profile. 5-HOB progressed into clinical studies in 2015 to be explored as a treatment for muscle wasting and muscle weakness.

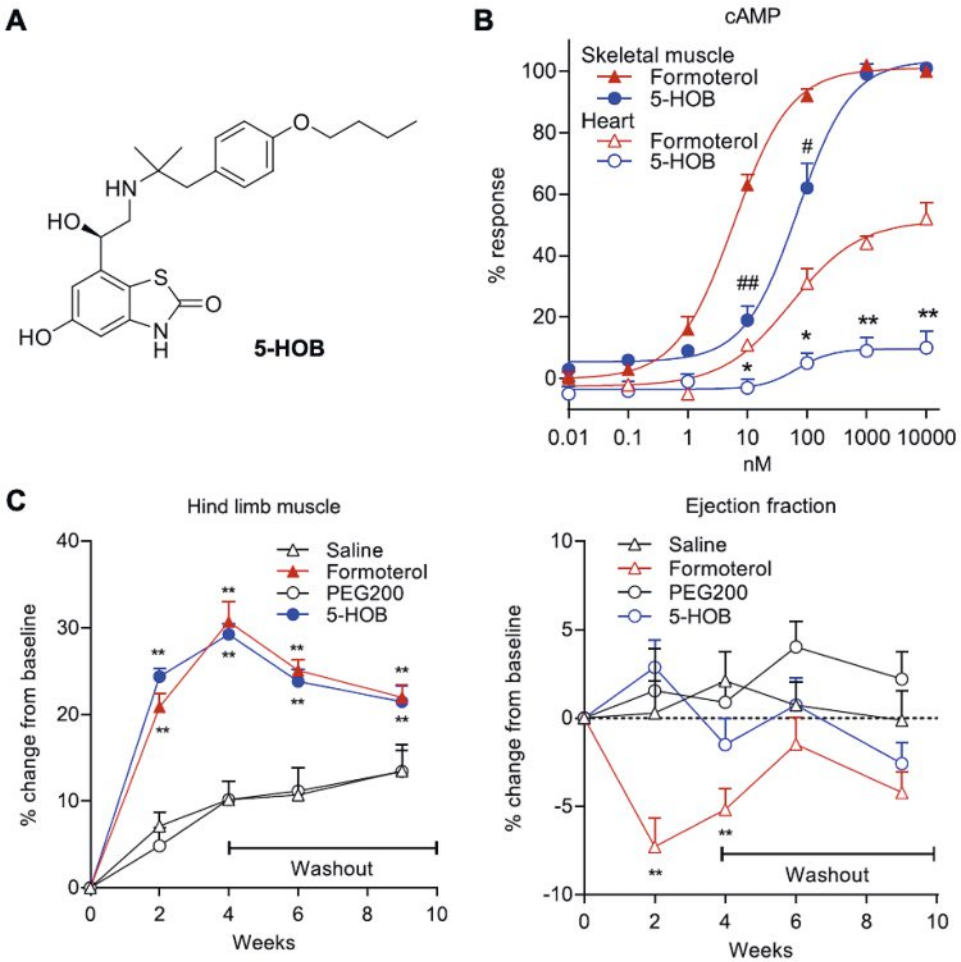


Fig. 15. *In vitro* and *in vivo* studies highlighting the relationship between muscle and cardiovascular readouts for 5-HOB compared to formoterol: (A) Structure of 5-HOB; (B) Effect of 5-HOB and formoterol on cAMP production in membranes isolated from Wistar rat gastrocnemius muscle and from the heart. Percentages of cAMP response were determined relative to the Emax of formoterol in skeletal muscle membranes. Data shown are means ± standard error of the mean of three independent experiments. *P 0.05; **P 0.01 on efficacy of 5-HOB versus formoterol in heart membranes and #P 0.05; ##P 0.01 in skeletal muscle membrane (T-test, unpaired); (C) Time-course changes of hind limb muscle mass and ejection fraction in Wistar rats. Changes in hind limb muscle and ejection fraction were evaluated by magnetic resonance imaging during treatment with 5-HOB and formoterol once-daily subcutaneously at 0.03 mg/kg for 4 weeks and during washout for 5 weeks. Values are means ± standard error of the mean (n = 6–8). **P 0.01 *vs* vehicle control (Holm-Sidak test following two-way repeated measurement ANOVA). Used with permission of *American Society for Pharmacology & Experimental Therapeutics* from 'Pharmacological Characterization of a Novel 5-Hydroxybenzothiazolone-Derived β_2-Adrenoceptor Agonist with Functional Selectivity for Anabolic Effects on Skeletal Muscle Resulting in a Wider Cardiovascular Safety Window in Preclinical Studies', Magdalena Koziczak-Holbro, Dean F. Rigel, Bérengère Dumotier, David A. Sykes, Jeffrey Tsao, Ngoc-Hong Nguyen, Julian Bösch, Marie Jourdain, Ludivine Flotte, Yuichiro Adachi, Michael Kiffe, Moïse Azria, Robin A. Fairhurst, Steven J. Charlton, Brian P. Richardson, Estelle Lach-Trifilieff, David J. Glass, Thomas Ullrich, Shinji Hatakeyama, 2 / 369, **2019**; permission conveyed through Copyright Clearance Center, Inc.

4. Conclusion

The β_2-adrenoceptor has been an almost ever-present target of interest throughout my medicinal chemistry career and has been central to my education in receptor pharmacology and the design of drugs for inhaled delivery. Over the past 25 years the understanding of how GPCRs function has grown immensely, and we have been able to explore and contribute to some of these concepts through the β_2-adrenoceptor. These activities have yielded three clinical candidates, one of which has gone on to become a marketed drug and being part of these successful drug discovery efforts has proven to be immensely rewarding. However, on a personal level it has been the scientific interactions and opportunities to explore and learn about how these systems operate and how they can be manipulated that has been the most satisfying. Hopefully, these examples have highlighted some of the opportunities that are open to medicinal chemists to collaborate across a diverse range of drug discovery areas through the design and delivery of interesting molecules.

Abbreviations

ANOVA, analysis of variance; BID, bis in die; BTSA, bis(trimethylsilyl)acetamide; cAMP, cyclic adenosine monophosphate; CHI_{IAM}, chromatographic hydrophobicity index; COPD, chronic obstructive pulmonary disease; CL_{int}, intrinsic clearance; $(DHQD)_2PHAL$, hydroquinidine 1,4-phthalazinediyl diether; DMF, dimethylformamide; DoA, duration of action; $EC_{50/80}$, half-/80%-maximal effective concentration; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IAM, immobilised artificial membrane; IC_{80}, 80%-maximal inhibitory concentration; K_d, equilibrium dissociation constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; OoA, onset of action; P, probability value; P_{app} A-B, apparent permeability apical to basolateral; PD, pharmacodynamic; PK, pharmacokinetic; PPB, plasma protein binding; PRN, pro re nata; QD, quaque die; SAR, structure activity relationship; S_N2, bimolecular nucleophilic substitution; THF, tetrahydrofuran; TPP, target product profile; UDP, uridine diphosphate glucose; UGT, UDP-glucuronosyltransferase.

Acknowledgements

I feel extremely fortunate to have been continually involved in engaging and exciting projects throughout my scientific career to date. However, what has been the most rewarding and fulfilling part of compiling this article has been the opportunity to reflect on the interactions I have had throughout this time. The individuals I have had the pleasure to share these discoveries with, both as colleagues and collaborators. These people have shaped the way I do science and inspired me to achieve more than I considered possible at the outset and to all of them go my warmest thanks. In particular, I would like to thank Bernard Cuenoud and Alexandre Trifilieff from the early encounters with the β_2-adrenoceptor, Brian Cox for his support throughout a large part of the work described in this article, and especially David Sykes and Steven Charlton for educating me in receptor pharmacology.

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